**Blm10 facilitates nuclear import of proteasome core particles**

Marion H. Weber russ, Anca F. Savulescu, Julia Jando, Thomas Bissinger, Amnon Harel, Michael H. Glickman, Cordula Enenkel

*Corresponding author: Cordula Enenkel, University of Toronto*

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**Transaction Report:**

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

1st Editorial Decision 23 November 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please excuse the delay in its evaluation due to limited referee availability. We have now received the input of three experts, which I am enclosing copied below. As you will see, all three referees acknowledge the implication of Blm10 as a dedicated proteasome nuclear import factor as potentially very interesting and important. At the same time, however, both referees 2 and 3 remain unconvinced that your current set of data offers sufficiently strong and definitive support to allow such conclusions. I am afraid that these concerns (which I will not repeat in detail here as they are very clearly stated in the referees' reports) in our view preclude publication of this work in our journal - given the major implications of the claim of Blm10 being a dedicated karyopherin it would appear to be essential that it is backed up by very decisive and unambiguous experimental evidence, which in our referees' opinion does not seem to be the case. I therefore see little choice but to return the manuscript to you with the message that we cannot proceed further with its publication at this point, but hope that you will nevertheless find the suggestions and insightful comments provided by the referees helpful for your further work on this interesting topic. Should this allow you to obtain further data substantiating the notion of proteasome import mediated by Blm10, we would remain open to looking once more at an extended version of this manuscript; however this would have to be treated as a new submission - also with regard to the novelty at the time of resubmission - and only be sent back to our referees if we felt that the key issues for supporting the conclusions have been thoroughly addressed. I am sorry we cannot be more positive at this stage, but would nevertheless like to thank you having had the opportunity to consider this work for The EMBO Journal.
REFEREE REPORTS

Referee #1:

It has been reported that in non-dividing yeast cells CP and RP are sequestered into motile cytosolic proteasome storage granuli (PSG). Besides the RP, there exist additional adaptor complexes to bind to the proteasome with Blm10 the latest being discovered. Although structural data on the CP:Blm10 complex are available the biological function of Blm10 is still mysterious. The presented work elucidates major functional implementations on Blm10 within various cell stages, thus, this referee enthusiastically followed the well performed experiments and their accurate interpretations and drawn conclusions. In particular the strength and novelty of the work is shown by:

a) PSG phenotypes are not displayed in Blm10 KO cells grown to stationary phase. Hereby, the CP is localized in the cytosol. Contrary, sequestration of RP components into PSG is independent on Blm10.

b) Overexpression of Blm10 results in RP:CP dissociation thus causing cell cycle arrest (as expected previously).

c) Addition of fresh medium to quiescent yeast cells induced mobilization of the CP from the PSG to the nucleus within a few minutes whereas in Blm10 KO cells the translocation takes hours - interesting!

d) Indication that Blm10 is a CP specific karyopherin - This referee is not convinced by "de facto"; additional experiments for further investigation in this interesting topic have to be performed, but this is not demanded for the current work. However, the conclusion that kyropherin has no major impact on nuclear import of the CP is in evidence by the presented results.

e) Nuclear import of CP is Ran-dependent in yeast and Blm10 is able to form complex with Ran-GTP and the nuclear pore protein Nup53 - nice data!

To summarize: the manuscript is clearly written and the drawn conclusions appropriate. Therefore, this referee recommends publication of the work by Weberruss et al. in EMBO Journal. Minor revisions have to be addressed which are listed below; additional experiments are not required by this referee.

a) CP-nomenclature was used; it would be advicable to cite the crystal structure of yeast CP as well as the current murine cCP and iCP since the discussion also compares the presented data with mammalian systems. yCP, cCP and iCP could be introduced in the intro in 2-3 sentences.

b) p17: data not shown: this statement is inappropriate and the results should be mentioned at least in the supplementary information. This referee is not a fan of papers which are citing to work where the data are not shown.

c) It was impossible by this referee to follow up the data received on the Blm10W2921A mutant. Therefore, this part needs revision. In accordance to the previous chapters a sentence of the most important outcome would be of help at the end of the paragraph.

