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Structure of a dominant-negative helix-loop-helix transcriptional regulator suggests mechanisms of autoinhibition

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1st Editorial Decision 06 June 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees find the structure of HHM and its activation to be potentially interesting. However, the referees raise a number of major issues, these concern the HHM mutant and functional data and also the consideration of an alternative model for binding to a target. Both these concerns are important and central to the main conclusions of the study and must be satisfactorily addressed before the manuscript can be further considered for The EMBO Journal. Nevertheless, given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

bHLH protein are an extremely important class of transcription factor, that usually binds to DNA as homo or heterodimers. The ID class of proteins are HLH proteins that lack the DNA-binding basic domain and act as dominant negative regulators of bHLH proteins. The authors of this paper have managed to finally determine the structure of an ID protein, in what they believe is an autoinhibited inactive state. They also propose a model for activation based on major conformational changes to bring about binding to bHLH targets, and test this model using molecular dynamics simulations and mutagenesis.

The structure itself is very interesting, obtained after having to take a number of innovative steps to determine the structure to 2.5 angstrom resolution (good for something of this size). The structure determination work seems solid and the data are interpreted at an appropriate level for the resolution obtained. The positioning of the HLH domain, where each helix contributes to a different 5-helix bundle in the V-shaped monomer, is very different to the structure expected for a "typical" bHLH dimer. The authors suggest that on binding to target bHLH proteins, the N and C bundles must bind each other to compensate for exposed hydrophobics, which is supported to some extend by molecular dynamics simulations which show some movement in the direction predicted over the time course of the simulations.

Is it possible to run the MD simulations for longer and/or at higher temperatures to take this further or does everything just unfold?
Why is it not possible for the 5-helical bundle to rearrange to make a four helical bundle?

The authors then make a couple of mutants designed to disrupt the interactions between the HLH and N/C bundles and test them for binding to protein targets (bHLH, another ID proteins and a cyclin D protein) using co-IP experiments) and then in a differentiation assay. These experiments don't convince me of the model because there is no structural/biophysical characterization of the mutants. I might be more convinced if the mutants were shown to be folded and monomeric and not just binding to everything presented to them because they are misfolded and/or sticky.

Would also like to see some data showing that the protein is a monomer in solution (e.g. Analytical ultracentrifugation or MALLS or SAXS)and some discussion about crystal contacts - as it is quite important for the message that the putative autoinhibited state is a monomer.

minor comments;
The introduction seems overly long

Order of the figures is out of synch - Fig 1 is followed by fig 3C then 2B in the text

Would like to see the positions of the mutated residues (Cys198/300) on a structure.

The title slightly overstates the case - might be better as something like:
"Structure of a dominant-negative helix-loop-helix transcriptional regulator suggests mechanisms of autoinhibition"

Referee #2

Seto et al. (Nureki) EMBO J.

The authors report the first structure of a free-standing dominant negative HLH protein, HHM. On this basis, they then predict a docking model of Olig1-bHLH with the HLH segment of HHM, and associated conformational changes. Finally, they then evaluate the effects of two HHM mutations that are predicted to weaken the interactions between HLH and the N and C bundles, on the interaction of HHM with HLH proteins and D-type cyclin, and on myogenic differentiation.

Based on my background, I am not well positioned to evaluate the structural aspects, but rather will focus on Fig. 6 and 7. Unfortunately these data are very minimal, and could easily be more expanded to better address the message that the authors want to convey. Besides being too minimal, they also have substantial problems.

- The data in Fig. 6 do not allow any conclusion and need to be redone. All bands shown on gel, with a few exceptions, are strongly overexposed, thus not allowing quantification of the interaction. In addition, the expression of the tagged proteins is not equal in each of the panels, although this is somewhat obscured by the overexposure. The quantification was derived from measuring the surface of the (overexposed) protein band, which is unorthodox but is maybe the only way one can do it when the gels are so overexposed. Instead, the authors should have much less intense/exposed gel bands that allow them to quantify band intensity (and not surface). The conclusions of this experiment should await the new data.

- Fig. 7 and associated text: The authors make a conclusion about the time course of differentiation, and state that the mutants accelerate the induction of myosin heavy chain expression, but no time course is shown. All data are shown for only one time point. Therefore, I do not see the basis for this conclusion. Furthermore, the differentiation of wt and N169E HHM is quantitatively similar. So, how can one then state that the differentiation is higher for the N169E mutant? The authors then conclude that the two mutants appear to perturb differentiation, although it is unclear what is meant, unless they merely refer to the increased differentiation. Finally, the authors state that this effect of the mutants may occur through binding to Ids, leading to reformation of E12/47-MyoD complexes, but have no data to show, even though these are very doable experiments.

In conclusion, with respect to Fig. 6 and Fig. 7, more work is needed and the conclusions may need to be re-evaluated.

Referee #3

This is a very interesting, and unexpected structure for a putative HLH protein. It is therefore worthy of publication in EMBO. The paper should be of general interest because of the importance of bHLH transcription factors, and the role played by inhibitory factors like the Id sub-family in modulating transcriptional activity. Unlike Id, which interacts with ubiquitously expressed class I bHLH factors, HHM interacts with class II bHLH proteins and therefore has cell-specific or path-specific effects. The main question I have is whether the structure is interpreted correctly. The authors take as given that the putative HLH domain of HHM will interact with the HLH of the bHLH factor Olig1. Since the HLH domain of HHM is interacting with other helices in their structure, they interpret the current structure as auto-inhibited. The structure suggests the possibility of an alternative model, namely that the HLH domain of Olig1 might bind to the V-shaped HHM structure instead of the canonical HLH dimer. Since the authors do not provide any functional data supporting autoinhibition of HHM, I believe they should address the possibility of this alternate model.

Comments:

Have the authors considered the alternate hypothesis, that the HLH of Olig1 interacts with HHM in the conformation in their crystal? In that line of thinking:

1) Is there any functional evidence for autoinhibition? It appears from all of the examples given in the paper that HHM readily interacts with Olig1.

2) On p. 14 the authors refer to (Ikushima et al, 2008) as demonstrating that "the HLH region of HHM exclusively interacts with the HLH region of the class II bHLH transcription factor, Olig1..." In Supplement Figure 5, Ikushima, et al show GST pull-down assays with different deletions of Olig1 and HHM. The deletion mutants of HHM were not of the putative HLH domain exclusively (Supplementary Figure 5E), but the N-domain plus the HLH, or the C-domain plus the HLH. It is therefore possible that the HLH of Olig1 spans binding to both the N- and C-domains of HHM in the V-shaped structure.

