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Structural basis for the slow dynamics of the actin filament pointed end

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(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 24 August 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the excessive delay in getting back to you with a decision. As I told you, we experienced quite some difficulty in finding three appropriate referees for your manuscript - primarily due to the holiday season. However, I have finally received all three reports, which are enclosed below.

As you will see, all three referees recognise the high technical quality of your work as well as the interest in the topic, and are broadly in favour of publication. However, referees 1 and 3 both raise serious concerns with the interpretation of the data, and argue that the manuscript needs to be extensively rewritten to better reflect recent advances in the field. In particular, neither is convinced that your data provide a structural explanation for actin dynamics based on the directionality of treadmilling. It is clear these two referees have rather different interpretations themselves - presumably reflecting a degree of controversy in the field. Still, both referees make useful suggestions for re-focusing your manuscript, and a balanced discussion of possible alternative interpretations and models for actin dynamics would be important here. Referee 3 also points out a number of important citations missing: I would stress that EMBOJ has no limit in terms of the reference list, and would encourage you to cite the primary literature more extensively to better put your work into context.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. 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 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Apologies again for the delay, and thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript addresses the important topic of the different structures of the ends of the actin filament. The authors have an authorized expertise in structural analysis of F-actin and of the barbed-end associated regulators like capping protein. Their present goal is to elucidate the structural basis for treadmilling, the process that drives actin-based motility.
Summary : It is shown that the actin-actin lateral interactions between the two terminal subunits at the pointed ends differ from those in the core of the filament. A 12° tilt of sudomain 2 of the last subunit allows formation of a loop-loop contact between DNase binding loop of penultimate subunit P-1 and the hydrophobic plug of terminal subunit P. This structure accounts well for the slow kinetics at pointed ends. It is proposed that it accounts for the unidirectional treadmilling process.

Critique : Actin filaments are polar polymers. It is well known that the kinetic parameters for growth and depolymerization are different at the two ends, i.e. a dynamic polarity is associated with structural polarity. There is about one order of magnitude difference between barbed ends and pointed ends in the association and dissociation rate constants of G-actin. This is true in the presence of either ADP or ATP. The critical concentrations are identical in ADP, and they differ in ATP. Treadmilling depends on the energetic difference (meaning critical concentration difference) between the two ends, independently from the difference in dynamics at the two ends. I believe there is a misconception of the significance of the results in this paper. The authors do make an important discovery that the 12° tilt of the penultimate subunit at the P end accounts well for the slow association of an additional G-actin and for the slow dissociation rate as compared to the B end. However this does not account for the difference in critical concentration in ATP which is due to ATP hydrolysis. Hence the title, the introduction and discussion of the paper are misleading. Treadmilling would occur in the same direction in a putative case in which the barbed end would be less dynamic than the pointed end, provided that the excess of association events at steady-state would take place at the barbed ends, thus generating a lower critical concentration at the barbed than at the pointed end !
In conclusion, I do not agree with the sentence (3 rd page of Discussion) : ’An alternative model with the same set of Cc but with the dynamic pointed end and less dynamic barbed ends would conceivably reverse the treadmilling ‘. I do not agree either with the sentence in the next paragraph’ the present model explains only the direction of the movement ‘. The data in fact only explain the difference in dynamics of the two ends both in ADP and ATP, independently of ATP hydrolysis. Therefore these data cannot be taken to provide a structural explanation for treadmilling and

movement. The results are nevertheless important ! But the challenge is still open to understand the structural change linked to ATP hydrolysis and Pi release, which triggers treadmilling.
Finally I wish to make a small suggestion regarding the possible structural regulation of the pointed end dynamics by ADF. The ADF changes the structure (twist) of the filament, destabilizing actin-actin contacts and leading to faster pointed end depolymerization. Using the structure data from Amy Mc Gough (1998), is it possible to see whether the loop-loop interaction at pointed ends of standard filaments seen here would be abolished in over-twisted ADF-F-actin filaments ? If true, this observation would validate/strengthen the present structural model for slow dynamics at pointed ends.

Referee #2 (Remarks to the Author):

This is an important new contribution to our understanding of actin filament dynamics. The authors have produced a high quality 3D image of the pointed end of an actin filament and correlated the observed interactions between subunits with molecular dynamics calculations. Flexible parts of the actin monomer were identified by comparing a large number of crystal structures and movement was restricted to these loops in the energy calculations. The conclusion that there is a substantial difference in the conformation of the final subunit is clear and does not depend on whether the resolution achieved is actually ~23Å or ~19.5Å. However, the discussion of the problem of estimating resolution is relevant to more than this study of actin filaments and is in tune with a widespread feeling that the 'FSC=0.5 between half data-sets' criterion tends to underestimate the resolution in EM maps. In this case, the authors have compared their EM data with a near-atomic structure based on X-ray scattering data from actin filaments and have obtained a more gradual FSC curve, which makes more sense than the sudden steep drop seen by comparing two half sets of EM data. The different comparisons will be of interest throughout the EM image-analysis field. Although the EM data here are relatively low resolution compared with many current investigations, it is clear they are sufficiently well-defined to accurately dock high-resolution structures.

My understanding of Fig.2 is that the green curve was obtained by fitting the same monomer structure into each subunit of the EM map except that P was tilted *en bloc*. The blue curve shows how the agreement can improve when flexible loops are allowed to move. Please make this clearer in the figure legend or related text. Presumably most of the flexible movements occurred in subunits P-1 and P, even though 6 subunits were given freedom to move?

The story would be clearer from the abstract if it said that, at the pointed end, there is a transverse interaction between the flexible loops that are needed to make new longitudinal connections and therefore the pointed end is less dynamic both because the end subunit is less likely to leave and also because another new subunit is less likely to bind.

As the authors state, a full understanding of treadmilling will require additional structural information on the effect of changes to the bound nucleotide. I look forward to further installments.

Referee #3 (Remarks to the Author):

Narita et al. EMBO journal: Structural basis of the unidirectional movement of the actin filament

This work is a valuable and interesting addition to our understanding of the structure of actin filaments, providing the first structural insights about why the kinetic properties of the two ends of actin filament are different. I have a few technical questions and comments.

