THE EMBO JOURNAL

Manuscript EMBO-2011-77233

Structural basis of cargo recognition by the myosin-X MyTH4-FERM domain

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Review timeline:

Submission date:	08 February 2011
Editorial Decision:	19 March 2011
Revision received:	05 April 2011
Editorial Decision:	30 April 2011
Revision received:	03 May 2011
Accepted:	06 May 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 19 March 2011

Thank you for submitting your manuscript 'Structural basis of cargo recognition by the myosin-X MyTH4-FERM domain' for consideration by the EMBO Journal. It has now been seen by two referees whose comments are enclosed. As you will see, both referees express significant interest in your manuscript and are broadly in favour of publication, pending satisfactory minor revision.

Both referees rightly point out that the Wei et al. PNAS paper, which appeared one week after your submission, should be cited and discussed. Referee 1 in particular points out the significant advances your dataset represents over the other study.

Referee 1 also suggests that you add binding data with integrin - or at least a competition assay - in order to broaden the relevance of the study. We would NOT expect a detailed binding analysis or indeed structural information. However, a competition assay would be feasible in a short time frame, and we would like to encourage it strongly.

We are expecting a third referee report, but have sent this decision now as we cannot justify a further delay, in particular given the competitive situation. If the report arrives in time, we will forward it to you immediately. We would only expect you to address additional experimental issues if these are essential to the integrity and quality of the manuscript.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript that addresses the comments of both reviewers within TWO WEEKS.

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, please visit our website:
http://www.nature.com/emboj/about/process.html

Please note that we would pursue fast track publication upon the still hypothetical final acceptance, to ensure no loss of priority relative to the PNAS paper. However, we would request a rapid resubmission, to minimize time delays now. If you cannot complete the requested experiment in a short time frame or if your resubmission will take more time for other reasons, please contact us to discuss the situation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Review of "Structural basis of cargo recognition by the myosin-X MyTH4-FERM domain" by Hirano et al.

The unconventional myosins are a large and diverse group of actin-based motor proteins. They are apparently important, given the number of biological processes that they have been implicated in by a variety of approaches, but as yet what, exactly, they do in cells, and how they do it, is somewhat mysterious except in a handful of cases.

Myosin-10 is particularly interesting, in that it apparently has the ability to bind several different partners, and is involved in filapodia formation, meiosis and mitosis. Much of myosin-10's function is thought to stem from its MyTH4-FERM cassette. However, the FERM domain of myosin-10 is nowhere near as well understood as other FERM domains, and the MyTH4 domain is almost completely mysterious.

In the current study, Hirano et al., have addressed these points using a combined structural and interaction analysis. In particular, they have studied the interaction of the MyTH4-FERM cassette with two of its binding partners, the netrin receptor, DCC, and microtubules. The authors MAP the microtubule binding site to the Myth4 domain, and find that while microtubules and DCC bind different parts of the cassette, their interaction is nevertheless mutually exclusive. They have also studied the interaction of a fragment of DCC with the MyTH4-FERM domain in considerable detail and show that it binds to s specific subdomain of FERM (subdomain C) which is quite different than the mode and site of target binding found for other FERM domains.

This study is thorough and the data are convincing. While a structural analysis of the interaction of the myosin-10 MyTH4-FERM domain with DCC was very recently published (Wei et al., 2011. Proc. Natl. Acad Sci USA 108:3572-3577) the current study is, simply put, better:

1. In the Wei et al study, the DCC peptide crystals were derived from fusions of the DCCP3 and aas 1503-2047 of human myosin-10. In the current study, in contrast, crystals were formed from separate proteins providing more confidence in the results.

2. In the Wei et al. study, only DCC-MyTH4-FERM interaction was studied and there was no analysis of microtubule binding as in the current study. This is particularly important in that a) to date, only a single report has shown microtubule binding by myosin-10 MyTH4-FERM and that was in 2004 and with a Xenopus myosin-10 (Weber et al., 2004. Nature 431:325-329). The current study not only confirms that result in a different myosin-10 but also MAPs the binding site to the MyTH4 domain and identifies the relevant residues.

3. Further, in the current study, it was shown that the interaction of the MyTH4-FERM domain with microtubules and DCC are mutually exclusive. This is particularly important as myosin-10 is now

reported to have several different binding partners, implying that under different cellular conditions, a different subset of proteins may be associated with myosin-10. This study represents the very first to study the interaction of myosin-10 with two different binding partners and sets the stage for other such studies.