3) Could the binding studies in Figure 6 be interpreted in terms of changes in flexibility between the N- and C-bundles of HHM that might facilitate binding of the HLH domain of Olig1?

4) Did you look at the surface of the HHM structure, eg the electrostatic potential, to see if there was any possible binding site for the HLH of Olig1?

5) Is this structure homologous to any known structure? If so, is there anything to learn from homologous structures?

Specific comments:

6) p. 4 - does "inhibition of ETS1 by Id" or "indirect inhibition of Rb by Id1.." imply direct protein-protein interactions or do you mean suppression of gene expression? To me the word inhibition implies direct interactions with the protein and not the gene."

7) Table I and Table II: indent the sub-headings. For example under "Phasing power", indent "Iso" and "Ano".

8) Table II, the Average B-factor is 99.6. This value is high - is it really the average? Is this with or without the TLS refinement?

9) TLS refinement - somewhere in the paper provide more details about the TLS refinement. Show that TLS refinement helped lower the R-factor and R-free. Was the whole structure treated as one domain, or were the N- and C-terminal domains assigned different TLS values? The TLS domains should make some physical sense.

10) p. 9, line 12: In the paragraph starting: "Previous studies suggested that the acidic domain and the putative Leu zipper (LZ) motif follow the HLH motif..." - specify the residue numbers of the acidic and LZ domains in the text. Add these sequences to the table in Figure 3C.

11) p. 9, line 17: "Leu and Ser residues in this putative LZ motif..." - specify the residue numbers of the Leu and Ser residues. Are these residues shown in Figure 2B or some Figure?

12) p. 10, line 4: in place of (Fig. 2D), specify (Fig 2C, 2D), since N169 is shown in Fig. 2C.

13) p. 10 paragraph starting: "This conserved NKAAA motif reinforces..." - you could put some of the details of the interactions in the figure legend.

14) p. 11, line 7: states that the "C-terminal halves of helices H2 form a coiled-coil structure (Fig. 3B)". I thought HHM bound to class II bHLHs that do not have coiled-coils. Does HHM bind class III and class IV bHLHs with coiled coils?

15) p. 11 and 12- you discuss residues that pack in a canonical bHLH, and state that these residues "...participate in the hydrophobic core formation with the N and C bundles." It would be helpful to have a figure showing this comparison in detail, to see how the HLH domain of HHM is oriented in your structure. Figure 3C shows which residues form the hydrophobic core of canonical bHLH proteins. Where are these residues in the HLH domain of HHM? Are they packed against the N- and C-domains or are they solvent exposed?

16) p. 13, lines 18-19: compares HHM to Myc and Max. Shouldn't HHM be more like E47 since it is supposed to bind class II bHLH's. Why compare it to Myc and Max, which have leucine zipper that dominate dimerization activity.

17) Figure 4A - your model of HHM/Olig1 is atypical for bHLHs. As far as I know, bHLH partners have about the same length of Helix2. In your model alpha6 is much longer than H2. In addition, the basic domain of H1 would be disordered if it is not bound to DNA. This should be stated somewhere at least.

18) p. 17 I found it difficult to interpret the mutations you made in HHM.
i) First of all they were made in the HLH region, so they could easily affect interactions between HLH domains as well as interactions with the N- and C- bundles. Why not make mutations in alpha4 or alpha7 that would disrupt the N- and C-bundles but not the HLH?
ii) These mutations were fairly conservative and not so disruptive. Did you make other mutations too? Did they fit your model?

19) p. 17, last paragraph. The experiment of myogenic differentiation has too many variables to use as evidence for your model. Would you expect HHM to bind MyoD as well as Id? Could it be that wt HHM is more effective at binding MyoD than the mutants (instead of less effective at binding Id), and so reduces the number of differentiated cells more?
There are some controls missing from your experiment. You suggest that the point mutants of HHM affect the "time course" of myogenic differentation. You don't show a time course, only one snapshot. Is it possible that HHM affects the total number of cells that differentiate and not the rate of differentiation? How efficient was your adenovirus transfection - did you measure that?

20) Figure 7B: The stars represent significance, compared to what? I don't think it is compared to the LacZ sample. There is no control showing the amount of immunostaining without transfecting any plasmid. Also, does the LacZ sample show significantly less differentiation compared with the no plasmid control? Why would that be?

21) Figure 2: It would be helpful if you labeled the N- and C-termini of the helices in all of the figures. Also Figure 2C is oriented backwards from the other figures. Switch it so the N-bundle is on the right and the C-bundle is on the left.

22) Figure 3D and E: specify that H1 is colored orange, and H2 is colored purple. Again, why compare HHM with Myc and Max instead of class I and II bHLHs? It makes the most difference in the comparison at step V, since Myc and Max have a leucine zipper.

23) Figure 5 C and D: the Run B structure should be red as in parts A and B.

1st Revision - authors' response 21 September 2011

Thank you very much for your kind invitation to submit a revised version of our manuscript. We were delighted to see the generally supportive comments by the referees, and we appreciate their suggestions, which have significantly improved the manuscript. In light of their comments and suggestions, we have revised the manuscript as follows.

First of all, according to the suggestions by Referee #1, we performed analytical ultracentrifugation (AUC) experiments of wild-type HHM and its mutants, to investigate the dynamics of the HHM molecule in solution (also related to the comments by Referee #3). Intriguingly, the results of the sedimentation velocity and sedimentation equilibrium experiments of wild-type HHM showed that the HHM molecule exists as a monomer in slow equilibrium between the V-shaped conformation and the partially-unfolded, relaxed conformation, in which the N bundle, HLH region and C bundle are flexibly linked, facilitating the access to its specific transcription factor. Moreover, the

sedimentation velocity experiments also revealed that, in the HHM mutants (P166Y and N169E), the equilibrium is shifted to the relaxed conformation, as expected from their designs. These results strongly support our previous findings that the present V-shaped crystal structure is a snapshot of the autoinhibited form for transcription factor specificity, and that the relaxed conformation may facilitate the formation of the canonical HLH active dimer with transcription factors, which explains how the mutants compromised transcription factor specificity *in vitro* and *in vivo*. Therefore, we have shifted the discussion and the figure (the previous Figure 5) of the MD simulation to the Supplementary Information, and replaced them with a new chapter about the conformational dynamics of HHM, accompanied by the new Figure 5, describing the biochemical and physicochemical analyses and the bi-conformational equilibrium of HHM.