My main reservation is the scholarship in the manuscript itself, which is shockingly out of date. With some exceptions, the authors and their thinking seem to stuck in the 1980s, before the rate constants for virtually all of the actin assembly reactions, ATP hydrolysis and phosphate release were determined and before anyone observed the steady state behavior of actin filaments in real time. Consequently, their approach to framing questions and interpreting their new data are inappropriate.

Making treadmilling the focus of the paper is a bad idea. Actin filaments do treadmill at steady state in ATP as documented by direct observations (Fujiwara paper and Kuhn paper; neither cited), but it is very slow (<0.1 subunits/s). No one has ever documented that the turnover of actin filaments in cells is due to treadmilling. The prevailing belief (except for Carlier and Pantaloni) is that filaments largely turn over by severing followed by disassembly of the fragments. Saying "cytokinetic movement plays central roles in a wide spectrum of cellular functions" is perpetuating a myth about the relevance of treadmilling. Many other aspects of actin polymerization are more important and interesting.

On the other hand, recent work raised many important questions that might be addressed by the work in this manuscript. A more compelling motivation for the present work would to determine the structural basis for the kinetic differences between the two ends characterized by Fujiwara (2007). That paper clearly states the questions which needed to be answered by structural studies: "Remarkably, 10 times more phosphate is required to slow the depolymerization of the pointed end than the barbed end, suggesting a weak affinity of phosphate near the pointed end. ....Pi dissociates rapidly from the terminal subunits at both barbed and pointed ends and .... has a weaker affinity for the terminal subunit at the pointed end than other parts of ADP-actin filaments. These differences must arise from interactions of subunits near the end of the filament." How does this new work address these questions?

The authors do not consider the currently accepted explanation from Sept (JMB 1999) for the differences in the kinetics at the two ends of the filament. How might a combination of electrostatics and conformations (observed here) influence the reactions?

Abstract: I would recast the entire paper and the abstract on something different from treadmilling.

Nomenclature: Most publications on actin use the term "subunit" rather than "protomer".

p. 3: The authors' own paper (Iwasa 2008) is not the origin of the knowledge that polymerization activates the ATPase activity or even the source of the best measurements of the ATPase rates of monomeric or polymeric actin or phosphate release (none cited). The Korn (1982) review is very out of date, since it came before the measurements of any of the rate constants for subunit association and dissociation, ATP hydrolysis or Pi release. None of the references given supporting a role of actin filament treadmilling in biological processes actually measured treadmilling; in general these and other papers documented the assembly and turnover of actin filaments in cells, but none have ever demonstrated that the turnover is by treadmilling. Proteins that nucleate, cap and sever actin filaments dominate the behavior of actin in cells. All of the reactions promoted by these proteins occur much more rapidly than treadmilling. Contrary to the last sentence in the first paragraph, nothing has ever been shown to increase the rate of treadmilling per se. All of the regulatory proteins do something else. In fact, two of the proteins cited here (Arp2/3 complex and capping proteins) actually inhibit treadmilling by capping the pointed and barbed ends of filaments. Work from multiple labs over the past decade (not cited) showed that the only way that cofilin influences treadmilling is by severing to create more ends, rather than enhancing subunit dissociation from ends or the rate of treadmilling.

p. 4: I think that the authors meant to cite Fujiwara for "ATP hydrolysis and phosphate release tend to occur at the less dynamic pointed end". Saying "critical concentration of ATP-F-actin or ADPPi-F-actin is lower than that of ADP-F-actin" is wrong, because critical concentration refers to actin monomers not filaments. The authors meant to say the "critical concentrations of ATP-actin monomers or ADP-Pi-actin monomers are lower than ADP-actin monomers." It is misleading to say "this results in the stability of the barbed end", because at steady state the barbed end actually exchanges subunits much faster than the pointed end. Maybe the authors meant to say that the barbed end tends to elongate slowly at steady state in ATP, because the actin monomer concentration is slightly above the critical concentration.

I am not sure what the authors mean by "importance of the second element has not been fully appreciated". Do they mean that no one read or appreciated the Fujiwara paper, which lays out the whole system of reactions?

Figures: The differences in subunit P would be much more obvious with a difference map between

what is observed in the EM reconstruction and a space-filling model (not backbone trace) of the normal conformation along the filament.

I agree that "the results presented indicate that at the pointed end of the actin filament the end protomer P is tilted against the adjacent protomer P-1", but I think that the higher resolution details extracted here by modeling and MD simulations are less convincing. The models and conclusions would be more convincing with quantitative evaluations of the results throughout rather than subjective assessments such as "largely limited" and "consistency was significantly improved by the MD simulation" (p. 6).

The authors conclude "the conformational changes and the tighter contacts likely inhibit the two loops from the canonical formation binding to P+1". I agree that the conformation observed at the pointed end may explain why association and dissociation of subunits is slower than at the barbed end and why association at the pointed end not a diffusion limited reaction (Drenckhahn 1986; not cited), but I do not understand the meaning of "...inhibit the two loops from the canonical formation binding to P+1".

Using energetics to explain kinetics is dangerous and should be avoided here.

Fig. 4: I think that the legend has the colors backwards in C. I am not impressed that the MD model fits into the experimental map very well in D and E. This needs better objective, quantitative documentation.

Fig. 8: I am not sure why the authors did a simulation of steady state length fluctuations with arbitrary ratios of rate constants, when Fujiwara et al. actually measured these rate constants and made some calculations about the behavior of the two ends. The actual rate constants are not related by a simple ratio, so this approach has no justification. In my view, the authors should have thought about how their structural results might explain the open questions raised by Fujiwara and listed above, rather than testing an arbitrary hypothesis.

Methods: What nucleotide and divalent cation were bound to the actin in the MD simulations. What charge was used for the divalent cation? How was the choice of this charge justified? How were the actin subunits fit into the density maps - as rigid elements or as subdomains? What is the justification for this choice of fitting methods?

1st Revision - authors' response 08 November 2010

Comments by Referee#1

Comment:
Critique : Actin filaments are polar polymers. It is well known that the kinetic parameters for growth and depolymerization are different at the two ends, i.e. a dynamic polarity is associated with structural polarity. There is about one order of magnitude difference between barbed ends and pointed ends in the association and dissociation rate constants of G-actin. This is true in the presence of either ADP or ATP. The critical concentrations are identical in ADP, and they differ in ATP. Treadmilling depends on the energetic difference (meaning critical concentration difference) between the two ends, independently from the difference in dynamics at the two ends. I believe there is a misconception of the significance of the results in this paper. The authors do make an important discovery that the 12° tilt of the penultimate subunit at the P end accounts well for the slow association of an additional G-actin and for the slow dissociation rate as compared to the B end. However this does not account for the difference in critical concentration in ATP which is due to ATP hydrolysis. Hence the title, the introduction and discussion of the paper are misleading.