Referee #2:

This manuscript describes an interesting potential new function for the 20S proteasome core particle (CP)-binding protein Blm10. Specifically, this yeast HEAT-repeat protein is proposed to be a CP-specific karyopherin important for import of mature CP in stationary phase cultures that are fed fresh medium and remodelize proteasomes to the nucleus from cytoplasmic proteasome storage
granules (PSG). Blm10 is shown to bind both an FG repeat nucleoporin (Nup53) and RanGTP, as would be expected of a karyopherin.

I have several questions/concerns:

1. How do the authors reconcile the function of Blm10 as a karyopherin with the finding that Blm10 is needed to localize the CP to PSG? Their original observation (Fig. 1) was that blm10 cells show diffuse CP localization throughout the cell in stationary cells. Does Blm10 have a distinct function in CP aggregation into PSG?

2. From Fig. 2B, it appears that large but distinct RP supercomplexes accumulate in stationary phase. This is inferred by the minimal signal seen with intrinsic GFP fluorescence of a tagged CP subunit compared to the strong signal seen with anti-Rpt1 blotting. It would be preferable to compare these by similar assays since it is possible that b5-GFPs fluorescence is somehow quenched in the context of the RP-CP-RP in stationary phase. An anti-20S subunit blot should be done here. I am concerned, in other words, that the bands on the native gel are incorrectly assigned.

3. It was surprising that Ran-GTP did not dissociate the Blm10-CP complex if Blm10 were acting as other karyopherins are known to (supplementary information). Perhaps some additional factor is needed. But in light of this result, do the authors observe a ternary RanGTP-Blm10-CP complex? This would be predicted if Ran-GTP in the nucleus is necessary to release Blm10 and allow it to recycle to the cytoplasm.

4. In light of the failure of Ran-GTP to cause Blm10-CP dissociation, what is the argument that Blm10 is not simply a cargo protein rather than a karyopherin? Here it would be useful to show that Blm10-CP binds Nup53 better than CP alone. In the text the authors note the potentially interesting observation that the CP degrades the disordered regions of the nucleoporin (would be nice to see this), so they could not do the suggested experiment. But it should be possible to catalytically inactivate the CP to prevent this cleavage (and prove degradation is not coming from a contaminating protease).

Referee #3:
The proteasome is a protease involved in a wide range of cellular processes. It consists of a core particle (CP) associated with regulatory particles (RP) and activators. Blm10 functions as a proteasome activator. The subcellular localization of CP-RP is dynamic. In yeast, CP and RP are stored in cytoplasmic PSG granules during quiescence, whereas they move into the nucleus during proliferation.
The manuscript by Weberruss et al. shows that Blm10 is responsible for the recruitment of CP but not of RP to PSGs. The fact that Blm10 consists of HEAT repeats and that this protein, together with the CP, relocalizes under different conditions, suggested a similarity with karyopherins (or Importin-like proteins). Karyopherins are HEAT-repeat containing proteins dedicated to nucleocytoplasmic trafficking. With a combination of yeast genetics, localization studies and biochemistry, the authors show that Blm10 facilitates nuclear import of CP and that Blm10 can bind to RanGTP (Gsp1GTP in yeast) and to Nup53, a FG-repeat containing nucleoporin. Moreover, they show that blm10 cells became more sensitive to DNA damage.
The manuscript reports a novel function of the proteasome activator Blm10 in the cytoplasmic localization and in nuclear import of proteasome core particles that has not been suggested before. In this respect the research described is new and interesting.

A major concern, however, is whether the experimental evidence provided is sufficient to draw the specific conclusions:
- Blm10 facilitates nuclear import of CP
- Blm10 behaves like a dedicated importin/karyopherin beta

The paper is understandably written. However, some parts should be clarified. From several statements the reader is brought to think that all HEAT repeat proteins are karyopherins and that Ran binding proteins are HEAT repeat proteins. This is misleading and should be clarified (see also point 2).
Major issues:

1) Subcellular localization of CPs:

To better appreciate the position of the yeast cell nuclei, a nuclear marker should be shown in all pictures. This would allow to unequivocally assess the subcellular localization of the factors examined.