4. In the current study, the crystallographic analyses of the MyTH4-FERM-DCC peptide interaction were directly supported with in vitro binding studies. Virtually no such data were presented in the study by Wei et al.

5. In the current study, a local conformational changes was detected in subdomain C upon DCC binding. This was missed by Wei et al.

In summary, it is likely that while Wei et al., may have published slightly sooner, the work by Hirano et al. is likely to be the one with more impact.

That being said, it would nevertheless be useful if the authors tested the generality of their findings against an additional binding partner. For example, the myosin-10 MyTH4-FERM domain is reported to bind integrin via interaction of the FERM domain with the NPXY motif of the integrin (Zhang et al., 2004. Nat. Cell Biol. 6:523-31). It would therefore be of great interest to know if the integrin peptide competes with DCC for binding to the MyTH4-FERM and to know if, like DCC, the interaction is mutually exclusive with microtubule binding. No structural analysis would be necessary for this analysis but it would nonetheless be extremely informative.

There are also several minor points that need to be addressed.

The Azoury et al., 2008 paper did not have any information concerning myosin-10
Supplementary figure S2--the third sequence is labeled xMyo-X but the legend says it is dMyo-X. As there is no Drosophila myosin-10 presumably this is Xenopus.
Pg. 6, line 5--Figure 1D should be 1C
Pg. 6, line 9--Figure 1C should be 1D
Pg. 9, line 17--Should not be PtdIns(4,5)P3 as this has a phosphate missing
Pg. 12, line 27--Figure 6E should be 5E
Pg. 13, line 2--Figure 6F should be 5F

Referee #2 (Remarks to the Author):

The manuscript by Hirano et al. reports the structure of the myosin X MyTH4-FERM domains in complex with the cytosolic P3 domain of the netrin receptor DCC. It is known that myosin X is a processive motor, which can transport its cargo molecules to specific cellular destination. A number of cargo molecules of myosin X have been reported including DCC, and it was found that MYTH4-FERM domain of myosin X binds to DCC. It is also known that myosin X plays an important role in spindle assembly during meiosis and consistently MYTH4-FERM domain binds to microtubules. The authors performed high resolution crystal structural analysis (2.55 A) of MYTH4-FERM domain of myosin X and provided an atomic structure of MYTH4-FERM domain with bound DCC peptide.

I found the work to be strong, in general, and the determined crystal structure of MYTH4-FERM domain of myosin X provides important structural information to understand the nature of interaction between the motor proteins having MYTH4-FERM domain and their specific cargo molecules.

A major concern is that the construct used in this study has 20AA deletion in the subdomain B of the FERM domain. The authors describe that MYTH4-FERM domain was degraded during purification to yield the two fragments, Frag-1 and Frag-2. They claim that these two fragments were co-purified and able to bind the DCC peptide. They also mention that the binding affinity of the deletion construct has the affinity to the DCC P3 similar to that of non-deletion construct, presumably the mixture of Frag-1 and Frag-2. Since the binding to DCC peptide is primarily determined by the subdomain C of the FERM domain, it is reasonable that the affinity is not much different between the deletion construct and non-deletion construct. Moreover, the binding affinity for DCC peptide is

compared between the degraded products of Frag-1 + Frag-2 and the 20 AA deletion construct, and it is anticipated that the former may also lose some amino acids by degradation. The authors should describe the predicted AA sequence of Frag-1 having molecular mass of 46,864. A critical question is if the deletion influences the overall structure of MYTH4-FERM or FERM. The authors need to address this point.

Quite recently, the atomic structure of MYTH4-FERM domain of myosin X with bound DCC P3 peptide was published. The work reported by Wei et al. (2011) is significantly overlapped with this manuscript. I believe that the present work is done independently, but it is desirable to DISCUSS the obtained results with the reports by Wei et al.