Treadmilling would occur in the same direction in a putative case in which the barbed end would be less dynamic than the pointed end, provided that the excess of association events at steady-state would take place at the barbed ends, thus generating a lower critical concentration at the barbed than at the pointed end !

In conclusion, I do not agree with the sentence (3 rd page of Discussion) : 'An alternative model with the same set of Cc but with the dynamic pointed end and less dynamic barbed ends would conceivably reverse the treadmilling '. I do not agree either with the sentence in the next paragraph 'the present model explains only the direction of the movement '. The data in fact only explain the difference in dynamics of the two ends both in ADP and ATP, independently of ATP hydrolysis. Therefore these data cannot be taken to provide a structural explanation for treadmilling and movement. The results are nevertheless important !

Response:
We should have emphasized the point that the critical concentrations at both ends are the same if the bound nucleotide is the same. This is because the free energy difference between the G-actin state and the F-actin state is independent from the end where the actin molecule associates or dissociates. We added the details as the Supplementary Figure S1 and added further explanation in the introduction.
It is true that the apparent critical concentration difference between the two ends drives the treadmilling movement. However, this apparent difference comes from the difference of the bound nucleotide, ADP at the pointed end and ADPPi or ATP at the barbed end. The nucleotide difference originates from the rate difference between the two ends, and the difference in critical concentration is not an intrinsic property that gives rise to the morphological differences of the two ends (Figure 9 and discussion section). The slower end tends to have ADP while the other end tends to have ADPPi or ATP. In conclusion, the rate difference, whose origin is described in this manuscript, is the origin of the direction of the treadmilling movement. To emphasize the importance of the rate difference, we added Figure 9F. A more dynamic pointed end than the barbed end would reverse the direction of the movement.

Comment:
Finally I wish to make a small suggestion regarding the possible structural regulation of the pointed end dynamics by ADF. The ADF changes the structure (twist) of the filament, destabilizing actin-actin contacts and leading to faster pointed end depolymerization. Using the structure data from Amy Mc Gough (1998), is it possible to see whether the loop-loop interaction at pointed ends of standard filaments seen here would be abolished in over-twisted ADF-F-actin filaments? If true, this observation would validate/strengthen the present structural model for slow dynamics at pointed ends.

Response:
This is a good suggestion. Although we have investigated this possibility, we have unfortunately realized that the relative position of the two loops is not changed substantially by the change in the helical parameter caused by cofilin binding. Therefore, destabilization of the filament by cofilin might be explained by a different mechanism. The right figure shows top views of two adjacent subunits with the normal helical parameters (A), and with the altered helical parameters with cofilin (B). The residues in the space-filling model are the hydrophobic plug (in red) and the DNase I binding loop (in blue).

Comments by Referee #2

Comment:
This is an important new contribution to our understanding of actin filament dynamics. The authors have produced a high quality 3D image of the pointed end of an actin filament and correlated the observed interactions between subunits with molecular dynamics calculations. Flexible parts of the actin monomer were identified by comparing a large number of crystal structures and movement was restricted to these loops in the energy calculations. The conclusion that there is a substantial difference in the conformation of the final subunit is clear and does not depend on whether the

resolution achieved is actually ~ 23 Å; or ~19.5Å. However, the discussion of the problem of estimating resolution is relevant to more than this study of actin filaments and is in tune with a widespread feeling that the 'FSC=0.5 between half data-sets' criterion tends to underestimate the resolution in EM maps. In this case, the authors have compared their EM data with a near-atomic structure based on
X-ray scattering data from actin filaments and have obtained a more gradual FSC curve, which makes more sense than the sudden steep drop seen by comparing two half sets of EM data.

Response:
Deciding on which criterion should be taken to estimate the resolution of an EM map is a difficult problem. We share the same feeling as the reviewer that the FSC = 0.5 criterion underestimates the resolution. Therefore, we have removed the words 'widely accepted' to explain the FSC = 0.5 criterion from the first paragraph of the results section. With regard to the comparison of the EM maps with an atomic structure, the gradual dumping of the FSC curve may indicate an advantage. However, it is challenging to detect overfitting of the atomic model to the EM map from the corresponding FSC curve alone; which might cause overestimation of the resolution. Therefore it is difficult to say this is better than the half data-setsí criterion. It is, however, safer to use both criteria and we have presented both FSC curves in Figure 2.

Comment:
*My understanding of Fig.2 is that the green curve was obtained by fitting the same monomer structure into each subunit of the EM map except that P was tilted *en bloc*. The blue curve shows how the agreement can improve when flexible loops are allowed to move. Please make this clearer in the figure legend or related text.*

Response:
This suggestion is completely correct. We have revised the text following this suggestion.

Comment:
Presumably most of the flexible movements occurred in subunits P-1 and P, even though 6 subunits were given freedom to move?

Response:
As we described in the Materials and Methods section, all of the atoms of P, P-1, P-2, P-3 and P-4 were given freedom to move in the energy minimization steps. In the energy minimization step, the movement of the residues was not large. In the molecular dynamics step, the alpha carbons were fixed except for the alpha carbons in the two loops.

Comment:
The story would be clearer from the abstract if it said that, at the pointed end, there is a transverse interaction between the flexible loops that are needed to make new longitudinal connections and therefore the pointed end is less dynamic both because the end subunit is less likely to leave and also because another new subunit is less likely to bind.

Response:
We have followed the suggestion and changed the abstract to explain our results in a little more detail.

Comments by Referee #3

Comment:
My main reservation is the scholarship in the manuscript itself, which is shockingly out of date. With some exceptions, the authors and their thinking seem to be stuck in the 1980s, before the rate constants for virtually all of the actin assembly reactions, ATP hydrolysis and phosphate release were determined and before anyone observed the steady state behavior of actin filaments in real time. Consequently, their approach to framing questions and interpreting their new data are inappropriate.

