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## Mechanism of Nucleotide Sensing in Group II Chaperonins

Jose H. Pereira, Corie Y. Ralston, Nikolai R. Douglas, Ramya Kumar, Tom Lopez, Ryan P. McAndrew, Kelly M. Knee, Jonathan A. King, Judith Frydman and Paul D. Adams

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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 June 2011

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Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, we experienced difficulties with the availability of suitable and willing referees. In the meantime, three referees have now evaluated the manuscript and their comments are shown below. As you will see, while referee 1 and referee 2 are more positive, referee 3 is not in favour of publication of the study here. On balance, we have come to the conclusion to give you the chance to address the referees' concerns in a revised version of this manuscript. However, additional functional/biochemical experimentation will be needed along the lines put forward by all three referees and all actual concerns need to be addressed or responded to in an adequate manner and to the satisfaction of the referees. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version and the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:  
<http://www.nature.com/emboj/about/process.html>

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of 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.

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

Yours sincerely,

Editor  
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper describes the crystal structures of an archaeal chaperonin in different nucleotide states. In addition, a limited amount of biochemical and kinetic data is provided. The paper contributes to our understanding of the conformational changes that occur upon ATP binding and hydrolysis and how they might propagate around the ring and is very well written. Additional insight would be obtained from more extensive kinetic analysis but that is probably beyond the scope of this study. A revision must, however, address the first point below.

1. Figure 5 - the comparison between the rates of wt and the mutants is valid only if the ATP concentration corresponds to  $V_{max}$  for all them. This can (and should) be shown by doing the assays also at a higher (say two-fold) concentration to test whether the rate does not increase. The comparison also requires that the protein concentrations of wt and the mutant are the same.

2. ATPase data for the E164A mutant by itself would allow in combination with the data for wt, K161A and the double mutant to determine whether K161 and E164 interact with each other indirectly (i.e. double-mutant cycle analysis).

Minor comments:

1. p. 16, 4 lines from bottom - what do the authors mean by 'these residues are not involved in the ATP hydrolysis mechanism' given that they make direct interactions with nucleotide? How do the authors define mechanism?
2. Legend to Fig. 4A - The position of the nucleotide in the Figure should be stated.

Referee #2 (Remarks to the Author):

In the manuscript "Mechanism of nucleotide sensing in group II chaperonins", the authors report a high resolution structure of the *Methanococcus marisaludis* group II chaperonin (Cpn) and biochemical experiments for the identification of the nucleotide sensing loops in the subunits. The results advance our understanding of this chaperone system. Some of the conclusions need to be further supported and some points need to be clarified in the manuscript.

Points to be addressed:

1. The binding site for substrate proteins is described as a loop close to helix-1 in the open structure of Cpn and a mechanism for substrate release is discussed upon formation of the closed conformation of Cpn. However, reference to the literature or experiments (like crosslinking) to support the assumption that the binding site is exclusively located in this loop close to helix-11 are missing.
2. The role attributed to Lys161 is not clear. On the one hand it is described as interacting with the

gamma phosphate of ATP (page 8), and on the other hand it is described as forming a salt bridge with Asp60 in the AMP-PNP-bound state of Cpn (page 9). However, only the gamma phosphate interaction is shown in figure 2B while Asp60 is not depicted at all. This point needs clarification also on the structural level.

3. The authors perform a protease cleavage assay to test for the opening and closing of Cpn upon binding of different nucleotides. ATP and ADP-ALFx induce a closed conformation. Experiments with ADP are missing. These should be added to the manuscript.

4. The authors show a less efficient protection against protease cleavage of the identified nucleotide sensing loop mutants. The authors conclude that the dynamics of the Cpn mutants are altered. ATPase activity assays revealed reduced turnover rates. To further analyze the differences between the mutants and wild type Cpn, the KM values for ATP would be important to know in order to be able to exclude an effect of the mutations on the affinity for the nucleotide. Additionally, the analysis of the E164A single mutant seems important for being able to pinpoint the effects observed in the K161A/E164A double mutant. A table summarizing the ATPase data could be useful.

5. The nucleotides used to crystallize the open and closed conformations of Cpn should be mentioned in figure 1.

Referee #3 (Remarks to the Author):

The manuscript submitted by Pereira et al. described the crystal structure of a group II chaperonin in several nucleotides bound states. There are observations that are potentially significant, such as the nucleotide-sensing loop (NSL) in this complex. But, the paper is written without adequate depth and rigor to demonstrate how significant this new finding is. In addition, it is rather poorly written, often confusing. Specific comments are listed below. In total, this manuscript does not attain the level of significance and rigor to merit publication in EMBO Journal.

Major points:

1. Most of the new findings in the manuscript are derived from the crystal structures of mutant chaperonin, called Cpn-rls (release loop for the substrate). First of all, I have a concern about the use of the mutant to draw the conclusion. Regarding the point, confusing is that supplemental information contains wild-type Cpn structure complexed with AMPPNP, which has been appeared in previous paper by the authors. Why did the authors stick to the mutant Cpn throughout the manuscript? Another confusion is that biochemical analyses (Fig. 5) used wild type chaperonin and the derivatives. Lack of consistency significantly weakens the impact of the new finding.

2. Biochemical analyses are insufficient to confirm the significance of NSL. Both proteinase K protection and ATP hydrolysis assay are too indirect, or even ambiguous, to demonstrate the biological significance of NSL. Instead, I recommend direct measuring of affinities between nucleotides and chaperonin. For the functional significance, chaperonin-assisted folding experiment using NSL mutants should be conducted.

3. The authors do not describe anything about allosteric property in the nucleotide-bound chaperonin complexes although they described a lot in introduction section. I am interested in the mode of inter-ring communication, if any, which might be important for the significance of NSL.

Minor points:

4. Introduction section contains many sentences that are not connected to the topics in the manuscript. For example, description on group III chaperonins and allosteric properties should be omitted or written concisely. Instead, the section should summarize previous studies on chaperonin structures complexed with nucleotides.

5. The relation between Asp60, previously assigned critical residue on ATP hydrolysis, and NSL is

unclear. At least, the authors should explicitly show the position of those regions and discuss about the role on chaperonin function.

6. The quality of ATPase assay in Fig. 5C is very low. In particular, first several data points are too fluctuating.

7. In Fig. 5B, ATPase activity of G160S mutant is low compared to K161A or K161A/E164A mutants. However, all of PK protection experiments using those mutants are appeared to be the same (Fig. 5A). If the ATPase deficiencies in the mutants are related to the partial digestion by PK, there might be difference in the extent of digestion in a long time-scales.