1st Revision - authors' response 05 April 2011

As to referees comments

Referee #1 *(Remarks to the Author):*

>That being said, it would nevertheless be useful if the authors tested the generality of their findings against an additional binding partner. For example, the myosin-10 MyTH4-FERM domain is reported to bind ***integrin*** *via interaction of the FERM domain with the* ***NPXY motif*** *of the integrin (Zhang et al., 2004. Nat. Cell Biol. 6:523-31). It would therefore be of great interest to know if the integrin peptide competes with DCC for binding to the MyTH4-FERM and to know if, like DCC, the interaction is mutually exclusive with microtubule binding. No structural analysis would be necessary for this analysis but it would nonetheless be extremely informative.*

As suggested we performed competition experiments with integrin. As we expected, DCC binding interferes integrin binding and also integrin binding interferes microtubule binding (page 13, bottom and Figures 5G and H). We speculate that myosin-X has two alternative functions of the primary motor function carrying cargos or a linker function linking an actin filament and a microtubule.

> There are also several minor points that need to be addressed.
>The Azoury et al., 2008 paper did not have any information concerning myosin-10

Thanks for pointing out and we deleted this reference.

>Supplementary figure S2--the third sequence is labeled xMyo-X but the legend says it is dMyo-X. As there is no Drosophila myosin-10 presumably this is Xenopus.

Yes, Xenopus is correct. We corrected the legend.

>Pg. 6, line 5--Figure 1D should be 1C
>Pg. 6, line 9--Figure 1C should be 1D
>Pg. 9, line 17--Should not be PtdIns(4,5)P3 as this has a phosphate missing
>Pg. 12, line 27--Figure 6E should be 5E
>Pg. 13, line 2--Figure 6F should be 5F

Many thanks to the referee for pointing out our mistakes. We corrected figure numbers and the phospholipid as PtdIns(3,4,5)P3.

Referee #2 *(Remarks to the Author):*

>A major concern is that the construct used in this study has 20AA deletion in the subdomain B of the FERM domain. The authors describe that MYTH4-FERM domain was degraded during purification to yield the two fragments, Frag-1 and Frag-2. They claim that these two fragments were co-purified and able to bind the DCC peptide. They also mention that the binding affinity of the deletion construct has the affinity to the DCC P3 similar to that of non-deletion construct, presumably the mixture of Frag-1 and Frag-2. Since the binding to DCC peptide is primarily determined by the subdomain C of the FERM domain, it is reasonable that the affinity is not much different between the deletion construct and non-deletion construct. Moreover, the binding affinity for DCC peptide is compared between the degraded products of Frag-1 + Frag-2 and the 20 AA deletion construct, and it is anticipated that the former may also lose some amino acids by

degradation. The authors should describe the predicted AA sequence of Frag-1 having molecular mass of 46,864. ***A critical question is if the deletion influences the overall structure of MYTH4-FERM or FERM.*** *The authors need to address this point.*

The observed molecular mass (46,864 Da) and N-terminal analysis indicate that Frag-1 corresponds to residues 1486-1888 (calculated 46,855 Da). Since Frag-2 corresponds to residues 1893-2058, only 4 residues (LRRS) are missing from our fragments. Thus, it is a reasonable assumption that Frag-1and Frag-2 form a complex similar to the native protein (Figure S1 legend).

In our purification, we failed to separate the non-fragmented protein from the Frag-1+Frag-2 complex. However, all our protein samples of non-fragmented protein contaminated with different amounts of Frag-1+Frag-2 exhibit a similar affinity to the DCC peptide, suggesting the non-fragmented protein and the co-purified Frag-1+Frag-2 complex possess a similar affinity. Therefore, our protein with 20aa-deletion is expected to have the affinity similar to the non-fragmented protein from the non-deletion construct.

Moreover, in the recent PNAS paper, Wei *et al*. prepared a non-degraded protein of the myosin-X MyTH4-FERM cassette from a slightly different non-deletion construct (residue 1503–2047) and also a protein having 36-residue deletion (1871-1906). Based on comparison of their ITC data, they found that this deletion has no impact on the DCC-binding affinity.

All these data support that our 20-AA deletion of the non-conserved flexible loop is not important on DCC binding. Nevertheless, this does not exclude a small possible change in the conformational pliability or the fine structure of the cassette or the FERM domain. At present, we did not detect any significant structural or functional perturbation in our study.

>Quite recently, the atomic structure of MYTH4-FERM domain of myosin X with bound DCC P3 peptide was published. The work reported by Wei et al. (2011) is significantly overlapped with this manuscript. I believe that the present work is done independently, but it is desirable to DISCUSS the obtained results with the reports by Wei et al.

See above our response to the Editor's comment.