Making treadmilling the focus of the paper is a bad idea. Actin filaments do treadmill at steady state in ATP as documented by direct observations (Fujiwara paper and Kuhn paper; neither cited), but it is very slow (<0.1 subunits/s). No one has ever documented that the turnover of actin filaments in cells is due to treadmilling. The prevailing belief (except for Carlier and Pantaloni) is that filaments largely turn over by severing followed by disassembly of the fragments. Saying "cytokinetic movement plays central roles in a wide spectrum of cellular functions" is perpetuating a myth about the relevance of treadmilling. Many other aspects of actin polymerization are more important and interesting.

Abstract: I would recast the entire paper and the abstract on something different from treadmilling.

None of the references given supporting a role of actin filament treadmilling in biological processes actually measured treadmilling; in general these and other papers documented the assembly and turnover of actin filaments in cells, but none have ever demonstrated that the turnover is by treadmilling. Proteins that nucleate, cap and sever actin filaments dominate the behavior of actin in cells. All of the reactions promoted by these proteins occur much more rapidly than treadmilling. Contrary to the last sentence in the first paragraph, nothing has ever been shown to increase the rate of treadmilling per se. All of the regulatory proteins do something else. In fact, two of the proteins cited here (Arp2/3 complex and capping proteins) actually inhibit treadmilling by capping the pointed and barbed ends of filaments. Work from multiple labs over the past decade (not cited) showed that the only way that cofilin influences treadmilling is by severing to create more ends, rather than enhancing subunit dissociation from ends or the rate of treadmilling.

Response:
We do not completely share this opinion. It is true that the treadmilling of actin alone is too slow to account for the actin turnover in live cells. However, this does not mean we must exclude the possibility of the treadmilling movement accelerating in some way in the cell. If it is true that cofilin influences treadmilling by severing to create more ends without capping the ends, cofilin will enhance the polymerization of actin because the polymerization rate at a newly created barbed end by cofilin is much faster than the depolymerization rate at the simultaneously created pointed end. In this case, to maintain the amount of the G-actin pool in the cell, another system for accelerating depolymerization is obviously required, which might accelerate the treadmilling. We still consider the accelerated treadmilling movement as a very good model to explain many movements of the actin filaments in the cell such as in philopodia or pollen tubes because we currently do not have any better model.
However, the detailed mechanism of the actin dynamics in the cell is still unclear and there are no obvious experiments which show the treadmilling movement in the cell, although there are no obvious experiments that deny it. We agree that it is not adequate to say ‘Treadmilling movement plays important roles in the cell’ and we changed the Introduction to reflect this.
We have maintained the use of ‘treadmilling’ in the manuscript because it represents the most typical intrinsic dynamics of the actin filament which includes the most important properties of the actin filament: polymerization, depolymerization, the ATP hydrolysis and the dynamic difference between the two ends.

Comment:
p. 3: The authors' own paper (Iwasa 2008) is not the origin of the knowledge that polymerization activates the ATPase activity or even the source of the best measurements of the ATPase rates of monomeric or polymeric actin or phosphate release (none cited). The Korn (1982) review is very out of date, since it came before the measurements of any of the rate constants for subunit association and dissociation, ATP hydrolysis or Pi release.

Response:
The reviewer is correct that our paper is not the first to report that polymerization activates ATPase. We have referred to another paper, Pollard & Weeds, 1984. We have also cited an updated review on actin treadmilling, Bugyi & Carlier, 2010.

Comment:
On the other hand, recent work raised many important questions that might be addressed by the

work in this manuscript. A more compelling motivation for the present work would to determine the structural basis for the kinetic differences between the two ends characterized by Fujiwara (2007). That paper clearly states the questions which needed to be answered by structural studies: "Remarkably, 10 times more phosphate is required to slow the depolymerization of the pointed end than the barbed end, suggesting a weak affinity of phosphate near the pointed end. ....Pi dissociates rapidly from the terminal subunits at both barbed and pointed ends and .... has a weaker affinity for the terminal subunit at the pointed end than other parts of ADP-actin filaments. These differences must arise from interactions of subunits near the end of the filament." How does this new work address these questions?

Response:
We agree that the question described above is important. However, in order to address the question, the structure around the bound Pi should be obtained with some clarity, particularly the -helices should be unambiguously traced. For this to be possible, at least 8 Å resolution is required. Therefore, it remains to be performed in future research.

Comment:
The authors do not consider the currently accepted explanation from Sept (JMB 1999) for the differences in the kinetics at the two ends of the filament. How might a combination of electrostatics and conformations (observed here) influence the reactions?

Response:
We do not deny the possibility that long-ranged electrostatic interactions could affect the association rate; however, the calculation of the simulation is required to be updated by using the current F-actin model. This is because the interface of the current model between the subunits is largely different from the model they used. We have added sentences in the discussion section to refer to this possibility.

Comment:
Nomenclature: Most publications on actin use the term "subunit" rather than "protomer"

Response:
We have followed this suggestion.

Comment:
p. 4: I think that the authors meant to cite Fujiwara for "ATP hydrolysis and phosphate release tend to occur at the less dynamic pointed end". Saying "critical concentration of ATP-F-actin or ADPPi-F-actin is lower than that of ADP-F-actin" is wrong, because critical concentration refers to actin monomers not filaments. The authors meant to say the "critical concentrations of ATP-actin monomers or ADP-Pi-actin monomers are lower than ADP-actin monomers." It is misleading to say "this results in the stability of the barbed end", because at steady state the barbed end actually exchanges subunits much faster than the pointed end. Maybe the authors meant to say that the barbed end tends to elongate slowly at steady state in ATP, because the actin monomer concentration is slightly above the critical concentration.

Response:
It is completely correct that the critical concentration refers to actin monomers. We have changed the sentences to reflect this important point.

Comment:
I am not sure what the authors mean by "importance of the second element has not been fully appreciated". Do they mean that no one read or appreciated the Fujiwara paper, which lays out the whole system of reactions?

Response:
We meant that most people think that the treadmilling movement is driven by the critical concentration difference between the two ends and pay much less attention to the dynamic difference between the two ends. We wanted to emphasize that the dynamic difference is the origin of the apparent critical concentration difference between the two ends and it determines the direction of the movement. However, the phrase "importance of the second element has not been fully appreciated" was obviously not clear and did not explain this point. Consequently, we have deleted this phrase.