The comments by referee #1.

Q1

Is it possible to run the MD simulations for longer and/or at higher temperatures to take this further or does everything just unfold?

We extended the length of simulation runs A and B up to 100 ns. No structural changes from the final snapshot in the initial version of the manuscript (*i.e.* 40 and 60 ns) were observed, suggesting that the structures were converged at those points. Based on the results, we updated Supplementary Figure S4 (previous Figure 5) and the Supplementary Discussion.

Q2

Why is it not possible for the 5-helical bundle to rearrange to make a four helical bundle?

We stated that two of the three simulations (runs A and C) resulted in the eight-helix bundle. As shown in Supplementary Figure S4C (previous Figure 5C), the final snapshot of run B did not form the eight-helix bundle, but an elongated four-helix bundle, which may correspond to the form that the referee mentioned. Moreover, as stated above, we shifted the figure and the discussion of the MD simulation to the Supplementary Information, and replaced them with the new Figure 3 and the discussion of the conformational dynamics of HHM (pp. 13–15).

Q3

The authors then make a couple of mutants designed to disrupt the interactions between the HLH and N/C bundles and test them for binding to protein targets (bHLH, another ID proteins and a cyclin D protein) using co-IP experiments) and then in a differentiation assay. These experiments don't convince me of the model because there is no structural/biophysical characterization of the mutants. I might be more convinced if the mutants were shown to be folded and monomeric and not just binding to everything presented to them because they are misfolded and/or sticky.

The wild-type and mutant HHM proteins exhibited the same behaviors in the gel filtration analyses. We then analyzed the HHM mutants (P166Y and N169E) by AUC. The results showed that neither the wild-type nor mutant HHM proteins formed aggregates. Furthermore, the wild-type HHM exists in slow equilibrium between the V-shaped and relaxed conformations, whereas the two mutants mainly adopt the relaxed conformation. We have added the results and discussion of the AUC analyses in a new section of the main text (pp. 13–15).

Q4

Would also like to see some data showing that the protein is a monomer in solution (e.g. Analytical ultracentrifugation or MALLS or SAXS) and some discussion about crystal

contacts - as it is quite important for the message that the putative autoinhibited state is a monomer.

As for the crystal packing, there is no crystal contact that is possibly involved in the dimer formation. Furthermore, as stated above, we analyzed the wild-type HHM by sedimentation equilibrium experiments, and found that HHM exists as a monomer in solution (accordingly, we have modified the main text; p.14, l.8 – p.15, l.13).

Q5

The introduction seems overly long

We have shortened the introduction by removing the unrelated descriptions, as the referee suggested.

Q6

Order of the figures is out of synch - Fig 1 is followed by fig 3C then 2B in the text

We have removed the reference to Figure 3C (the present Figure 4C; p.8, l.3), since the sequence similarity is not a main point in this section. The sequence similarity and the comparison shown in Figure 3C (the present Figure 4C) are intensively discussed in the subsequent sections (p.11–13).

Q7

Would like to see the positions of the mutated residues (Cys198/300) on a structure.

We have added a new figure showing the mutated residues (Supplementary Figure S1).

Q8

The title slightly overstates the case - might be better as something like…

We changed the title as the referee suggested.

The comments by referee #2.

Q1

– The data in Fig. 6 do not allow any conclusion and need to be redone. All bands shown on gel, with a few exceptions, are strongly overexposed, thus not allowing quantification of the interaction. In addition, the expression of the tagged proteins is…

According to this suggestion, we repeated these experiments and have presented the data in the new Figure 6. Band intensities (IP:FLAG, IB:Myc) were quantified under unsaturated conditions and calibrated by the amounts of the inputs (IB:Myc) and HHM/HHM mutants (IP:FLAG, IB:FLAG).

Q2

– Fig. 7 and associated text: The authors make a conclusion about the time course of differentiation, and state that the mutants accelerate the induction of myosin heavy chain expression, but no time course is shown. All data are shown for only…

According to the reviewer's suggestion, we included the data at 36 h after the induction of differentiation (medium change). We saw a tendency of enhanced myogenic marker expression in

the two mutants, although it was not statistically significant. In the new figure, the 36 h data and 48 h data are derived from the same set of experiments. We omitted the 36 h data in the original manuscript because the difference was not statistically significant. We agree that the values of the WT and N169E samples appear to be similar. However, we performed a statistical analysis by the Tukey-Kramer test. Forty-eight hours after the induction of differentiation, the MHC expression in the N169E sample was significantly higher than that of the LacZ control, while that of the WT sample was not. We revised Figure 7B to clarify the sample sets that are compared in the test, and enhanced the description in the Legend to Figure 7B.

Q3 *The authors then conclude that the two mutants appear to perturb differentiation, although it is unclear what is meant, unless they merely refer to the increased differentiation.*

We just intended to say that normal myogenic differentiation was affected by the HHM mutants. To clarify this point, we now avoid the use of "perturb", and simply mention that the mutants "accelerate myogenic differentiation".

Q4 *Finally, the authors state that this effect of the mutants may occur through binding to Ids, leading to reformation of E12/47-MyoD complexes, but have no data to show, even though these are very doable experiments.*

We tried to detect an increased amount of the E12/47-MyoD complex in MHC expressing cells, using the *in situ* PLA system. We used an anti-E12/47 antibody (SC763, Santa Cruz) in combination with an anti-MyoD antibody (ab16148, abcam), but could not successfully detect the complex, probably due to experimental difficulties. As described in the main text, we agree that the Id-mediated mechanism is just a possibility. Therefore, we ask the referee to allow us not to demonstrate this experimentally.

The comments by referee #3.

Q1

Is there any functional evidence for autoinhibition? It appears from all of the examples given in the paper that HHM readily interacts with Olig1.