1st Revision - authors' response

06 October 2011

Referee #1:

*This paper describes the crystal structures of an archaeal chaperonin in different nucleotide states. In addition, a limited amount of biochemical and kinetic data is provided. The paper contributes to our understanding of the conformational changes that occur upon ATP binding and hydrolysis and how they might propagate around the ring and is very well written. Additional insight would be obtained from more extensive kinetic analysis but that is probably beyond the scope of this study. A revision must, however, address the first point below.*

*1. Figure 5 - the comparison between the rates of wt and the mutants is valid only if the ATP concentration corresponds to Vmax for all them. This can (and should) be shown by doing the assays also at a higher (say two-fold) concentration to test whether the rate does not increase. The comparison also requires that the protein concentrations of wt and the mutant are the same.*

The Cpn has a measured  $K_m$  of  $5.8 \pm 0.3 \mu\text{M}$  (Reissmann et al., 2007). Therefore the initial ATPase activity assays using the ATP concentration of  $1000 \mu\text{M}$  (1mM) correspond to  $V_{\text{max}}$  of the protein. The ATPase assays were carried out at the same protein concentration ( $0.25 \mu\text{M}$ ) for all Cpn variants. However, now we have also performed additional ATPase activity assays using a 2-fold increased ATP concentration (2mM). These experiments confirm that the ATPase rate does not increase at higher ATP concentration. ATPase activity plots for Cpn-WT, Cpn-G160S, Cpn-K161A, and Cpn-E164A using a 2-fold increased ATP concentration (2mM) have been added to Figure 4B. In addition to the plots showing the ATPase activity, a table summarizing the data with S.E.M (Standard Error of the Mean) has also been included (Table-II).

Figure 4B

Table-II – ATPase activity for Cpn-WT, Cpn-G160S, Cpn-K161A, and Cpn-E164A.

	1mM ATP	2mM ATP
Cpn-WT	$15.38 \pm 0.4031$	$12.49 \pm 1.018$
Cpn-G160S	$0.205 \pm 0.2522$	$-0.189 \pm 0.5272$
Cpn-K161A	$4.018 \pm 0.2700$	$3.732 \pm 0.3664$
Cpn-E164A	$2.122 \pm 0.2837$	$1.137 \pm 0.2834$

ATPase activity measured as ATP hydrolyzed/minute ( $\mu\text{M}\cdot\text{min}^{-1}$ ), with standard error of the mean.

The follow sentence has been added to manuscript (Page 11). “In order to compare the ATPase activity amongst Cpn-WT and the NSL mutants, we performed the assay using an ATP concentration of 1mM, corresponding to  $V_{max}$  of the Cpn. Additionally, the assay was performed with a 2-fold increase in ATP concentration (2mM) yielding ATPase rates consistent with those observed at 1mM ATP (Fig. 4b and Table-II).”

2. ATPase data for the E164A mutant by itself would allow in combination with the data for wt, K161A and the double mutant to determine whether K161 and E164 interact with each other indirectly (i.e. double-mutant cycle analysis).

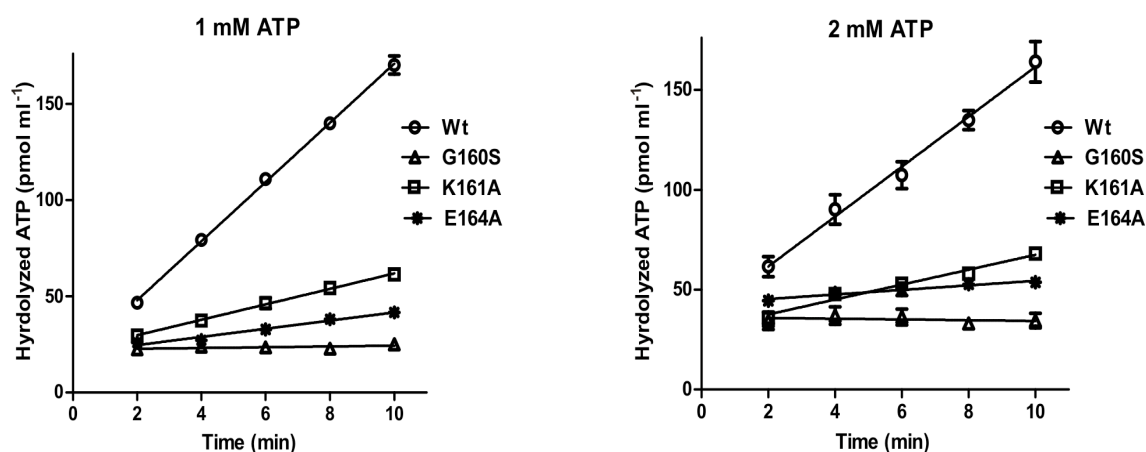
The single mutant Cpn-E164A was created and it has a significant reduction in ATPase activity as observed in the other NSL mutants. The follow discussion has been added to manuscript (Page 12) “The Cpn-E164A mutant also shows a strong reduction in its ATPase activity compared to Cpn-WT. Intriguingly, Glu-164 is located approximately 15 Å away from the nucleotide-binding site in either the AMP-PNP state or ADP state. Consequently, residue Glu-164 has no contact with nucleotide in either conformation and cannot be directly involved in nucleotide sensing or ATP hydrolysis. However, Glu-164 does make direct contact with the residues of the neighboring subunits in the AMP-PNP state (Fig. 5). Therefore, the reduction in ATPase activity for the Cpn-E164A mutant suggests this residue plays a role in coupling lateral subunits in the ring to achieve optimal ATPase activity.”

#### Minor comments:

1. p. 16, 4 lines from bottom - what do the authors mean by 'these residues are not involved in the ATP hydrolysis mechanism' given that they make direct interactions with nucleotide? How do the authors define mechanism?

The mechanism of ATP hydrolysis for group II chaperonin has been described previously by Ditzel and collaborators (Ditzel et al, 1998) using the first crystal structure of this class of protein. Based on the proposed mechanism of ATP hydrolysis, the NSL residues Gly-160 and Lys-161 were not identified as being involved directly in the hydrolysis of the  $\gamma$ -phosphate - residues Asp-60 and Asp-386 play the central role in this activity. In our manuscript we emphasize that the NSL is important for sensing the nucleotide state, but the ATP hydrolysis can still occur even with the NSL mutant forms. Therefore the sentence on Page 16, “These residues are not involved in the ATP hydrolysis