Comment:
Figures: The differences in subunit P would be much more obvious with a difference map between what is observed in the EM reconstruction and a space-filling model (not backbone trace) of the normal conformation along the filament.

Response:
We agree and have added Supplementary Figure S2 showing the difference in the density of the P subunit from the normal conformation.

Comment:
I agree that "the results presented indicate that at the pointed end of the actin filament the end protomer P is tilted against the adjacent protomer P-1", but I think that the higher resolution details extracted here by modeling and MD simulations are less convincing. The models and conclusions would be more convincing with quantitative evaluations of the results throughout rather than subjective assessments such as "largely limited" and "consistency was significantly improved by the MD simulation" (p. 6).

Fig. 4: I think that the legend has the colors backwards in C. I am not impressed that the MD model fits into the experimental map very well in D and E. This needs better objective, quantitative documentation.

Response:
We added Supplementary Figure S2 to show how the fitting of the atomic model was improved by the MD simulation. We also inserted a phrase to explain how significant the interface was limited without the tilting of P.

Comment:
The authors conclude "the conformational changes and the tighter contacts likely inhibit the two loops from the canonical formation binding to P+1". I agree that the conformation observed at the pointed end may explain why association and dissociation of subunits is slower than at the barbed end and why association at the pointed end not a diffusion limited reaction (Drenckhahn 1986; not cited), but I do not understand the meaning of "...inhibit the two loops from the canonical formation binding to P+1".

Response:
We have added phrases to explain this in the text. What we wished to describe is that a specific conformation at the pointed end must inhibit polymerization, as indicated by the reviewer.

Comment:
Using energetics to explain kinetics is dangerous and should be avoided here.

Response:
We have followed the suggestion and removed sentences which referred to energetics from the corresponding part (page 7).

Comment:
Fig. 8: I am not sure why the authors did a simulation of steady state length fluctuations with arbitrary ratios of rate constants, when Fujiwara et al. actually measured these rate constants and made some calculations about the behavior of the two ends. The actual rate constants are not related by a simple ratio, so this approach has no justification. In my view, the authors should have thought about how their structural results might explain the open questions raised by Fujiwara and listed above, rather than testing an arbitrary hypothesis.

Response:
As we described in the discussion section, we would like to emphasize that the slower rates at the pointed end, without any other difference between the two ends, accounts for the direction of the treadmilling movement. The parameters determined by Fujiwara et al. are realistic but complicated. It is useful to introduce the parameter r to make the system simpler and make the important points clearer. We added one sentence to explain this in the legend of Figure 9.
To emphasize the importance of the rate difference further, we have added Figure 9F with r = 2. In this model, the rates at the pointed end are larger than those at the barbed end, and the treadmilling direction is inverted.

Comments:
Methods: What nucleotide and divalent cation were bound to the actin in the MD simulations. What charge was used for the divalent cation? How was the choice of this charge justified?

Response:
We have added sentences to describe this. We used a model with Ca2+ and ADP because we do not have any other atomic model of F-actin. We do not believe that this affects the result significantly because the flexible region in the molecular dynamics simulation does not interact with the nucleotide or the divalent cation.

Comments
How were the actin subunits fit into the density maps - as rigid elements or as subdomains? What is the justification for this choice of fitting methods?

Response:
Each actin subunit was fitted as a rigid body to the EM map without any structural change. We have added sentences to describe this. The rigid body fitting and the molecular dynamics simulation were sufficient to construct an atomic model which fits well to the EM map up to the resolution limit, around 20 ≈ (Figure 2 and the last part of the Results section).

2nd Editorial Decision 16 December 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75229. Please let me first apologise for the time taken to get back to you with a decision - this was due to the late return of one of the reports. However, all three referees have now seen the revised version of your manuscript, and their comments are appended below. As you will see, referee 2 is now fully satisfied with the revision, but referees 1 and 3 - while fully supportive of publication here - still have a number of concerns with the discussion of the results that I would like to ask you to address before we can accept the manuscript.

Referee 1 still finds that you need to discuss more clearly the importance of ATP hydrolysis rate for determining treadmilling activity. He/she also points out a couple of places where citations need to be changed or added.

Referee 3, on the other hand, is still dissatisfied that you discuss your results almost exclusively in the context of actin treadmilling, and asks again that you broaden your discussion to other aspects of

actin dynamics. I would once again encourage you to take this comment on board when revising the text of your manuscript. He/she also requests again that you make use of the kinetic parameters defined by Fujiwara et al in your modelling of actin dynamics. If you are able to do this, it would clearly be valuable to use the known parameters rather than a simplified version in these analyses.

I would therefore ask you to revise your manuscript according to the referees' comments. Please get in touch if you have any questions or concerns regarding this final revision.

Best wishes,
Editor

The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

General comments
This paper clearly shows the details of the structure of the pointed end of the actin filament assembled from CaATP-actin. The resolution is impressive and the work accounts for a lot of biochemical observations and will have important bearings. The revised version is improved and makes more clearly appear that ATP hydrolysis and not only the kinetic difference between the two ends is at the origin of treadmilling. I still have a few concerns regarding the expression of the significance of the results within the treadmilling function of the filaments (see detailed comments)