Our new biochemical GST pull-down analysis, following the post-translational separation of the GST-tagged N bundle, HLH linker and C bundle, showed that the HLH region and the C bundle were not co-precipitated with the N bundle, which suggests that the interactions between the HLH region and the N and C bundles are not permanent, but transient. Further analytical ultracentrifugation experiments revealed that both the wild-type and mutant HHM proteins are monomers, and the wild-type HHM exists in slow equilibrium between the V-shaped and relaxed conformations. In contrast, the mutants destabilizing the V-shape mainly adopt the relaxed conformation, in which the N bundle, HLH region and C bundle are flexibly linked, facilitating the access to transcription factors. Therefore, we have concluded that the present static V-shaped crystal structure represents the autoinhibited form.

Q2

On p. 14 the authors refer to (Ikushima et al, 2008) as demonstrating that "the HLH region of HHM exclusively interacts with the HLH region of the class II bHLH transcription factor, Olig1..." In Supplement Figure 5, Ikushima, et al show GST…

There is misunderstanding in the referee's interpretation of our previous paper (Ikushima et al., 2008). The results of the pull-down experiments showing that the HLH region of HHM exclusively interacts with Olig1 were shown in Supplementary Figures 5C and D, and not 5E in our previous paper. The schematic drawings in Figure 5E show the constructions used in the luciferase assay, and are not related to these pull-down experiments.

Q3

Could the binding studies in Figure 6 be interpreted in terms of changes in flexibility between the N- and C-bundles of HHM that might facilitate binding of the HLH domain of Olig1?

Our new analytical ultracentrifugation experiment revealed that the mutants adopt the relaxed conformation, in which the N bundle, HLH region, and C bundle are flexibly linked, and no longer retain the V-shape, facilitating the access to Olig1, Id2 and NeuroD1. Therefore, the results in Figure 6 could be interpreted as the conformational equilibrium shift towards the relaxed form, as described above, rather than the flexibility in the scaffold of the V-shape.

Q4

Did you look at the surface of the HHM structure, eg the electrostatic potential, to see if there was any possible binding site for the HLH of Olig1?

We checked the surface electrostatic potential of HHM, but it seems difficult to predict the Olig1-binding site (shown in new Supplemental Figure S3A). Since the interaction between HHM and Olig1 should be based on a hydrophobic interaction, we mapped the surface hydrophobic residues on the molecular surface of HHM (Supplemental Figure S3B). This figure shows there is no hydrophobic cluster on the surface of the present crystal structure, suggesting that some structural reorganization should occur when HHM interacts with Olig1. We mentioned this point in the main text (p.10, l.23– p.11, l.3).

Q5

Is this structure homologous to any known structure? If so, is there anything to learn from homologous structures?

We performed a DALI search with the present crystal structure, and did not find any proteins with overall structural similarity to HHM.

Q6

p. 4 - does "inhibition of ETS1 by Id" or "indirect inhibition of Rb by Id1.." imply direct protein-protein interactions or do you mean suppression of gene expression? To me the word inhibition implies direct interactions with the protein and not the gene.

We have deleted the description from the introduction, since the detailed explanation of the Id family is not directly related to the present study.

Q7

Table I and Table II: indent the sub-headings. For example under "Phasing power", indent "Iso" and "Ano".

We modified Tables I and II as the referee suggested (p. 26).

Q8

Table II, the Average B-factor is 99.6. This value is high - is it really the average? Is this with or without the TLS refinement?

The *B*-factor produced by the phenix.refine program includes the contribution from the TLS parameters (i.e. U_{TLS} + $U_{individual}$), so the value has a tendency to become higher than that without the TLS refinement.

Q9

TLS refinement - somewhere in the paper provide more details about the TLS refinement. Show that TLS refinement helped lower the R-factor and R-free. Was the whole structure treated as one domain, or were the N- and C-terminal domains assigned different TLS values? The TLS domains should make some physical sense.

We treated the N- (1-167) and C-terminal (168-360) regions as independent TLS groups. As described in the Supplementary Discussion, the results of the MD simulation indicated the bending motion between the N- and C-terminal regions. Therefore, this TLS-group assignment seems to be physically reasonable. We have added the description of the TLS refinement in the Materials and Methods (p.21), as the referee suggested.

Q10

p. 9, line 12: In the paragraph starting: "Previous studies suggested that the acidic domain and the putative Leu zipper (LZ) motif follow the HLH motif..." - specify the residue numbers of the acidic and LZ domains in the text. Add these sequences to the table in Figure 3C.

The previous Figure 3C (the new Figure 4C) describes the amino acid sequence similarity among the HLH proteins. Since the acidic region and the LZ motif are far downstream from the HLH region, we cannot include them in this figure. Instead, the locations of the acidic and LZ regions are indicated in the schematic drawing of the primary structure of HHM in Fig. 1, which we believe is sufficient for the readers' understanding.

Q11

p. 9, line 17: "Leu and Ser residues in this putative LZ motif..." - specify the residue numbers of the Leu and Ser residues. Are these residues shown in Figure 2B or some Figure?

The residue numbers of the putative LZ motif have been added in the main text (p.8, l.16). These residues are also shown in Supplementary Figure S2. (The description of the LZ motif in the submitted version is based on the amino acid sequence of mouse Maid. The Ser residue in mouse Maid is changed to Cys in the human HHM, and so we corrected the description.)

Q12

p. 10, line 4: in place of (Fig. 2D), specify (Fig 2C, 2D), since N169 is shown in Fig. 2C.

We have modified the main text as the referee suggested (p.9, l.10).

Q13

Is p. 10 paragraph starting: "This conserved NKAAA motif reinforces..." - you could put some of the details of the interactions in the figure legend.1.

We have modified the figure legend of Figure 2D, as the referee suggested (p.28).

Q14

p. 11, line 7: states that the "C-terminal halves of helices H2 form a coiled-coil structure (Fig. 3B)". I thought HHM bound to class II bHLHs that do not have coiled-coils. Does HHM bind class III and class IV bHLHs with coiled coils?

We have modified the main text and figures to replace the wording “coiled-coil” with “two helix-bundle”, as the referee suggested (p. 11 and Figure 4).

Q15

p. 11 and 12- you discuss residues that pack in a canonical bHLH, and state that these residues “...participate in the hydrophobic core formation with the N and C bundles.” It would be helpful to have a figure showing this comparison in detail…

The hydrophobic residues, which are packed against the N and C bundles in the V-shaped crystal structure, are shown in Figure 2A and B. Therefore, we have corrected the reference to the figures in the above sentence from Figure 1B to 2A and B (p.10, l.15). In addition, we have added the residue numberings in Figure 2A and B for comparison.