The authors should comment on why, when Blm10 is deleted (Fig. 1B and 3A), a nuclear signal is still visible. One would expect that if nuclear import were impaired, the complex would be excluded from the nucleus.

In Fig. 1A, GFP signal from tagged CPs appears to be present in the nuclei of quiescent cells. Is this actual signal or is this background?

2) Sequence alignment of Blm10 with Yrb1:

In Fig. 4B, very short sequence stretches are aligned. From a bioinformatics point of view, such an alignment with tiny stretches of sequence and such weak and scattered consensus, is hardly meaningful.

Indeed in the first line, one and half HEAT repeat from Blm10 (all helical, from Sadre-Bazzaz et al., 2010; pdb id. 1VSY) is aligned with part of a -hairpin of Yrb1 (from Koyama and Matsuura, 2010; pdb id. 3m1i). Moreover, Yrb1 is the yeast hortologue of RanBP1: it contains a RanBD (Ran binding domain) and it is involved in nuclear export by facilitating RanGTP hydrolysis in the cytoplasm. However, RanBP1 is not an importin -like protein. Given that structurally these two proteins are very different, it is difficult to envision how the two proteins could associate similarly with Ran.

Karyopherins make extensive contacts with RanGTP that extend over several HEAT repeats at the N-terminal arch of the proteins. The Ran binding surface on karyopherins is large and complex. Typically, karyopherins consist of about 20 HEAT repeats and have a superhelical arrangement.

Blm10 consists of 32 HEAT repeats and shows a dome-like structure that caps the CP. The authors map the Ran-binding site at the very C-terminal region of the protein.

From the sequence and structural level, Blm10 is not an obvious importin -like protein although, similarly to importin and many other (non homologous) proteins, it contains HEAT repeats. The relevant comparison that the authors should discuss is between Blm10 and karyopherins. The Ran binding region (CRIME domain) is the most conserved across karyopherins: does Blm10 have any similarity in this region, for example with importin?

The structures of importin and of Blm10-CP are available. Based on the structural information, is it possible to envision how RanGTP would recognize the region mapped in the paper? Would RanGTP binding be possible in the context of the Blm10-proteasome complex?

Page 7: "...resembles the structure of karyopherin ." The references cited at this point are based on predictions from sequence. The crystal structure of Blm10-CP-Blm10 is available (see above) and should be used to compare with the available structures of karyopherins.

3) RanGTP binding:

The directionality of nucleo-cytoplasmic transport by karyopherins relies on RanGTP association either to import (Ran binding promotes import-cargo dissociation) or to export (Ran binding in a ternary complex promotes export-complexes formation). If Blm10 binds to RanGTP and not to RanGDP, its localization is affected by perturbing the Ran cycle and RanGTP addition does not stabilize putative transport receptor (Blm1)-cargo dissociation, it is unclear whether Blm10 might function as an export or as an import factor. The experiment performed to test dissociation of Blm10-CP complex by RanGTP is critical to provide important information on the putative function of Blm10 as a nuclear import receptor, namely on the directionality and on cargo release/association (Page 16, bottom; Suppl. Information Page 4). It should be shown and discussed.

The gel in Fig. 4D provides nice information on mapping the Ran binding region of Blm10. However, the gel/western windows are cut very close to the bands and it is difficult to judge
size/specificity. The experiment should be shown as in Fig. 4C.

4) Over-expression of Blm10 causes sequestration of CP into PSG:

The authors should discuss in more depth how this observation could be reconciled with Blm10 function in nuclear import.

Minor issues:

- The construct Blm10 C1804-2143 should be labeled consistently: it is also mentioned as Blm10-C1804-2143 or Blm10C.