Detailed comments
Introduction :
Evidence for filament treadmilling has been clearly demonstrated in stereocilia (works of Bechara Kachar) and in lamellipodia (Lai et al., 2008), as well as in other works (Mizuno and coworkers, Iwasa, etc...).
The review of Korn et al., Science 1987 would be more appropriate regarding the role of ATP hydrolysis than the older review of Korn et al. 1982. The original data showing the existence of a major ADP-Pi-F-actin transient (slow release of Pi) might be cited since they represent the basis of the issues discussed in the paper.
Page 4 : I agree that ATP hydrolysis and Pi release occur on F-actin at such rates that given the kinetic parameters at barbed and pointed ends, at steady state the barbed end and pointed ends do not have the same bound nucleotide, this is well acknowledged. I agree that this difference contributes in establishing the polarity and speed of treadmilling, this is also obvious. However, Wegner predicted treadmilling in 1976 without knowing nor making any assumptions on the mechanism of ATP hydrolysis, simply because ATP hydrolysis is an irreversible reaction which allows monomer-polymer exchanges to be not isoenergetic at the two ends. Hence according to Wegner, could not treadmilling in principle occur if ATP hydrolysis was mechanistically coupled to actin assembly, which would generate ADP bound to each end ?
There is no doubt that the unidirectional movement of the filament takes place toward the end at which actin polymerizes with the lowest critical concentration. The fact that ATP/ADP-Pi maintain strong actin-actin interactions determines the barbed end as the end at which actin polymerizes with the lowest critical concentration. Therefore ATP hydrolysis determines the direction of treadmilling. To be more specific, with CaATP-actin, ATP hydrolysis is very slow, CaATP-actin assembles with identical critical concentrations at the two ends and there is no teadmilling, although the same difference in kinetic parameters exist between the two ends. The present work, which actually is carried out with filaments assembled from CaATP-actin, shows that the structural difference between the two ends exists as well, consistent with the difference in kinetic parameters, yet no treadmilling exists in that case. In conclusion, the difference in kinetic parameters by itself is not sufficient to justify treadmilling if ATP hydrolysis is not considered. I think this should be more

clearly stated than it is in this version (although it is improved). For instance on page 4 ‘The apparent difference in the critical concentrations at the two ends is due to the nucleotide difference, which originates from the difference in the dynamics’ This sentence would be more accurate as follows : ‘ The apparent difference in the critical concentrations at the two ends is due to the nucleotide difference, which originates from the combination of difference in the dynamics and ATP hydrolysis rate’ .
Figure 9 : It is not clear how the concentration of G-actin ´ which is chosen so that the average length of the filament remains constant [a] has been determined. Obviously, this concentration is between the critical concentrations at the two ends, hence in the premices ATP hydrolysis is implied. Therfore it is not surprising that the filament treadmills. In case F the critical concentration at the pointed end is chosen smaller than at the barbed end, which reverses the treadmilling direction. Again this is no surprise.

I agree with the authors that ADF/cofilin enhances treadmilling by increasing the rate of filament disassembly, not by severing the filaments. Severing can only increase the number of ends, which in itself cannot increase the rate of treadmilling per filament, it only increases the global flux. In addition severing cannot in itself cause but transient depolymerization of ADP-actin, not the increase in stationary ATP-G-actin that feeds faster treadmilling (faster barbed end growth), as shown both in vitro and in vivo.

Referee #2 (Remarks to the Author):

In my view, the authors have satisfactorily answered the criticisms of the original version of their manuscript and the revised version is substantially improved. In particular, I agree with their point that morphological differences at the two ends produce the difference in critical concentration rather than vice-versa. This important point is now expressed more clearly.

Referee #3 (Remarks to the Author):

Narita et al. EMBO journal: Structural basis of the unidirectional movement of the actin filament

As noted in the first review, this work is a valuable and interesting addition to our understanding of the structure of actin filaments, providing the first structural insights about why the kinetic properties of the two ends of actin filament are different.

I made several suggestions about how to align the new work with current issues in the field. I am disappointed that the authors ignored some of this advice. A main point was (and is) that our understanding of actin assembly has progressed far beyond the simple idea of treadmilling, since virtually all of the actin assembly reactions have been characterized. In the presence of ATP the net result of lots of reactions at both ends of the filament is very slow treadmilling (<0.1 subunits/s). But by simplifying this to "treadmilling" the authors ignore the actual underlying reactions, which they could and should consider in the context of their novel structural data. A second shortcoming of this approach (including the title) is that their emphasis reinforces the misconception that treadmilling accounts for the turnover of actin filaments in cells. I continue to recommend that the emphasis be changed.

The authors argue "if it is true that cofilin influences treadmilling by severing to create more ends without capping the ends, cofilin will enhance the polymerization of actin because the polymerization rate at a newly created barbed end by cofilin is much faster than the depolymerization rate at the simultaneously created pointed end." This is true in a bulk sample without capping, but the issue addressed by the authors' own structural data is what happens at the ends of each filament.

Given this missed opportunity to address the underlying mechanisms rather than a special case, I advised the authors to drop their focus on treadmilling and address how their work establishes the structural basis for the kinetic differences between the two ends of the filament. The authors

responded that "to address the question, the structure around the bound Pi should be obtained with some clarity, particularly the α-helices should be unambiguously traced. For this to be possible, at least 8 Å resolution is required. Therefore, it remains to be performed in future research." Obviously, much better resolution will be required to determine the intramolecular details, but the discovery of structural differences at the two ends provides the first and only clues about basis for the kinetic differences of the two ends, which deserves attention by the authors rather than deferring the matter for the future.

I suggested that the authors use measured rate constants for their calculations about the behavior of the two ends. The authors replied "the parameters determined by Fujiwara et al. are realistic but complicated. It is useful to introduce the parameter r to make the system simpler and make the important points clearer." I find it difficult to rationalize simplifications when the actual parameters are known. Surely the computer does not worry about this complication and the simulations would be more realistic.

An example of an excellent use of the new data is "the specific conformational changes and the tighter contacts of the two loops at the pointed end likely inhibit the addition of the P+1 actin monomer" and I would add that this is one possible explanation for why the association rate constant is not diffusion limited like the barbed end. In the following sentence I would say "... the fortified loop-to-loop contact may explain the very slow dissociation rate constants at the pointed end, because it is necessary to break the contact for P to dissociate from the filament." This is more specific than "slows down the depolymerization rate", since depolymerization is the net result of many reactions including dissociation. More could be said about how the structure might relate to the other kinetic parameters.

On the other hand the main summary (in the following extended quote) considers what happens at steady state (a condition which may exist in vitro with purified proteins but not in cells), whereas the structural data provides a more general insights, since the rate constants for the reactions apply regardless of the actin monomer concentration: "The slower rate of polymerization at the pointed end due to the loop-to-loop interaction allows a high probability of ATP hydrolysis and phosphate release in the terminal subunit before incorporation of the next monomer, while many ATP-G-actin monomers are polymerized at the barbed end before the hydrolysis occurs. Therefore, the F-actin species at the pointed end should be gradually replaced by ADP-F-actin, which makes the critical concentration at the pointed end higher than the ATP-G-actin concentration in solution, resulting in depolymerization, while at the barbed end ATP-F-actin is predominant, thereby keeping the critical concentration lower than the ATP-G-actin concentration. Collectively, in the steady state, the same amount of depolymerization and polymerization occurs at the pointed end and the barbed end, respectively, and the actin filament moves toward the barbed end without changing its length. Computer simulations confirmed that the slower rates at the pointed end, caused by the loop-to-loop interaction without any other difference between the two ends, accounts for the unidirectional movement toward the barbed end (Figure 9). An alternative model with the same set of critical concentrations, but with the dynamic pointed end and the less dynamic barbed end, reverses the direction of treadmilling (Figure 9F)."