Q16

p. 13, lines 18-19: compares HHM to Myc and Max. Shouldn’t HHM be more like E47 since it is supposed to bind class II bHLH's. Why compare it to Myc and Max, which have leucine zipper that dominate dimerization activity.

The interaction interface of HHM for bHLH should be similar to that of the class I factors, as the referee pointed out. However, the main subject here is the structure of the loop region that is not involved in the interaction interface. The amino acid sequence of the loop region of HHM (NKAA) is more similar to those of v-Myc (EKAA) and Max (EKAS) than to that of E47 (KAQT), and thus we described that the structure of this region may be similar to those of v-Myc and Max. Therefore, we propose that the HLH region of HHM is a hybrid of the v-Myc-like loop region and the E47-like helix regions (H1 and H2). To clarify this point, we have modified the main text (p.12, l.20 – p.13, l.6).

Q17

Figure 4A - your model of HHM/Olig1 is atypical for bHLHs. As far as I know, bHLH partners have about the same length of Helix2. In your model alpha6 is much longer than H2. In addition, the basic domain of H1 would be disordered if it is not bound to DNA. This should be stated somewhere at least.

We have added the description of the structure of the helix H2 C terminus and basic region in the main text (p.13, l.8–11), as the referee suggested. In addition, we have modified Figure 4A to emphasize this point.

Q18

p. 17 I found it difficult to interpret the mutations you made in HHM…

According to the referee’s comment, we newly mutated Val271 and Val288 on a8 to Phe and Arg, respectively. These mutants are expected to destabilize the interactions between the HLH region and C bundles. These two mutants were overproduced in the soluble fraction, and were purified in the same manner as the wild-type. The previous two mutants, P166Y and N169E, exhibited stronger affinity with Id2 and NeuroD1 as well as with Cyclin D1. Therefore, these two mutations on a7 may shift the conformational equilibrium to the relaxed conformation to access non-specific transcription factors, in a similar manner to the previous P166Y and N169E mutations on the HLH region. In terms of the mutant selection, the P166Y and N169E mutations shifted the conformational equilibrium to the relaxed conformation, probably due to the destabilization of the V-shaped conformation, which was verified by our new analytical ultracentrifugation experiment, and they are now considered to be the ideal mutations to support our new model (Figure 5B).

Q19

p. 17, last paragraph. The experiment of myogenic differentiation has too many variables to use as evidence for your model. Would you expect HHM to bind MyoD as well as Id? Could it be that wt HHM is more effective at binding MyoD than…

We found that neither the wild-type HHM nor HHM mutants interacted with MyoD. Thus the Id-mediated mechanism, which we present, appears more likely.

There are some controls missing from your experiment. You suggest that the point mutants of HHM affect the "time course" of myogenic differentation. You don't show a time course, only one snapshot.

According to the reviewer's suggestion, we included the data at 36 h after the medium change, showing similar results. In the new figure, the 36 h data and 48 h data are derived from the same set of experiments. We omitted the 36 h data in the original submission, because the difference was not statistically significant.

Is it possible that HHM affects the total number of cells that differentiate and not the rate of differentiation?

Under the conditions used, almost all of the cells undergo differentiation, although they are not completely synchronized. We thus think that HHM affects the rate of differentiation.

How efficient was your adenovirus transfection - did you measure that?

Under the conditions used, b-galactosidase activity was detected in 100% of the LacZ-infected cells.

Q20

Figure 7B: The stars represent significance, compared to what? I don't think it is compared to the LacZ sample. There is no control showing the amount of immunostaining without transfecting any plasmid…

We compared the HHM infected samples to the LacZ sample by the Tukey-Kramer test. We described this in the legend to Figure 7B.

In comparison to the LacZ sample with the no-infection control, the cell differentiation was delayed in the LacZ sample. We also observed a differentiation delay in cells infected with the empty adenovirus vector. We concluded that the delay in differentiation occurs by the adenovirus infection, although the precise mechanism is unclear at present. We thus compared the rates of differentiation among adenovirally-infected samples.

Q21

Figure 2: It would be helpful if you labeled the N- and C-termini of the helices in all of the figures. Also Figure 2C is oriented backwards from the other figures. Switch it so the N-bundle is on the right and the C-bundle is on the left.

We have added the arrows indicating the directions of the helices in Figure 2. As for Figure 2C, we would prefer not to change the viewing direction, since the loop of the HLH region hides the interaction between the N and C bundles, which is the main point of this figure.

Q22

Figure 3D and E: specify that H1 is colored orange, and H2 is colored purple. Again, why compare HHM with Myc and Max instead of class I and II bHLHs? It makes the most difference in the comparison at step V, since Myc and Max have a leucine zipper.

We have modified Figure 3D and E to color H1 and H2 accordingly, and to show the interaction interface between NeuroD1 and E47, as the referee suggested.

Q23

Figure 5 C and D: the Run B structure should be red as in parts A and B.

We have modified Supplementary Figure S4 (previously Figure 5) as the referee suggested.

In addition, we changed the order of the authors, considering their contributions to this revision.

2nd Editorial Decision 21 October 2011

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. I have now received the final report from the three original referees who evaluated your study and I enclose their comments below. As you will see from their comments, the concerns regarding the structural aspects of the work have been satisfactorily addressed, however, referee #2 finds that additional analysis is still required for the biological data to support the importance of the proposed mechanism. It is the policy of The EMBO Journal to only allow a single round of revision, however, given the positive support of two referees and the willingness of referee #2 to look at the manuscript once more means that we are willing to allow a second round of review in this case. However, it is important that the remaining issues raised by referee #2 are addressed prior to publication, if this cannot be done within this round of revision we will not be able to proceed further with publication. Nevertheless, at this stage we would like to invite you to submit a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

I think the authors have done a good job in assessing my concerns with the original manuscript. I do like that the MD simulations have moved into the supplementary data to make room for the analytical centrifugation studies.
A couple of very minor points about that new data though - in the results sections where the sedimentation equilibrium data are presented please cite the theoretical molecular weight in the text next to the molecular weight estimated by AUC. In the methods section, watch the SI units (h not hrs) and note the formatting of V-bar isn't correct in the pdf.