**B)**

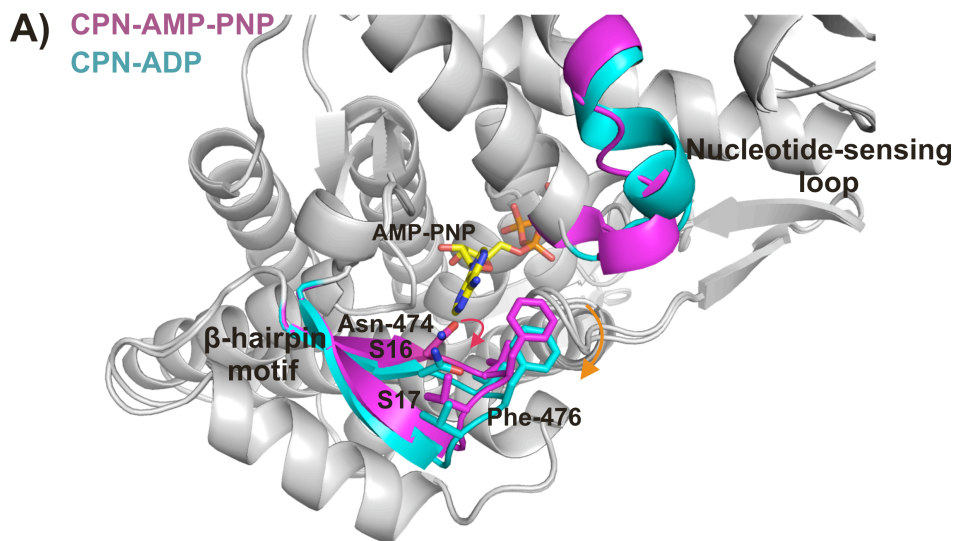


mechanism, as determined by protease digestion assays with NSL mutant proteins.” has been changed to “The NSL residues are not essential for ATP hydrolysis and lid closure, as determined by protease digestion assays with NSL mutant proteins.”

2. Legend to Fig. 4A - The position of the nucleotide in the Figure should be stated.

The position of the nucleotide is now specified in the new Figure 3A (former Fig.4A).

Figure 3A



Referee #2:

*In the manuscript "Mechanism of nucleotide sensing in group II chaperonins", the authors report a high resolution structure of the Methanococcus maripaludis group II chaperonin (Cpn) and biochemical experiments for the identification of the nucleotide sensing loops in the subunits. The results advance our understanding of this chaperone system. Some of the conclusions need to further supported and some points need to be clarified in the manuscript.*

*Points to be addressed:*

*1. The binding site for substrate proteins is described as a loop close to helix-11 in the open structure of Cpn and a mechanism for substrate release is discussed upon formation of the closed conformation of Cpn. However, reference to the literature or experiments (like crosslinking) to support the assumption that the binding site is exclusively located in this loop close to helix-11 are missing.*

The reference describing that the substrate-binding site for Group II chaperonin is located close to helix-11, based on crosslinking experiments, has been added to manuscript (Page 2, Supplemental Material), "The substrate binding site for a group II chaperonin and one of its client proteins has been identified and has been mapped to a location along helix-11 in the apical domain (Spiess et al, 2006). This, in combination with the open and closed structures of Cpn (Zhang et al, 2010; Pereira et al, 2010) led to a hypothesis of how the substrate is released into the central chamber (Douglas et al, 2011)."

Reference

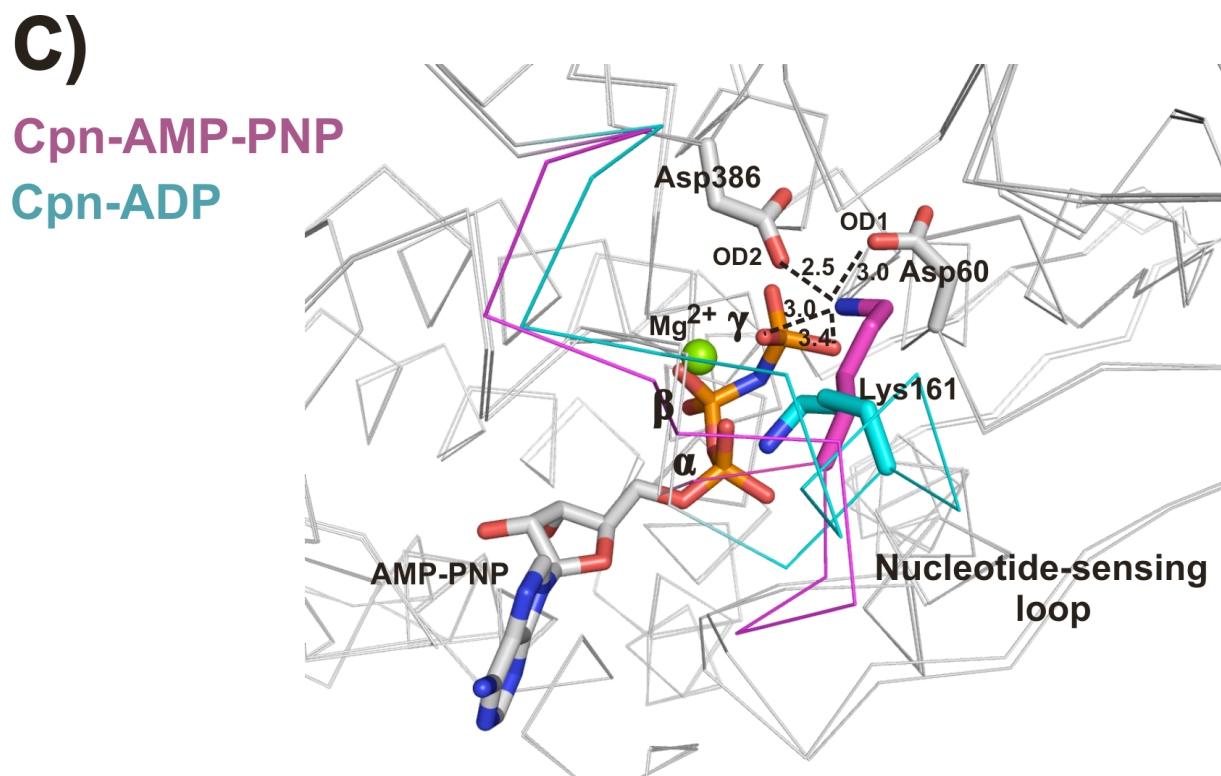
Spiess C, Miller EJ, McClellan AJ, Frydman J (2006) Identification of the TRiC/CCT substrate binding sites uncovers the function of subunit diversity in eukaryotic chaperonins. *Molecular Cell* 24: 25-37.