I asked about the charge of divalent cation used in simulations. The authors responded that that used calcium with a +2 charge. They state "We do not believe that this (assumption) affects the result significantly, because the flexible region in the molecular dynamics simulation does not interact with the nucleotide or the divalent cation." I would advise more caution, since others have found that the charge does make a difference in the dynamics of actin family proteins and that a charge closer to +1 gives more reliable simulations.

2nd Revision - authors' response 28 January 2011

Point-by-Point Response to Reviewers

Comments by Referee #1

Comment:

Evidence for filament treadmilling has been clearly demonstrated in stereocilia (works of Bechara Kachar) and in lamellipodia (Lai et al., 2008), as well as in other works (Mizuno and coworkers, Iwasa, etc...).

The review of Korn et al., Science 1987 would be more appropriate regarding the role of ATP hydrolysis than the older review of Korn et al. 1982. The original data showing the existence of a major ADP-Pi-F-actin transient (slow release of Pi) might be cited since they represent the basis of the issues discussed in the paper.

Response:

We thank the reviewer for the advice. In the revised manuscript we have included the suggested citations, i.e., Korn et al., 1987, Lai et al., 2008 and Manor and Kachar., 2008.

Comment:

Page 4 : I agree that ATP hydrolysis and Pi release occur on F-actin at such rates that given the kinetic parameters at barbed and pointed ends, at steady state the barbed end and pointed ends do not have the same bound nucleotide, this is well acknowledged. I agree that this difference contributes in establishing the polarity and speed of treadmilling, this is also obvious. However, Wegner predicted treadmilling in 1976 without knowing nor making any assumptions on the mechanism of ATP hydrolysis, simply because ATP hydrolysis is an irreversible reaction which allows monomer-polymer exchanges to be not isoenergetic at the two ends. Hence according to Wegner, could not treadmilling in principle occur if ATP hydrolysis was mechanistically coupled to actin assembly, which would generate ADP bound to each end ? There is no doubt that the unidirectional movement of the filament takes place toward the end at which actin polymerizes with the lowest critical concentration. The fact that ATP/ADP-Pi maintain strong actin-actin interactions determines the barbed end as the end at which actin polymerizes with the lowest critical concentration. Therefore, ATP hydrolysis determines the direction of treadmilling.

To be more specific, with CaATP-actin, ATP hydrolysis is very slow, CaATP-actin assembles with identical critical concentrations at the two ends and there is no teadmilling, although the same difference in kinetic parameters exist between the two ends. The present work, which actually is carried out with filaments assembled from CaATP-actin, shows that the structural difference between the two ends exists as well, consistent with the difference in kinetic parameters, yet no treadmilling exists in that case.

In conclusion, the difference in kinetic parameters by itself is not sufficient to justify treadmilling if ATP hydrolysis is not considered.

I think this should be more clearly stated than it is in this version (although it is improved). For instance on page 4 'The apparent difference in the critical concentrations at the two ends is due to the nucleotide difference, which originates from the difference in the dynamics ' This sentence would be more accurate as follows : 'The apparent difference in the critical concentrations at the two ends is due to the nucleotide difference, which originates from the combination of difference in the dynamics and ATP hydrolysis rate '

Response:

We agree with the reviewer, it is correct that ATP hydrolysis is essentially required for the unidirectional movement. It was clearly shown by investigations of Ca-actin, whose ATPase is very slow and treadmilling movement is undetectable. However, it does not necessarily mean that ATP hydrolysis determines the direction of the treadmilling movement. Assuming the ATP hydrolysis occurs everywhere in the filament and the Pi release rate is identical at both ends, the actin filament does not move (Fig 9B,D). Furthermore, our simulation clearly showed that the difference in the polymerization and depolymerization rates is enough to determine the direction of treadmilling (Fig9E,F). This was essentially predicted by Wegner, 1976. ATP hydrolysis provides the energy for this unidirectional movement. In the revised manuscript, we have included a phrase to describe the importance of the ATPase in the second paragraph on page 9: ATP hydrolysis is essential to provide energy for movement.

Comment:
Figure 9 : It is not clear how the concentration of G-actin 'which is chosen so that the average length of the filament remains constant ' has been determined. Obviously, this concentration is between the critical concentrations at the two ends, hence in the premices ATP hydrolysis is implied. Therfore it is not surprising that the filament treadmills. In case F the critical concentration at the pointed end is chosen smaller than at the barbed end, which reverses the treadmilling direction. Again this is no surprise.

Response:
Please note that the critical concentration is determined by the nucleotide bound to the actin subunit. We did not change the critical concentration between Fig. 9D, E and F. The only difference was r, the ratio of dynamics rate at the pointed end against that at the barbed end. Thus, r does not change the critical concentration at the ends. The simulation clearly showed that r determines the direction of the movement, not ATP hydrolysis.

Comments by Referee #3

Comment:
I made several suggestions about how to align the new work with current issues in the field. I am disappointed that the authors ignored some of this advice. A main point was (and is) that our understanding of actin assembly has progressed far beyond the simple idea of treadmilling, since virtually all of the actin assembly reactions have been characterized. In the presence of ATP the net result of lots of reactions at both ends of the filament is very slow treadmilling (<0.1 subunits/s). But by simplifying this to "treadmilling" the authors ignore the actual underlying reactions, which they could and should consider in the context of their novel structural data. A second shortcoming of this approach (including the title) is that their emphasis reinforces the misconception that treadmilling accounts for the turnover of actin filaments in cells. I continue to recommend that the emphasis be changed.

Given this missed opportunity to address the underlying mechanisms rather than a special case, I advised the authors to drop their focus on treadmilling and address how their work establishes the structural basis for the kinetic differences between the two ends of the filament.