Overall I'm not fully convinced that the model presented for the different states is correct, but I think it will take some structures of the hetero-complexes to convince me. But, I do think that the authors have provided enough data for me to accept that it may be feasible, which I think is sufficient for publication. To me the focus is the structure itself and I do think it is a very interesting structure for an important class of proteins.

Referee #2 (Remarks to the Author):

Ishii et al. (Nureki) EMBO J.

The authors report the first structure of a free-standing dominant negative HLH protein, HHM. On this basis, they then predict a docking model of Olig1-bHLH with the HLH segment of HHM, and associated conformational changes. Finally, they then evaluate the effects of two HHM mutations that are predicted to weaken the interactions between HLH and the N and C bundles, on the interaction of HHM with HLH proteins and D-type cyclin, and on myogenic differentiation.

Based on my background, I am not well positioned to evaluate the structural aspects, but rather will focus on Fig. 6 and 7. I thank the authors for responding to these comments. Consequently new data were provided for Fig. 6 and Fig. 7. Unfortunately these data remain minimal, and could easily be better more expanded to better address the message that the authors want to convey. Unfortunately, they are even too minimal to support the conclusions, and, consequently, the conclusions are not sufficiently supported by the data. I seems that the authors tried to present the minimal "biological" data to complement their structural data, but they are just not sufficient to allow the conclusions.

- The data in Fig. 6 were nicely redone, and indeed the bands are no longer overexposed. However, the conclusion of this figure that the "mutations relaxed the binding specificity for transcription factors and enhanced the binding with cyclin D1" cannot be made based on the data shown. Most importantly, in evaluating the interaction with cyclin D1, the expression of wt HHM is much lower than that of the mutants; so no conclusion can be made with respect to the binding of HHM to cyclin D1. Additionally, that the mutations relaxed the binding specificity for the transcription factors is also tenuous, and maybe true for its affinity for Id2, but is certainly not reflected in the P166Y HHM binding to NeuroD1. As I am sure the authors agree, the data are just not sufficient to make such broad statement. Furthermore, how can one even make a statement of that nature when the loading of IP'd HHM proteins differs between lanes, and only one level of HHM is evaluated against one level of the transcription factor or cyclin D1 texted.

- Fig. 7 and associated text: Again the data are too minimal to allow the conclusions made. (1) The authors conclude that the HHM mutants accelerate myogenic differentiation, but (a) only two HHM mutants were tested, whereas four mutants were tested in Fig. 6, (b) only the intensity of immunostaining is scored and this is at best semi-quantitative (rather than the more quantitative qRT-PCR or immunblotting), and this was, furthermore, done only for one marker, (c) only the P166Y mutant, but not the other mutant showed an effect, compared to wt HHM, whereas the N169E mutant scores similarly to wt HHM. (2) The authors furthermore conclude that these mutants accelerate differentiation, by acting as dominant negative HLH proteins with relaxed binding specificity, but this relaxed binding specificity, based on Fig. 6, was questioned above, nor is there a reason presented to conclude that the effect on myogenic differentiation relates to the relaxed specificity. (3) Finally, as acknowledged by the authors in the rebuttal, they do not have a mechanism that could account for the effects, if any, on differentiation.

In conclusion, with respect to Fig. 6 and Fig. 7, not much progress was made, when compared to the previous version, even though the data in Fig. 6 are better. More work is needed to allow conclusions.

Referee #3 (Remarks to the Author):

While there are many more experiments that could be done to demonstrate the proposed model, I think that the authors have accomplished a significant piece of work and that the structure deserves

to be published without further delay. The authors have proposed a model interpreting their structure in light of the expected formation of an HLH-dimer. This model will continue to be tested in the future. I therefore think the paper should be published without asking the authors for further experiments.

One comment that I'm still unclear about. If the model is correct, then the HLH of HHM should interact with the bHLH of olig1 or NeuroD directly. I saw several examples in references to interactions between HHM N-bundle-HLH or HHM HLH-C-bundle, leaving the possibility that the HLH of olig1 interacts with the N-bundle or C-bundle and not the HLH. I think your supplementary data from Ikushima H et al (2008) EMBO J 27:2955-65, Figure S5 D, may have tested the HLH of HHM alone for interaction with Olig1. If so, that is very convincing and seems worth emphasizing.

Comments:

Figure 1: It would help to number the amino acids for each region on Fig 1A.

p. 10, line 2: reference for the Myc/Max structure is missing

p. 13: Equilibrium between the V-shaped and relaxed conformations:
I do not find the TeV protease cleavage experiment very convincing. If you cleave the loops of a protein, you increase the entropy of the unfolded state dramatically - especially if you cleave 2 loops. It is difficult to estimate the size of this entropy gain, and so difficult to know how well the HLH domain would have to bind to see the interaction in a gel. It is even possible that the cleaved protein unfolds completely (the MD showed it was no longer stable). CD would have at least demonstrated that the N-bundle and C-bundle remain folded. I think this approach should not be emphasized and does not strengthen the argument.

Figure 3 D- in the caption you reference the Myc/Max structure, but in the figure you show the NeuroD/E47 structure. Also on p. 11 line 12 you refer to the E47/NeuroD structure but the reference (Nair and Burley, 2003) is to Myc/Max. Nowhere do you reference the E47/NeuroD structure.

p. 21 , line 17: v-bar not written right

p. 22 - protein binding assays in HEK293 cells - did you use just the bHLH of Olig1 and NeuroD, or full length proteins? You say you synthesized NeuroD, from oligos, suggesting you used only the bHLH domain.

Table II: Units of B-factor should be A^2, not A. The average B-factor is high (99.6). I assume this is the overall B-factor (U(isotropic) + U(TLS)), reported by Phenix. I have also observed high overall B-factors in a structure, though the Phenix people claim their B-factors should not be higher than calculated by other programs. One question is whether you have over-estimated the resolution. Given the high B-factor, it would make sense to display electron density of the critical region in the supplementary data. In particular electron density for the contacts discussed between the C-terminus of alpha 5, the N-terminus of helix alpha 6 and the N- and C-bundles.

2nd Revision - authors' response 16 February 2012

The comments by referee #1.