*2. The role attributed to Lys161 is not clear. On the one hand it is described as interacting with the gamma phosphate of ATP (page 8), and on the other hand it is described as forming a salt bridge with Asp60 in the AMP-PNP-bound state of Cpn (page 9). However, only the gamma phosphate interaction is shown in figure 2B while Asp60 is not depicted at all. This point needs clarification also on the structural level.*

The function attributed to Lys-161 is as an ATP sensor, as we described on page 8 of the manuscript. To help clarify how Lys-161 acts as an ATP sensor from the NSL, we have included a detailed structural analysis on Page 7, which includes the catalytic residues Asp-60 and Asp-386, visualized in the AMP-PNP-bound state: "The Cpn-AMP-PNP state shows two residues located in

the NSL that interact directly with the phosphates of the bound nucleotide. The residues Gly-160 and Lys-161 make direct interactions with the  $\alpha$  and  $\gamma$ -phosphate, respectively. In the Cpn-ADP state, the main-chain carbonyl group of residue Gly-160 is no longer interacting with the  $\alpha$ -phosphate and Lys-161 lacks the contact with the  $\gamma$ -phosphate (Fig. 1b). The position assumed by Lys-161 in the Cpn-AMP-PNP state, due to the presence of the  $\gamma$ -phosphate, promotes an interaction of this residue via salt-bridges with the two catalytic residues Asp-60 and Asp-386 (Fig. 1c). The position of Lys-161 in the Cpn-AMP-PNP state may also be important for neutralizing the charges around the  $\gamma$ -phosphate group. The hydrolysis of the  $\gamma$ -phosphate group promotes the rearrangement of residue Lys-161, however, this conformational change does not affect the position of the core catalytic residues Asp-60 and Asp-386, which show similar locations in both the Cpn-AMP-PNP and Cpn-ADP states. The rearrangement of Lys-161, associated with the absence of the  $\gamma$ -phosphate, supports the theory that Lys-161 acts as an ATP sensor. We propose that this is a general mechanism for group II chaperonins since this residue is strictly conserved within this class of chaperonins (Fig. 2)". In addition to this structural discussion, an extra figure (Fig. 1c) has been added to illustrate all of the interactions involving the NSL residue Lys-161.

Figure 1C



3. The authors perform a protease cleavage assay to test for the opening and closing of Cpn upon binding of different nucleotides. ATP and ADP-ALFx induce a closed conformation. Experiments with ADP are missing. These should be added to the manuscript.

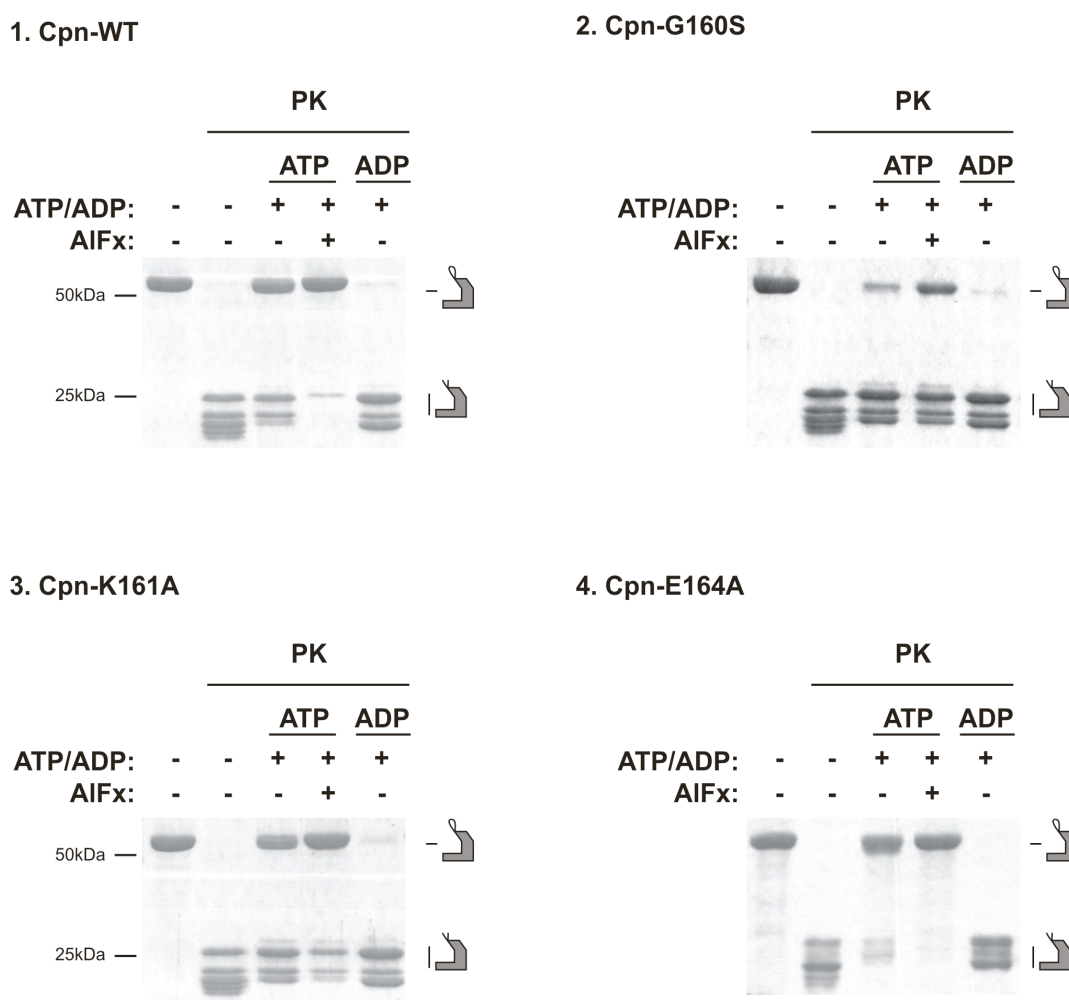
Proteinase-K digestion results for Cpn-WT and for all NSL mutants using ADP have been added to the manuscript (Figure 4a) (Page 11) "The Cpn-WT and Cpn mutants were incubated with either ADP, ATP, or the ATP hydrolysis mimics ATP•AlFx. Incubation with ADP (Fig 4a, compare +ADP lanes) did not protect the Cpn from PK digestion, however, the presence of ATP, or



ATP•AIFx promoted either full or partial protection (See Fig. 4). Despite mutations in the NSL region, all mutants were still capable of changing conformation to the closed, PK protected, state. This analysis strongly suggests that residues Gly-160, Lys-161 and Glu-164 are not absolutely required for ATP hydrolysis, since the mutants (Cpn-G160S, Cpn-K161A, and Cpn-E164A) are still capable of sampling the closed conformation when incubated with nucleotide or the hydrolysis mimic. However, we do observe variations in the levels of PK digestion of the mutants, compared to Cpn-WT, suggesting that these residues are critical for linking the ATPase activity of the complex to the conformational cycling from the open to closed state.”

Figure 4a

A)



4. The authors show a less efficient protection against protease cleavage of the identified nucleotide sensing loop mutants. The authors conclude that the dynamics of the Cpn mutants are altered. ATPase activity assays revealed reduced turnover rates. To further analyze the differences between the mutants and wild type Cpn, the KM values for ATP would be important to know in order to be able to exclude an effect of the mutations on the affinity for the nucleotide. Additionally, the analysis of the E164A single mutant seems important for being able to pinpoint the effects observed in the K161A/E164A double mutant. A table summarizing the ATPase data could be useful.