- Fig S2: a decrease of RP-CP in the last lane, lower panel is apparent. One would expect an increase of particle formation over time. Can the authors comment?
Dear Dr. Vodermaier,

thank you for considering a second view on the extended version of our manuscript “Blm10 facilitates nuclear import of proteasome core particles” for publication in “The EMBO Journal”.

We thank the referees for their time to review our work and their insightful comments that encouraged us to strengthen the manuscript and streamline our results.

Major changes were made concerning the alignment of Blm10 and β karyopherin (Fig. 4B). Fig. 4B was replaced by Fig. S5B and a model in Fig. 6. As suggested by the reviewers additional new experiments were added in Figs. 4E and 4G. Fig. 4E shows that Blm10 facilitates binding of the catalytic core (CP) to nucleoporin Nup53. Fig. 4G shows a ternary complex of Blm10-CP with Ran-GTP.

We addressed all points of the reviewer as outlines below:

Referee #1 (Remarks to the Author):

It has been reported that in non-dividing yeast cells CP and RP are sequestered into motile cytosolic proteasome storage granuli (PSG). Besides the RP, there exist additional adaptor complexes to bind to the proteasome with Blm10 the latest being discovered. Although structural data on the CP:Blm10 complex are available the biological function of Blm10 is still mysterious. The presented work elucidates major functional implementations on Blm10 within various cell stages, thus, this referee enthusiastically followed the well performed experiments and their accurate interpretations and drawn conclusions. In particular the strength and novelty of the work is shown by: a) PSG phenotypes are not displayed in Blm10 KO cells grown to stationary phase. Hereby, the CP is localized in the cytosol. Contrary, sequestration of RP components into PSG is independent on Blm10.
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c) Addition of fresh medium to quiescent yeast cells induced mobilization of the CP from the PSG to the nucleus within a few minutes whereas in Blm10 KO cells the translocation takes hours - interesting!

d) Indication that Blm10 is a CP specific karyopherin - This referee is not convinced by "de facto"; additional experiments for further investigation in this interesting topic have to be performed, but this is not demanded for the current work. However, the conclusion that karyopherin has no major impact on nuclear import of the CP is in evidence by the presented results.

As outlined in my response to reviewer #3 we searched for sequence similarities between Blm10 and karyopherin β. We could not detect the karyopherin β-typical CRIME motif but a conserved stretch of acidic amino acids and scattered tryptophans that might serve as initial docking site for Ran-GTP binding to Blm10. Thus, we refrain from naming Blm10 a de facto β karyopherin (see response to reviewer #3).

e) Nuclear import of CP is Ran-dependent in yeast and Blm10 is able to form complex with Ran-GTP and the nuclear pore protein Nup53 - nice data!

To summarize: the manuscript is clearly written and the drawn conclusions appropriate. Therefore, this referee recommends publication of the work by Weberruss et al. in EMBO Journal. Minor revisions have to be addressed which are listed below; additional experiments are not required by this referee.

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Thanks for clarifying this point. The references were added in the introduction.

b) p17: data not shown: this statement is inappropriate and the results should be mentioned at least in the supplementary information. This referee is not a fan of papers which are citing to work where the data are not shown.

p.17: We now show the experiment in Fig. 4G.

To test whether recombinant Ran-GTP is sufficient to dissociate Blm10-bound CP in vitro, GFP-labelled CP was purified from yeast under native conditions and incubated with purified Blm10 (Lehmann et al, 2008). By that way, Blm10-CP-
Blm10 was reconstituted as shown by native PAGE and fluoroimaging of the GFP-moieties of the CP. Ran-GTP / Gsp1Q71L-GTP was incubated with Blm10-CP-Blm10. The reaction was subjected to native PAGE and probed for Ran / Gsp1 by immunoblotting. It turned out that Ran-GTP may bind to Blm10-CP-Blm10 in a ternary complex but is not sufficient to dissociate Blm10-CP-Blm10 suggesting that the in vitro reconstitution does not yet mimic the in vivo situation (Fig. 4G).

c) It was impossible by this referee to follow up the data received on the Blm10W2921A mutant. Therefore, this part needs revision. In accordance to the previous chapters a sentence of the most important outcome would be of help at the end of the paragraph.