A couple of very minor points about that new data though - in the results sections where the sedimentation equilibrium data are presented please cite the theoretical molecular weight in the text next to the molecular weight estimated by AUC. In the methods section, watch the SI units (h not hrs) and note the formatting of V-bar isn't correct in the pdf.

According to the comments from the referee, we have cited the theoretical molecular weight of HHM in the result section, modified the SI units, and re-formatted V-bar in the method section.

The comments by referee #2.

During our revision, we noticed that we mixed up HHM mutants. We actually used V278R, but not V288R in our experiments. We apologize for this and would like to correct it in the revised manuscript. However, we think that these mix-ups do not affect our conclusion because both V277R and V288R mutants are expected to disrupt the interaction between helix a4/8 and the C-bundles.

The data in Fig. 6 were nicely redone, and indeed the bands are no longer overexposed. However, the conclusion of this figure that the "mutations relaxed the binding specificity for transcription factors and enhanced the binding with cyclin D1" cannot be made based on the data shown. Most importantly, in evaluating the interaction with cyclin D1, the expression of wt HHM is much lower than that of the mutants; so no conclusion can be made with respect to the binding of HHM to cyclin D1.

We appreciate the reviewer's comment. We carefully repeated the experiments to compare bindings at more equal expression levels. As the referee pointed out, binding with Cyclin D1 was not strikingly different between wild type HHM and four mutants. Accordingly, we revised the main text and deleted related descriptions from concluding remarks.

Additionally, that the mutations relaxed the binding specificity for the transcription factors is also tenuous, and maybe true for its affinity for Id2, but is certainly not reflected in the P166Y HHM binding to NeuroD1. As I am sure the authors agree, the data are just not sufficient to make such broad statement. Furthermore, how can one even make a statement of that nature when the loading of IP'd HHM proteins differs between lanes, and only one level of HHM is evaluated against one level of the transcription factor or cyclin D1 tested.

It was quite difficult to get equal expression between wild type HHM and four HHM mutants, together with equal expression of partner binding proteins. We think that our new data are improved, although not perfect, and enough to conclude that binding with NeuroD1 was stronger in HHM mutant proteins than in wild type HHM.

Fig. 7 and associated text: Again the data are too minimal to allow the conclusions made.
(1) The authors conclude that the HHM mutants accelerate myogenic differentiation, but
(a) only two HHM mutants were tested, whereas four mutants were tested in Fig. 6,

According to this comment, we examined the effects of all 4 mutants together with wild type HHM.

(b) only the intensity of immunostaining is scored and this is at best semi-quantitative (rather than the more quantitative qRT-PCR or immunblotting), and this was, furthermore, done only for one marker,

Thank you very much for this important as well as helpful comment to which we totally agree. Our previous immunostaining data was semi-quantitative. We re-prepared all the adenoviral constructs using a new vector system because adenoviral constructs prepared with the previous system was toxic to C2C12 cells, which made it difficult to evaluate cell differentiation quantitatively. Now this problem is overcome by use of the new vector system and we present qRT-PCR data on myosin heavy chain and myogenin. In the previous version, we examined protein

expression at 36 and 48h after induction, but here we measured mRNA expression at earlier time points. At 12 h after induction of differentiation, there was no significant difference in marker expressions between samples. At 24 h, HHM mutants enhanced marker expression while wild type HHM did not. At 36 h, difference was less clear in expression of myogenin and not evident in expression of myosin heavy chain. Thus we are presenting data at 12 and 24 h after induction of differentiation.

(c) only the P166Y mutant, but not the other mutant showed an effect, compared to wt HHM, whereas the N169E mutant scores similarly to wt HHM.

We think that this concern is addressed in our new data using qRT-PCR. We thank the referee for suggesting the use of qRT-PCR.

(2) The authors furthermore conclude that these mutants accelerate differentiation, by acting as dominant negative HLH proteins with relaxed binding specificity, but this relaxed binding specificity, based on Fig. 6, was questioned above, nor is there a reason presented to conclude that the effect on myogenic differentiation relates to the relaxed specificity.

New Figure 6 now indicates that four mutants all have relaxed binding specificity. We anticipated some perturbation of cellular processes if helix-loop-helix proteins with such relaxed binding specificity are ectopically expressed in cells. We examined this anticipation using an in vitro myogenic differentiation assay and obtained consistent results. These findings support our idea that the N- and C-bundle of HHM plays an important role in limiting its binding specificity, which is predicted from our structural study, and we think that is enough for our purpose.

(3) Finally, as acknowledged by the authors in the rebuttal, they do not have a mechanism that could account for the effects, if any, on differentiation.

As the referee pointed out, we do not have definite mechanism at present. Of course, it would be nice if we can demonstrate reformation of endogenous E12/47-MyoD complex. We tried to detect the physical interaction between endogenous E12/47 and MyoD in C2C12 cells by co-precipitation assay. We prepared C2C12 cell lysates from 10 cm dish after induction of differentiation (24 h), immunoprecipatated proteins with anti-MyoD antibody (Abcam, AB16148) followed by immunoblotting using anti-E47 antibody (SantaCruz, N649) but detected no reliable signals. Immunoprecipitation with anti-E12/E47 could not be used because MyoD migrate close to Ig heavy chain, making detection of MyoD after immunoprecipitation difficult. In the paper demonstrating inhibition of E12/47-MyoD complex by Id, in vitro translated proteins were used (Benezra et a, Cell 61, 49-59, 1990), thus not applicable to endogenous proteins.

However, we observed that HHM and its mutants failed to interact with MyoD (Supplementary Figure S6). In contrast, HHM mutants, but not wild type HHM, efficiently interacted with Id2 (Figure 6). Id proteins are known to disrupt E12/E47-MyoD complex to inhibit its transcriptional activity (Benezra et al., 1990). Thus, the HHM mutants are likely to bind and inhibit the Ids, which originally suppress the E12/47-MyoD complex, accelerating myogenic differentiation by the reformation of the complex between E12/47 and MyoD (described in the main text, page 18, line 10 – page 18, line 16).

We think it important to show differential behavior of wild type HHM and HHM mutants, all four of which have relaxed biding specificity to helix-loop-helix proteins and acceleration of myogenic marker expression. We feel that the precise mechanism is beyond the scope of this paper.

We thus ask the referee to understand the situation and allow us not to address this concern perfectly.

The comments by referee #3.