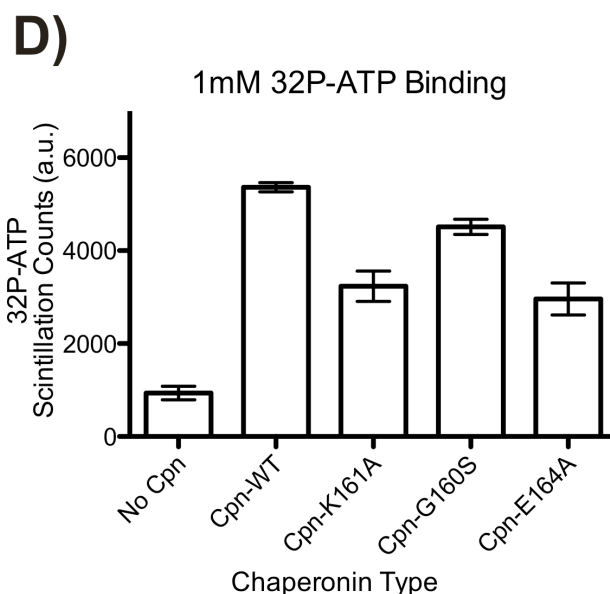
Affinity measurements determining Km are outside the scope of the work presented in our manuscript. However, chaperonins are highly allosteric protein machines. Subunits within each ring are coupled though positive cooperativity in ATP binding, while negative cooperativity between



rings inhibits ATP binding. Therefore, ATP binding affinity depends on communication between individual subunits. ATP binding assays were performed (Figure 4d) and the following discussion was added to (Page 13).

“The NSL mutants all show a reduced ATPase activity. To probe the influence of the NSL on the affinity for ATP we performed ATP binding assays using radiolabelled ATP (Fig. 4d). The ATP binding to one subunit enhances ATP association with subunits in the same ring (Reissmann et al, 2007). Therefore, ATP binding affinity is dependent on communication between subunits. Both Cpn-K161A and Cpn-E164A mutants show a modest decrease in ATP binding affinity compared to Cpn-WT (Fig. 4d). Intriguingly, the Cpn-G160S mutant displayed an ATP binding affinity close to wild type, but greatly lowered ATPase activity (Fig. 4c). This result, in conjunction with the proteinase-K assay, suggests that the binding of ATP by Cpn-G160S is similar as Cpn-WT but the timing for the conformation cycle is changed.

The reduced ATP affinity for the Cpn-K161 mutant may result from a combination of disruption of communication of  $\gamma$ -phosphate state between subunits, and the loss of direct stabilizing interactions between the lysine nitrogen and the ATP  $\gamma$ -phosphate. The Cpn-E164A mutant has a diminished capacity for ATP binding. The location of residue E164 some 15 Å from the active site argues that this effect cannot be a result of direct interaction with the ATP. This further supports a role for E164 in intra-ring communication and a potential impact on positive cooperativity within the ring arising



from the mutation.”

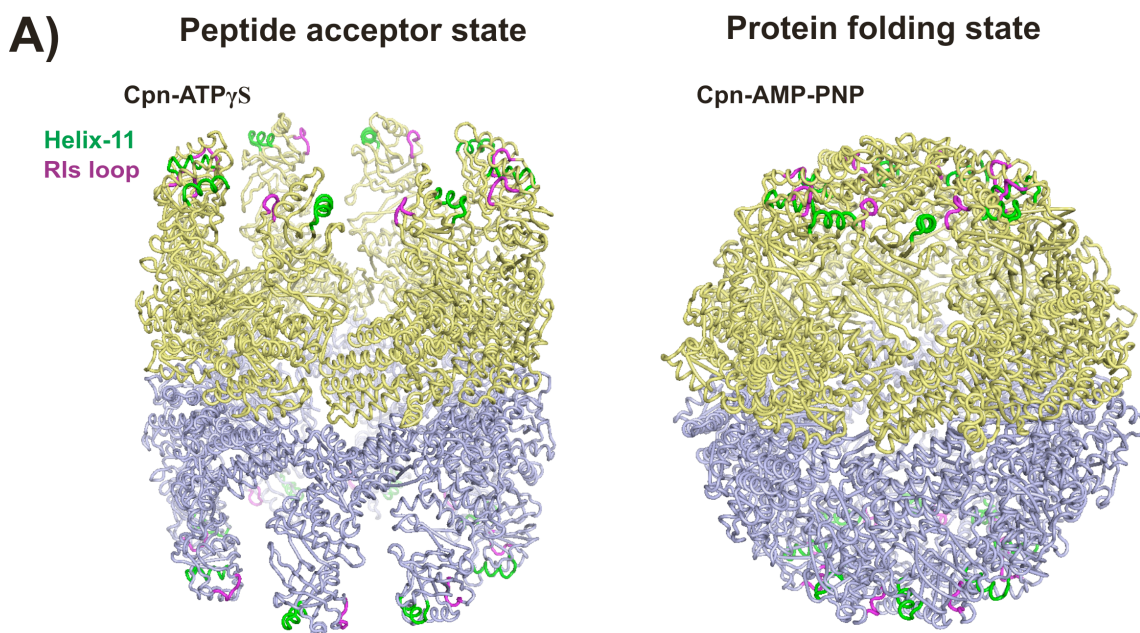
Additionally, the ATPase activity of the chaperonin was measured at both 1 and 2mM ATP, and the resulting ATPase rates are nearly the same (with the exception of Cpn-E164A which, if anything, decreases at higher ATP concentration). If it were the case that we had drastically altered ATP binding in the mutants, this would likely be reflected in the ATPase measurements at higher ATP concentration.

In the revised manuscript we focus our discussion on the single mutant, E164A, rather than the double mutant (K161A/E164A), as the latter does not add anything more to our analysis. We have therefore moved the description of the K161A/E164A double mutant to the Supplemental Materials.

Additionally, we have added a table summarizing the ATPase results for the Cpn-WT and all the NSL mutants for easier comparison of the ATPase rates (Table II).

5. The nucleotides used to crystallize the open and closed conformations of Cpn should be mentioned in figure 1.

The nucleotides used in the open and closed conformations of Cpn structures have been added to Figure S1A.