Since Ran binding to karyopherins is mediated by more than 30 residues, it is not surprising that the single site mutation W2021A in full length Blm10 (2143 aa) had no effect on nuclear import. Therefore, we skipped this experiment.

Referee #2 (Remarks to the Author):

This manuscript describes an interesting potential new function for the 20S proteasome core particle (CP)-binding protein Blm10. Specifically, this yeast HEAT-repeat protein is proposed to be a CP-specific karyopherin important for import of mature CP in stationary phase cultures that are fed fresh medium and remobilize proteasomes to the nucleus from cytoplasmic proteasome storage granules (PSG). Blm10 is show to bind both an FG repeat nucleoporin (Nup53) and RanGTP, as would be expected of a karyopherin.

I have several questions/concerns:

1. How do the authors reconcile the function of Blm10 as a karyopherin with the finding that Blm10 is needed to localize the CP to PSG? Their original observation (Fig. 1) was that blm10 cells show diffuse CP localization throughout the cell in stationary cells. Does Blm10 have a distinct function in CP aggregation into PSG?

We think that Blm10 has an additional function in PSG formation. We performed a synthetic genetic array with Brenda Andrews at the UofT in search for genes required for PSG formation. Blm10 is among the hit genes. In addition to Blm10 about 30 genes seem to be involved in PSG formation suggesting that PSG formation is orchestrated by several proteins. We are at the beginning to understand this phenomenon.

2. From Fig. 2B, it appears that large but distinct RP supercomplexes accumulate in stationary phase. This is inferred by the minimal signal
seen with intrinsic GFP fluorescence of a tagged CP subunit compared to the strong signal seen with anti-Rpt1 blotting. It would be preferable to compare these by similar assays since it is possible that b5-GFPS fluorescence is somehow quenched in the context of the RP-CP-RP in stationary phase. An anti-20S subunit blot should be done here. I am concerned, in other words, that the bands on the native gel are incorrectly assigned.

Thanks for this helpful comment. We agree that our assignment of RPN and RPnn species is unexpected, although RP oligomerisation upon ATP depletion is already reported (Kleinjen et al.). Metabolomics revealed that the ATP level significantly decreases during quiescence (Laporte et al 2010 JCB) which parallels with RP-CP disassembly and PSG formation. We mention this in the discussion on p.19.

We arranged new Fig. S2 by extracting wt native PAGE gels from Figs. 2A and B that log and stat phase are shown in parallel. An anti-CP (a6, Pre4) blot was added showing that the CP and RP species are correctly assigned. Only few RP-CP species were left in stat phase. Most CP migrates as free and Blm10-bound species consistent with previous reports (Bajorek et al). Since RP is stable during stat phase and not dissociated into single subunits, we conclude that RP oligomerisation also occurs in vivo when ATP concentrations decline.

3. It was surprising that Ran-GTP did not dissociate the Blm10-CP complex if Blm10 were acting as other karyopherins are known to (supplementary information). Perhaps some additional factor is needed. But in light of this result, do the authors observe a ternary RanGTP-Blm10-CP complex? This would be predicted if Ran-GTP in the nucleus is necessary to release Blm10 and allow it to recycle to the cytoplasm.

p17: We did the experiment and show it in new Fig. 4G.

To test whether recombinant Ran-GTP is sufficient to dissociate Blm10-bound CP in vitro, GFP-labelled CP was purified from yeast under native conditions and incubated with purified Blm10 as described before (Lehmann et al, 2008). By that way, Blm10-CP-Blm10 was reconstituted as shown by native PAGE and fluoroiimaging of the GFP-moieties of the CP. Gsp1Q71L-GTP was incubated with Blm10-CP-Blm10. The reaction was subjected to native PAGE and probed for Ran / Gsp1 by immunoblotting. It turned out that Ran-GTP may bind to Blm10-CP-Blm10 in a ternary complex but is not sufficient to dissociate Blm10-CP-Blm10 suggesting that the in vitro reconstitution does not yet mimic the in vivo situation (Fig. 4G).