Referee #3:

A potentially interesting aspect of this work is the competitive binding experiments presented in Fig. 5. MyTH-FERM binding to the acidic tubulin tail is completely abolished by the addition of DCC-P3 peptide (at 1:1 molar ratio with the MyTH-FERM; Fig. 5E). This is unexpected because of the apparently distinct binding sites for the two peptides, although they might be near to each other, and the lack of any significant change in the overall crystal structure of the MyTH-FERM when DCC-P3 is bound. In contrast, DCC-P3 does not significantly inhibit the binding of microtubules themselves to the MyTH-FERM, possibly because of larger binding surface of the microtubules.

> It should be noted that it is difficult to assess the extent of tubulin binding inhibition due to absence of quantification of the binding in the different samples - loss of MyTH-FERM binding to tubulin in the presence of DCC-P3 is not obvious in the gels presented especially when the bands in the pellet are smeared and there are variable amounts of tubulin in the sups and pellet to begin with.

The binding assay was re-examined with constant amounts of tubulins but without smeared bands. The result was presented in the new figure 5F with a quantitative analysis that clearly shows loss of MyTH4-FERM binding to microtubules in the presence of the DCC P3 peptide.

>However, the results do raise the question of whether the competition between the acidic tail and DCC-P3 is due to some unusual behavior of the tubulin and/or DCC-P3 peptides or if the results could be revealing a conformational state that is not seen in the crystal - a possibility that is certainly worth further study.

Our present data suggest no significant changes in the overall structure on DCC binding. However, the acidic tail or microtubule bindings may induce some induced-fit changes in the overall structure. We also speculated that negatively-charged residues of the N-terminal flanking region of the DCC P3 peptide may cause electrostatic repulsion with the tubulin acidic tails and the negatively-charged surfaces of microtubules. We know that integrin b also has such negatively-charged residues at the N-terminal flanking region of the NPXY motif. These are indeed interesting questions in our next structural and biophysical studies.

Minor comments -

>The reference to Azoury et al. (pg. 4) for the role of M10 in spindle assembly might not be appropriate based on the text. That paper studied the role of actin in spindle positioning but not that of M10 - perhaps the authors intended to say that both actin and M10 contribute to this process, in which case the citation would make sense.

The reference may be misleading and was deleted from the text.

>Reference should also be made to the nice work of Toyoshima & Nishida (EMBOJ, 2007) on the role of M10 in integrin-dependent spindle orientation.

We added this interesting reference in the text (page 4).

>There are a few places in the manuscript where the authors refer the reader to the wrong figure (top of pg 6, bottom of pg. 12, top of pg. 13).

We corrected these mistakes.

>There is no explanation for the region circled in yellow in Fig. 2B. Presumably this is the positively charged area discussed in the manuscript - this should be explained in the figure legend.

The circle indicates the positively-charged patch, which is shown to be the microtubule binding site. We added this explanation in the legend.

>The legend for Fig. 2C indicates that the Ub is shown in dark grey when it appears to be pink.

We corrected this mistake.

>Supp Fig. 2 is a useful alignment of M10 MyTH-FERM sequences. A clear marker should be placed at the location of each of the indicated numbers in the alignment so the reader can more easily find numbered residues of interest.

We put a dot to indicate the location of each residue number.

2nd Editorial Decision 30 April 2011

Thank you for submitting your revised manuscript
I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal pending the following small additions:

1) Like for figure 5 F, please also add quantification bar charts for fig 5E, G and H.
I also think the discussion in response to ref 3 (p6 author response) should be added to the paper.
You must also indicate what the error bars in fig 5F denote in the figure legend, including how often the experiment was repeated independently. The same will apply for the quantifications to 5E, G and H.
2) Please add the discussion made in response to ref 3 on page 6 of your rebuttal to the discussion (in abbreviated form if you prefer).

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

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - authors' response 03 May 2011

Please find enclosed our revised manuscript entitled "**Structural basis of cargo recognition by the myosin-X MyTH4-FERM domain**" by Hirano *et al.*, for publication in *The EMBO Journal.*

We added quantification bar charts for the data of figures 5E, G and H. The figure legends contain description of error bars, which are standard deviations of each mean value from three

independent experiments. Also, we added our discussion about possible mechanisms of the binding interference (in response to referee #3) in DISCUSSION (page 16, 6 lines at the bottom).

We thank you for your kind cooperation for swift publication of our work.

Sincerely yours,