Referee #3:

*The manuscript submitted by Pereira et al. described the crystal structure of a group II chaperonin in several nucleotides bound states. There are observations that are potentially significant, such as the nucleotide-sensing loop (NSL) in this complex. But, the paper is written without adequate depth and rigor to demonstrate how significant this new finding is. In addition, it is rather poorly written, often confusing. Specific comments are listed below. In total, this manuscript does not attain the level of significance and rigor to merit publication in EMBO Journal.*

*Major points:*

*1. Most of the new findings in the manuscript are derived from the crystal structures of mutant chaperonin, called Cpn-rls (release loop for the substrate). First of all, I have a concern about the use of the mutant to draw the conclusion. Regarding the point, confusing is that supplemental information contains wild-type Cpn structure complexed with AMPPNP, which has been appeared in previous paper by the authors. Why did the authors stick to the mutant Cpn throughout the manuscript? Another confusion is that biochemical analyses (Fig. 5) used wild type chaperonin and the derivatives. Lack of consistency significantly weakens the impact of the new finding.*

The purpose of using the mutant Cpn-rls to study the conformational change around the nucleotide-binding site is because the crystals of Cpn-rls diffracted at significant higher resolution compared to the Cpn-WT crystals. At this resolution it is possible to make significantly better interpretations of the detailed structural changes. However, realizing that the changes could be somehow related to the mutants we also solve, and describe in the manuscript, the crystal structures of Cpn-WT in complex with nucleotides in order to confirm that the conformational changes observed between the high-resolution structures of Cpn-rls in complex with AMP-PNP and ADP (pre- or post-hydrolysis states) are not a result of the rls-loop mutations. Comparison of Cpn-WT-AMP-PNP and Cpn-WT-ADP confirmed the same conformational changes observed in Cpn-rls, albeit at lower crystallographic resolution. Therefore, our conclusions are not solely based on the mutant structures, and are corroborated by the same analysis of the wildtype structures.

We have clarified the text in response to the referee's comment by rewriting the section that introduces the crystals structures of wildtype and mutant chaperonin, and emphasizing that we used both structures in the manuscript. In addition, we have been moved the detailed discussion about the Cpn-rls mutant to Supplemental Material. The follow sentence was added to the paper (Page 5),

“Fortuitously, when we obtained crystals of a substrate release mutant (Douglas et al, 2011) of the chaperonin (Cpn-rls) in complex with AMP-PNP, we observed a significantly higher resolution of X-ray diffraction (see Table-I for details). This allowed us to identify the detailed effect of different nucleotide states on the Cpn structure. However, we also solved the structure of the Cpn-WT protein in complex with ADP in order to eliminate the possibility that the conformational changes were actually a result of the rls loop mutations. A detailed structural analysis of Cpn-rls structures can be found in Supplemental Material section. Briefly, we observed that conformational changes in the nucleotide region are preserved between wildtype and rls-mutant, the only minor differences in the latter being in the region of the rls loop in the apical domain (Supplemental Fig. S1b). We therefore use the more general Cpn nomenclature in the remainder of this manuscript when discussing the structural changes, referring to both wildtype and rls-mutant structures.”

*2. Biochemical analyses are insufficient to confirm the significance of NSL. Both proteinase K protection and ATP hydrolysis assay are too indirect, or even ambiguous, to demonstrate the biological significance of NSL. Instead, I recommend direct measuring of affinities between nucleotides and chaperonin. For the functional significance, chaperonin-assisted folding experiment using NSL mutants should be conducted.*

Our study is focused on understanding the structural changes we observe as related to different nucleotide states. To support the structures described here, biochemical analyses, such as ATPase activity and dynamics of lid closure by PK digestion assays, were undertaken. The ability of the complex to undergo ring closure is probed directly by the PK digestion assays. Lid closure is critical for the chaperonin folding action, making the PK digestion assay a valuable indicator of ability to fold substrates. The work outlined in the manuscript is intended to provide insight into the coupling of the ATPase activity to the conformation changes in the chaperonin from the open to closed state. An investigation into how these dynamics are coupled to the folding of substrates inside the chamber is currently underway. However, at present the substrate folding/dynamics in the chaperonin chamber is outside the focus (and scope) of our work.

To determine the impact of the mutants on nucleotide binding we have performed filter-binding assays with radiolabelled ATP (Figure 4d), as discussed above in “Referee #2, point 4”

*3. The authors do not describe anything about allosteric property in the nucleotide-bound chaperonin complexes although they described a lot in introduction section. I am interested in the mode of inter-ring communication, if any, which might be important for the significance of NSL.*

A detailed study of the allosteric properties of the chaperonin would be interesting we agree,. However, our goal in this manuscript is to identify the NSL and verify its role in sensing nucleotide at a structural and biochemical level. Therefore, we have modified our introduction to the allosteric properties of Group II chaperonins by concisely re-writing part of the Introduction (Page 4), “The ring movements involved in the folding cycle of group II chaperonins are coordinated in time and space via a complex allosteric regulation (Reissmann et al, 2007). ATP binding to one subunit enhances ATP association with subunits in the same ring, whereas ATP binding to one ring inhibits ATP association with the subunits of the adjacent ring. The synchronized ring action depends on communication between individual subunits (Horovitz et al, 2001).” Concerning inter-ring communication, the following sentences have been added (Page 16), “Consistent with all structures of Group II chaperonins obtained to date, we observe symmetric complexes, and there are no differences at the inter-ring interface comparing the Cpn-ADP and Cpn-AMP-PNP states. To observe a difference between the upper and lower rings it is likely that the complex needs be trapped in a state with different nucleotide species in each ring.”

#### Minor points:

*4. Introduction section contains many sentences that are not connected to the topics in the manuscript. For example, description on group III chaperonins and allosteric properties should be omitted or written concisely. Instead, the section should summarize previous studies on chaperonin structures complexed with nucleotides.*

The description of the Group III chaperonins has been omitted,

The allosteric properties of Group II chaperonins have been re-written concisely in the introduction section (as discussed in the previous point).