One comment that I'm still unclear about. If the model is correct, then the HLH of HHM should interact with the bHLH of olig1 or NeuroD directly. I saw several examples in references to interactions between HHM N-bundle-HLH or HHM HLH-C-bundle, leaving the possibility that the HLH of olig1 interacts with the N-bundle or C-bundle and not the HLH. I think your supplementary data from Ikushima H et al (2008) EMBO J 27:2955-65, Figure S5 D, may have tested the HLH of HHM alone for interaction with Olig1. If so, that is very convincing and seems worth emphasizing.

As the referee pointed out, our previous experiments showed the HLH domain of HHM alone is sufficient for the interaction with Olig1 and we have emphasized the results in the main text (page 13., line 11)

Figure 1: It would help to number the amino acids for each region on Fig 1A.

We have added the number of the amino acids.

p. 10, line 2: reference for the Myc/Max structure is missing

We noticed that we mixed up the figure and the reference. The figure 3A shows the E47/NeuroD1 structure and we have corrected the reference for it. We apologize for this and have corrected it in the revised manuscript.

p. 13: Equilibrium between the V-shaped and relaxed conformations:
I do not find the TeV protease cleavage experiment very convincing. If you cleave the loops of a protein, you increase the entropy of the unfolded state dramatically - especially if you cleave 2 loops. It is difficult to estimate the size of this entropy gain, and so difficult to know how well the HLH domain would have to bind to see the interaction in a gel. It is even possible that the cleaved protein unfolds completely (the MD showed it was no longer stable). CD would have at least demonstrated that the N-bundle and C-bundle remain folded. I think this approach should not be emphasized and does not strengthen the argument.

Since, as pointed by the referee, we could not exclude a possibility that the removal of the loops itself induces the structural change, we have weakened the argument in the revised main text (page 14, lines 5-6). Nevertheless, we believe that the protein structure would remain intact if domains strongly interact with each other. For example, the structure of retinoblastoma was solved with the cleavage of an internal loop connecting two domains (Lee, et al., Nature, Vol. 391, pp859-865, 1998), meaning that cleaving a loop itself does not always increase the entropy to unfold a protein. Combined with the ultracentrifugation experiments, we concluded that the HLH region of HHM does not exist as the rigid V-shaped structure but as the equilibrium between the V-shaped and relaxed conformations.

Figure 3 D- in the caption you reference the Myc/Max structure, but in the figure you show the NeuroD/E47 structure. Also on p. 11 line 12 you refer to the E47/NeuroD structure but the reference (Nair and Burley, 2003) is to Myc/Max. Nowhere do you reference the E47/NeuroD structure.

As mentioned above, we mixed up the figure and the reference. The figure 3A shows the E47/NeuroD1 structure and referred to it (Longo A et al., Biochemistry, 2008). We apologize for this and have corrected it in the revised manuscript.

p. 21 , line 17: v-bar not written right

We have re-formatted the v-bar appropriately.

p. 22 - protein binding assays in HEK293 cells - did you use just the bHLH of Olig1 and NeuroD, or full length proteins? You say you synthesized NeuroD, from oligos, suggesting you used only the bHLH domain.

We used full length Olig1 and full length NeuroD1 for our experiments. The full length cDNA encoding mouse NeuroD1 was synthesized from oligos.

Table II: Units of B-factor should be A^2, not A. The average B-factor is high (99.6). I assume this is the overall B-factor (U(isotropic) + U(TLS)), reported by Phenix. I have also observed high overall B-factors in a structure, though the Phenix people claim their B-factors should not be higher than calculated by other programs. One question is whether you have over-estimated the resolution. Given the high B-factor, it would make sense to display electron density of the critical region in the supplementary data. In particular electron density for the contacts discussed between the C-terminus of alpha 5, the N-terminus of helix alpha 6 and the N- and C-bundles.

We have corrected the units of B-factor to $Å^2$, and added the figure of electron density for the contacts region between the C-terminus of alpha 5, the N-terminus of helix alpha 6 and the N- and C-bundles as the supplementary figure 3, according to the referee's comment.

Although the average B-factor is high, the completeness, I/s(I), and R_{sym} values in the last shell are 91.6%, 2.46, and 0.371, respectively, which are generally acceptable values as resolution cutoff. Furthermore, we compared the average B-factors after performing refinement with different resolution cutoff values (as shown in the Table below). The results showed that the average B-factor still remained over 90 $Å^2$, after reducing the cutoff to 3.5 Å resolution. Therefore, we concluded that the high average B-factor is NOT an artifact introduced by incorrect resolution cutoff, but reflects intrinsic flexibility of the structure of HHM (The dynamic nature of HHM structure was also demonstrated by UAC experiments).

Resolution (Å)	TLS	R_{work}	R_{free}	Average B-factor ($Å^2$)
50-2.5	on	0.222	0.261	99.6
50-3.0	on	0.200	0.250	96.8
50-3.5	on	0.180	0.239	93.1
50-2.5	off	0.253	0.299	84.9
50-3.0	off	0.232	0.282	89.2
50-3.5	off	0.209	0.281	81.8

3rd Editorial Decision 05 March 2012

Thank you for submitting your re-revised manuscript for our consideration. It has now been seen once more by the original referee 2 (see comments below), and I am happy to inform you that this reviewer is now satisfied and the paper therefore in principle acceptable for publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, we will however still require some important changes to the manuscript text, which I kindly ask you to incorporate, and to send us a modified text file by email as soon as possible. In this modified text, please

- include the PDB accession codes for the structural work reported

- include the author contribution statement you already sent us
- also include a brief Conflict of Interest statement
- rewrite selected passages in the text that currently seem to be near-verbatim copies from some of your previous publications: the passage on pages 4/5 from 'HHM appears to be involved...' to '...remains to be elucidated' (cf. Seto et al Acta Cryst 2009); and the 2 paragraphs on AUC methods on pages 22/23 starting at 'The molecular mass distribution function...' and ending on the next page '...using the SEDNTERP program' (cf Akhter et al BBA 2007, Kumar Sarkar et al BBA 2006). This is simply to exclude any possibility of self-plagiarism accusations at later, post-publication stages.

Once we will have received a modified manuscript file with these changes, we should then hopefully be able to swiftly proceed with formal acceptance and production of the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors have now adequately addressed my concerns. Their conclusions are now better backed up by the data provided, and more carefully stated.