The authors responded that "to address the question, the structure around the bound Pi should be obtained with some clarity, particularly the α-helices should be unambiguously traced. For this to be possible, at least 8 Å; resolution is required. Therefore, it remains to be performed in future research." Obviously, much better resolution will be required to determine the intramolecular details, but the discovery of structural differences at the two ends provides the first and only clues about basis for the kinetic differences of the two ends, which deserves attention by the authors rather than deferring the matter for the future.

Response:
We thank the reviewer for the helpful comments. In the revised manuscript, we have changed the emphasis on the rate difference between the ends, which largely influences all actin dynamics in the cell.

Comment:
The authors argue "if it is true that cofilin influences treadmilling by severing to create more ends without capping the ends, cofilin will enhance the polymerization of actin because the polymerization rate at a newly created barbed end by cofilin is much faster than the depolymerization rate at the simultaneously created pointed end." This is true in a bulk sample without capping, but the issue addressed by the authors' own structural data is what happens at the ends of each filament.

Response:
We agree with the reviewerís comment. It is very possible that the combination of cofilin and CP accelerate the depolymerization of the actin filament by increasing the number of depolymerizing ends. We consider that this is one mechanism that may accelerate treadmilling in the cell. To avoid

confusing the reader, we have removed the corresponding sentences in the main text.

Comment:
I suggested that the authors use measured rate constants for their calculations about the behavior of the two ends. The authors replied "the parameters determined by Fujiwara et al. are realistic but complicated. It is useful to introduce the parameter r to make the system simpler and make the important points clearer." I find it difficult to rationalize simplifications when the actual parameters are known. Surely the computer does not worry about this complication and the simulations would be more realistic.

Response:
We agree that the parameters archived by Fujiwara et al. are currently the most reliable. However, it was not enough to perform a realistic simulation, as they did not measure the rates with ATP. Fujiwara et al. only cited the parameters used for previous studies with ATP, however, the critical concentrations of the cited parameters at the two ends were significantly different, implying that they are inaccurate due to ATP hydrolysis during the measurements. Many other parameters also have large errors, for example, 0.2 ± 0.1 or 0.06 ± 0.03; therefore, we are not sure that the simulation based on their parameters is more useful than our current simulation with simplified parameters.

Comment:
An example of an excellent use of the new data is "the specific conformational changes and the tighter contacts of the two loops at the pointed end likely inhibit the addition of the P+1 actin monomer" and I would add that this is one possible explanation for why the association rate constant is not diffusion limited like the barbed end. In the following sentence I would say "... the fortified loop-to-loop contact may explain the very slow dissociation rate constants at the pointed end, because it is necessary to break the contact for P to dissociate from the filament." This is more specific than "slows down the depolymerization rate", since depolymerization is the net result of many reactions including dissociation. More could be said about how the structure might relate to the other kinetic parameters.

Response:
We agree with the reviewer and have included the suggested discussion.

Comment:
On the other hand the main summary (in the following extended quote) considers what happens at steady state (a condition which may exist in vitro with purified proteins but not in cells), whereas the structural data provides a more general insights, since the rate constants for the reactions apply regardless of the actin monomer concentration: "The slower rate of polymerization at the pointed end due to the loop-to-loop interaction allows a high probability of ATP hydrolysis and phosphate release in the terminal subunit before incorporation of the next monomer, while many ATP-G-actin monomers are polymerized at the barbed end before the hydrolysis occurs. Therefore, the F-actin species at the pointed end should be gradually replaced by ADP-F-actin, which makes the critical concentration at the pointed end higher than the ATP-G-actin concentration in solution, resulting in depolymerization, while at the barbed end ATP-F-actin is predominant, thereby keeping the critical concentration lower than the ATP-G-actin concentration. Collectively, in the steady state, the same amount of depolymerization and polymerization occurs at the pointed end and the barbed end, respectively, and the actin filament moves toward the barbed end without changing its length. Computer simulations confirmed that the slower rates at the pointed end, caused by the loop-to-loop interaction without any other difference between the two ends, accounts for the unidirectional movement toward the barbed end (Figure 9). An alternative model with the same set of critical concentrations, but with the dynamic pointed end and the less dynamic barbed end, reverses the direction of treadmilling (Figure 9F)."

Response:
We have rewritten the discussion section in the revised manuscript according to the suggestions by the reviewer. The treadmilling movement is treated as an example of actin dynamics in the revised version.

Comment:

I asked about the charge of divalent cation used in simulations. The authors responded that that used calcium with a +2 charge. They state "We do not believe that this (assumption) affects the result significantly, because the flexible region in the molecular dynamics simulation does not interact with the nucleotide or the divalent cation." I would advise more caution, since others have found that the charge does make a difference in the dynamics of actin family proteins and that a charge closer to +1 gives more reliable simulations.

Response:
In response to the reviewerís comment, we performed the same simulations using calcium with a +1.2 charge, and the results were not significantly different. The figure left presents the results of the simulations. The averaged structures of the four independent results using calcium with +2 charge and +1.2 charge are presented in blue and red backbone models, respectively. The viewing angle is the same as that of Figure 5. Similar results were found by (Dalhaimer et al., 2008) that also found that the charge of the cation did not significantly affect the whole structure.

References:
Dalhaimer, P., Pollard, T.D. and Nolen, B.J. (2008) Nucleotide-mediated conformational changes of monomeric actin and Arp3 studied by molecular dynamics simulations. J Mol Biol, 376, 166-183.

Acceptance letter 02 February 2011

Many thanks for submitting the revised version of your manuscript. I have now had the chance to look through it and your response to the previous round of reviews, and I am pleased to be able to tell you that we can now accept it for publication in the EMBO Journal. However, there are just a couple of issues from the editorial side that I need to ask you to deal with first.

I couldn't see whether you have deposited your structural data into the appropriate database (presumably EMDB). For acceptance, we do need to have the data publicly available and the accession codes provided. Please can you let me know if the data has been deposited and, if so, include the codes in the Materials and Methods section. If the data are not in the database, then this needs to be done as soon as possible.

As standard, we now require both Author Contributions and Conflict of Interest statements (below the Acknowledgements section).
If you could send me a new version of the text file including these various things, wecan upload it into our system. Once we have this, we will then be able to accept your manuscript formally for publication in the EMBO Journal.

Many thanks and best wishes,

Editor
The EMBO Journal