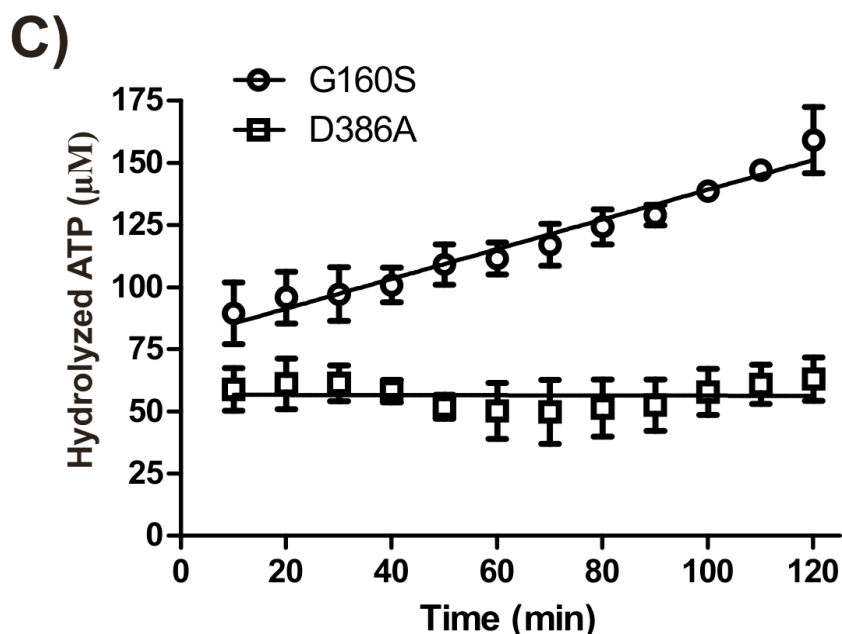
A brief description of previous studies of the Group II chaperonin complexed with nucleotide has been added to the Introduction section (Page 4), “Recently, a crystal structure of an open state Group II chaperonin in complex with ADP (Huo et al., 2010) showed a rotation of the apical domain by  $\sim 30^\circ$  compared to the open Cpn structure in complex with ATP $\gamma$ S nucleotide (Pereira et al., 2010). ATP-binding alone causes a counter-clockwise rotation of the apical domain. Subsequent ATP hydrolysis drives the subunits to close the chamber completely (Zhang et al., 2011).” Also, in the Results section (Page 15), “The crystal structure of a group II chaperonin in complex with ADP and AIF<sub>x</sub> from *Thermoplasma acidophilum* (Ditzel et al, 1998) provided crucial structural information about the ATP hydrolysis mechanism. The catalytic residues Asp-63 and Asp-398 (Asp-60 and Asp-386 in Cpn) contribute to stabilizing, by hydrogen bonds, the attacking nucleophile water used for ATP hydrolysis.”

5. The relation between Asp60, previously assigned critical residue on ATP hydrolysis, and NSL is unclear. At least, the authors should explicitly show the position of those regions and discuss about the role on chaperonin function.

In response to comments by referee #2, a detailed explanation of the interaction of Asp-60 and NSL (Lys-161) was added on Page 7. Also, an additional figure (Figure 1c) was created to illustrate the position of catalytic residues Asp-60 and Asp-386, NSL residues and nucleotide (AMP-PNP).

6. The quality of ATPase assay in Fig. 5C is very low. In particular, first several data points are too fluctuating.

A new long-time course ATPase experiments (2-hours) using the single mutant Cpn-G160S and the catalytic dead mutant Cpn-D386A has been performed. A new Figure 5c is included with much lower S.E.M.



7. In Fig. 5B, ATPase activity of G160S mutant is low compared to K161A or K161A/E164A mutants. However, all of PK protection experiments using those mutants are appeared to be the same (Fig. 5A). If the ATPase deficiencies in the mutants are related to the partial digestion by PK, there might be difference in the extent of digestion in a long time-scales.

The PK assay is a measure of the chaperonin's ability to access the open and closed states, and the relative residence time in each state. Therefore, for a chaperonin mutant that samples these two states more slowly (e.g. a slower ATPase rate), there will be a larger population of chaperonins in the open state at any given time. Indeed, as can be observed in figure 4a (Cpn-G160S) (former Fig. 5B) the PK digestion is qualitatively more pronounced than that observed in either the Cpn-K161A or Cpn-E164A (compare "+ATP" lane for mutants). The PK digestion, as a measure of the dynamic sampling of the open and closed state, which is driven by the ATPase activity, is consistent with our ATPase measurements (the Cpn-G160S mutant is a slower ATPase, and is digested more rapidly than the Cpn-K161A or Cpn-E164A). Performing PK digestions for longer time-scales will eventually digest the entire population of Cpn's as they all able to cycle in the presence of ATP. This would diminish the utility of the assay, as differences in the PK digestion would be flattened to the noise.

2nd Editorial Decision

31 October 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I would like to ask you to address the minor issues suggested by referee 1 (see below).

Furthermore, there are two editorial issues that need further attention:

First, please include the number of independent repeats for table II, figure 4B-D, supplementary figure S3B, C.

Second, in your supplementary materials section, you essentially include descriptions of 'supplementary analyses' similar to a bona fide results section. According to our policies, there should be no such supplementary results section. The Supplement should only contain supplementary figures, together with figure legends, and more detailed descriptions of materials and methods if needed. I therefore need to ask you to include descriptions of these analyses into the main body of the manuscript text, referring to the supplementary figures.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor  
The EMBO Journal

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REFeree COMMENTS

Referee #1:

Many of the issues that remain open in this paper could be resolved by a more comprehensive kinetic analysis (e.g. measuring binding/hydrolysis over a range of concentrations as opposed to one concentration). However, given the structural work described in this paper such an analysis may be beyond the scope of the present study. A further small revision that addresses the points below is necessary.

Comments:

1. p. 4 - AIFx is a mimic of the gamma-phosphate, and, therefore, it does not make sense to write ATP-AIFx. The analogue is ADP-AIFx.

2. The various group II chaperonin structures are discussed in the paper except for the recently determined one by Willison and co-workers. This omission should be corrected.
3. The decrease in the ATPase activity at higher ATP concentrations is by 50% in the case of E164A which is hardly a subtle effect. There is a small decrease also in the cases of the other variants (BTW, why the negative sign in the case of G160S?). A decrease in the ATPase activity at high ATP concentrations has been observed in the case of GroEL (Yifrach & Horovitz, 1995) but never before for group II chaperonins. The authors should comment on that.
4. p. 4 - The authors cite Reissmann et al. (2007) for allosteric regulation in group II chaperonins but the first papers were by Kafri et al. (2001, 2003) and they should be cited.
5. Fig. 4D - it is very dangerous to determine binding affinity on the basis of one ligand concentration. Errors in the concentrations of the cpns could easily distort the results.
6. Legend to Fig. 1C - I would write 'in the AMP-PNP state' and not 'on AMP-PNP state'.
7. The concentration of cpn is not mentioned in the Legend to Fig. 4 or in the Methods.

Referee #2:

The authors have addressed all queries raised and answered them in a satisfactory manner. The new data included in the revised version make the paper stronger and suitable for publication in the EMBO Journal.

Referee #3:

The authors have improved the manuscript significantly after the revision. All points I raised in my last review were addressed, and the manuscript has been modified accordingly. The revision was adequate and there are new results that improved the manuscript.

2nd Revision - authors' response

24 November 2011

Referee #1

*1. p. 4 - AlFx is a mimic of the gamma-phosphate, and, therefore, it does not make sense to write ATP-AlFx. The analogue is ADP-AlFx.*

We could not find any "ATP-AlFx" nomenclature on page 4. However, we changed a sentence on Page 11. "The Cpn-WT and Cpn mutants were incubated with either ADP, ATP, or ATP hydrolysis mimic ATP-AlFx" to "The Cpn-WT and Cpn mutants were incubated with either ADP, ATP, or ATP-AlFx".

*2. The various group II chaperonin structures are discussed in the paper except for the recently determined one by Willison and co-workers. This omission should be corrected.*

A description about the recently published structure of yeast CCT was added to the manuscript. Page 4, "A closed state structure of yeast chaperonin in complex with substrate (actin) revealed an asymmetry configuration of the rings (Dekker et al, 2011)".