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regions of the nucleoporin (would be nice to see this), so they could not do the suggested experiment. But it should be possible to catalytically inactivate the CP to prevent this cleavage (and prove degradation is not coming from a contaminating protease).

We thank the reviewer for this great idea. The experiment was straight forward in the presence of proteasome inhibitor. It is shown in new Fig. 4E.

Referee #3 (Remarks to the Author):
The proteasome is a protease involved in a wide range of cellular processes. It consists of a core particle (CP) associated with regulatory particles (RP) and activators. Blm10 functions as a proteasome activator. The subcellular localization of CP-RP is dynamic. In yeast, CP and RP are stored in cytoplasmic PSG granules during quiescence, whereas they move into the nucleus during proliferation. The manuscript by Weberruss et al. shows that Blm10 is responsible for the recruitment of CP but not of RP to PSGs. The fact that Blm10 consists of HEAT repeats and that this protein, together with the CP, relocates under different conditions, suggested a similarity with karyopherins (or Importin β-like proteins). Karyopherins are HEAT-repeat containing proteins dedicated to nucleo-cytoplasmic trafficking. With a combination of yeast genetics, localization studies and biochemistry, the authors show that Blm10 facilitates nuclear import of CP and that Blm10 can bind to RanGTP (Gsp1GTP in yeast) and to Nup53, a FG-repeat containing nucleoporin. Moreover, they show that blm10Δ cells became more sensitive to DNA damage.

The manuscript reports a novel function of the proteasome activator Blm10 in the cytoplasmic localization and in nuclear import of proteasome core particles that has not been suggested before. In this respect the research described is new and interesting.

A major concern, however, is whether the experimental evidence provided is sufficient to draw the specific conclusions:
- Blm10 facilitates nuclear import of CP
- Blm10 behaves like a dedicated importin/karyopherin beta

The paper is understandably written. However, some parts should be clarified. From several statements the reader is brought to think that all HEAT repeat proteins are karyopherins and that Ran binding proteins are HEAT repeat proteins. This is misleading and should be clarified (see also point 2). Major issues:

I greatly appreciate reviewer #3’s thoughtful comments regarding the structures of
Blm10-CP-Blm10, karyopherins and RanBP1. Reviewer #3 made us aware that something was odd with our alignment in Fig. 4B and that the structures and functions of Ran binding domains differ in RanBP1 and karyopherins. Due to the heterogeneity of Ran binding sites within karyopherins it is difficult to assign the Ran binding site in Blm10C. We removed Fig. 4B. Sequence alignments between Blm10 and β karyopherins are shown in Figs. S5B and 6. We also clarified that a HEAT repeat protein is not necessarily a β karyopherin.

p.13 we introduce Fig. 4 as following: Genetic interactions between Blm10 and Prp20, the Ran nucleotide exchange factor, and Blm10 and Rna1, the RanGTPase activating protein, were first hints that Blm10 senses the Ran-GTP gradient between the nucleo- and cytoplasm and might function like a karyopherin (Costanzo et al, 2010).

p.7
Blm10 belongs to the family of HEAT-repeat proteins such as karyopherins (Kajava et al, 2004), although the shared structural features do not necessarily mean that HEAT-repeat proteins function as karyopherins.

p. 22
Here, it is important to note that Ran-GTP binding by the HEAT repeat protein is mandatory for the directionality of nuclear transport of macromolecules.

We reorganized Fig. 4 and rewrote this part of the manuscript.

1) Subcellular localization of CPs:

To better appreciate the position of the yeast cell nuclei, a nuclear marker should be shown in all pictures. This would allow to unequivocally assess the subcellular localization of the factors examined. The authors should comment on why, when Blm10 is deleted (Fig. 1B and 3A), a nuclear signal is still visible. One would expect that if nuclear import were impaired, the complex would be excluded from the nucleus.