Dekker C, Roe SM, McCormack EA, Beuron F, Pearl LH, Willison KR (2011) The crystal structure of yeast CCT reveals intrinsic asymmetry of eukaryotic chaperonins. EMBO J. 30: 3078–3090

3. *The decrease in the ATPase activity at higher ATP concentrations is by 50% in the case of E164A which is hardly a subtle effect. There is a small decrease also in the cases of the other variants (BTW, why the negative sign in the case of G160S?). A decrease in the ATPase activity at high ATP concentrations has been observed in the case of GroEL (Yifrach & Horovitz, 1995) but never before for group II chaperonins. The authors should comment on that.*

- We revised the discussion about ATPase results for Cpn-E164 mutant. The “subtle” interpretation about the decrease of ATPase rate for Cpn-E164A was changed on the manuscript.
- The negative sign in front of Cpn-G160S is because the slope is small (and there is quite a bit of variability over a short time-scale). This is the reason why we did the longer time course ATPase assay for Cpn-G160S (Fig. 4C).
- A decrease of ATPase activity at high concentration of ATP for group I (Yifrach & Horovitz, 1995) was also observed for the group II chaperonin (Reissmann et al, 2007) and interpreted to be an indicator of negative cooperativity in the chaperonin. These 2 references were added to the paper.

Page 12, “A decrease in the ATPase activity at higher concentration was observed for the Cpn-E164A mutant (Table-II). This behavior has been observed previously in the Cpn-WT (Reissmann et al. 2007), as well as the group I chaperonin GroEL (Yifrach & Horovitz, 1995), and is interpreted to be an indicator of negative cooperativity in the chaperonin. Disrupting the lateral contact at residue E164 may alter communication between adjacent subunits within a ring, but further investigation will be required to determine if there is a link to inter-ring negative cooperativity.”.

1. Yifrach O, Horovitz A (1995) Nested cooperativity in the ATPase activity of the oligomeric chaperonin GroEL. *Biochemistry* 34: 5303–5308
2. Reissmann S, Parnot C, Booth CR, Chiu W, Frydman J (2007) Essential function of the built-in lid in the allosteric regulation of eukaryotic and archaeal chaperonins. *Nature Struct. Biol.* 14: 432–440

4. *p. 4 - The authors cite Reissmann et al. (2007) for allosteric regulation in group II chaperonins but the first papers were by Kafri et al. (2001, 2003) and they should be cited.*

The references Kafri et al. 2001 and 2003 have been added to the manuscript (Page 4). “The ring movements involved in the folding cycle of group II chaperonins are coordinated in time and space via a complex allosteric regulation (Kafri et al, 2001; Kafri & Horovitz, 2003; Reissmann et al, 2007)”.

1. Kafri G, Horovitz A (2003) Transient kinetic analysis of ATP-induced allosteric transitions in the eukaryotic chaperonin containing TCP-1. *J. Mol. Biol.* 326: 981–987
2. Kafri G, Willison KR, Horovitz A (2001) Nested allosteric interactions in the cytoplasmic chaperonin containing TCP-1. *Protein Sci.* 10: 445–449

5. *Fig. 4D - it is very dangerous to determine binding affinity on the basis of one ligand concentration. Errors in the concentrations of the cpns could easily distort the results.*

To get a completely affinity measurement, the reviewer is absolutely correct, a wide range of ATP concentrations are needed to determine the  $K_d$ . We believe these affinity measurements are outside the scope of the work presented in our manuscript. However, initial binding experiments were done in order to address the concerns about the influence of the NSL on the ATP binding. We revised discussion about the ATP binding results. Page 13, “The NSL mutants all show a reduced ATPase activity. To probe the influence of the NSL on the ATP binding we monitored the chaperonin capacity to bind radiolabelled nucleotide at a physiological ATP concentration (Fig. 4d). It has previously been shown that ATP binding to one subunit enhances ATP association with subunits in the same ring (Reissmann et al, 2007). Therefore, ATP binding affinity is dependent on communication between subunits. Both Cpn-K161A and Cpn-E164A mutants show a modest decrease in ATP binding affinity compared to Cpn-WT (Fig. 4d). Intriguingly, the Cpn-G160S mutant displayed comparable ATP binding to that seen in the Cpn-WT, despite a significant drop in ATPase activity (Fig. 4c). This result, in conjunction with the proteinase-K assay, suggests that the binding of ATP by Cpn-G160S is similar as Cpn-WT but the timing of the conformation cycle is changed.



The reduced ATP binding observed in the Cpn-K161 mutant may result from a combination of disruption of communication of  $\gamma$ -phosphate state between subunits, and the loss of direct stabilizing interactions between the lysine nitrogen and the ATP  $\gamma$ -phosphate. Additionally, the Cpn-E164A mutant has a diminished capacity for ATP binding. The location of residue E164 some 15 Å from the active site argues that this effect cannot be a result of direct interaction with the ATP. This further supports a role for E164 in intra-ring communication and a potential impact on cooperativity within the chaperonin arising from the mutation”.

6. Legend to Fig. 1C - I would write 'in the AMP-PNP state' and not 'on AMP-PNP state'.

The legend to Fig. 1C has been changed to 'in the AMP-PNP state'.

7. The concentration of cpn is not mentioned in the Legend to Fig. 4 or in the Methods.

The protein concentration for ATPase assay was added to the Materials and Methods section (Page 21). “In brief, after pre-incubation in ATPase buffer at 37°C, the chaperonin (0.25 $\mu$ M) was supplemented with 1mM and 2mM  $\alpha$ -[<sup>32</sup>P]-ATP.”.

Referee #2

The authors have addressed all queries raised and answered them in a satisfactory manner. The new data included in the revised version make the paper stronger and suitable for publication in the EMBO Journal.

Referee #3

The authors have improved the manuscript significantly after the revision. All points I raised in my last review were addressed, and the manuscript has been modified accordingly. The revision was adequate and there are new results that improved the manuscript.

Editorial Issues

1. Please include the number of independent repeats for table II, figure 4B-D, supplementary figure S3B, C.

All the data represent the results from 3 independent repeats. This information was added to the table II and to the legends of the Fig.4 and S3.

2. In your supplementary materials section, you essentially include descriptions of 'supplementary analyses' similar to a bona fide results section. According to our policies, there should be no such supplementary results section. The Supplement should only contain supplementary figures, together with figure legends, and more detailed descriptions of materials and methods if needed. I therefore need to ask you to include descriptions of these analyses into the main body of the manuscript text, referring to the supplementary figures.

All the discussion from supplementary materials section was moved to main body of the manuscript or to the legends of supplemental figures. The new supplementary materials section only contains figures and legends.