We tried to show for all mutants a nuclear staining with RFP-labelled histone H2A as in wild type (Fig. 1A). We added the double labeling of nuclei and CP for blm10Δ in Fig. 1B. Unfortunately, simultaneous labeling of H2A and proteasomal components in temperature-sensitive Ran cycle and nuclear import mutants grown to stationary phase interfered with cell viability. The morphology of the cells was completely disturbed at restrictive temperatures.

In Fig. 1A, GFP signal from tagged CPs appears to be present in the nuclei of quiescent cells. Is this actual signal or is this background?
The nuclear background signal in wt and blm10null cells is most likely derived from RP-CP remnants. In prolonged quiescence (2-3 weeks) all RP-CP assemblies are dissociated and sequestered into PSG as mentioned on p. 11. This observation is consistent with previous independent studies on proteasome assembly and localization reflecting the metabolic state of quiescence (Bajorek et al. Sagot et al.), see discussion p. 19.

2) Sequence alignment of Blm10 with Yrb1:

In Fig. 4B, very short sequence stretches are aligned. From a bioinformatics point of view, such an alignment with tiny stretches of sequence and such weak and scattered consensus, is hardly meaningful.

Indeed in the first line, one and half HEAT repeat from Blm10 (all helical, from Sadre-Bazzaz et al., 2010; pdb id. 1VSY) is aligned with part of a β-hairpin of Yrb1 (from Koyama and Matsuura, 2010; pdb id. 3m1i). Moreover, Yrb1 is the yeast hortologue of RanBP1: it contains a RanBD (Ran binding domain) and it is involved in nuclear export by facilitating RanGTP hydrolysis in the cytoplasm. However, RanBP1 is not an importinβ-like protein. Given that structurally these two proteins are very different, it is difficult to envision how the two proteins could associate similarly with Ran.

We agree with reviewer #3 and removed Fig. 4B.

Karyopherins make extensive contacts with RanGTP that extend over several HEAT repeats at the N-terminal arch of the proteins. The Ran binding surface on karyopherins is large and complex. Typically, karyopherins consist of about 20 HEAT repeats and have a superhelical arrangement. Blm10 consists of 32 HEAT repeats and shows a dome-like structure that caps the CP. The authors map the Ran-binding site at the very C-terminal region of the protein. From the sequence and structural level, Blm10 is not an obvious importinβ-like protein although, similarly to importinβ and many other (non homologous) proteins, it contains HEAT repeats.

The relevant comparison that the authors should discuss is between Blm10 and karyopherins. The Ran binding region (CRIME domain) is the most conserved across karyopherins: does Blm10 have any similarity in this region, for example with importinβ?

The structures of importinβ and of Blm10-CP are available. Based on the structural information, is it possible to envision how RanGTP would recognize the region mapped in the paper? Would RanGTP binding be possible in the context of the Blm10-proteasome complex?
We completely rewrote that part of the manuscript and show a new model how we envision the interaction of Blm10 with Ran GTP based on the PDB structures (new Figs. S5B and 6).

p. 15 result section
Thus, we investigated whether this region might confer Ran dependence on nuclear import of Blm10-bound CP by direct binding to Ran-GTP and searched for sequence similarities between β karyopherins and Blm10. Structural determinants for Ran-GTP binding to β karyopherins are the CRIME motif in the N-terminal arch and an acidic loop surrounded by scattered tryptophans (Vetter et al. 1999). Sequence alignment programs (J.Hein, ClustalV and ClustalW) did not reveal an N-terminal CRIME motif within Blm10. A β karyopherin-typical \(245^{\text{LKN}}247\) motif is located in the N-terminal region of Blm10 but from a bioinformatics point of view it is hardly meaningful and it is not accessible at the Blm10 surface. However, a stretch of acidic amino acid residues and scattered tryptophans which mediate Ran GTP binding to β karyopherins are accessible in the C-terminal region of Blm10 close to the penultimate tyrosine that is required for Blm10-CP interaction. Thus, we tested whether the C-terminal region of Blm10 is involved the initial contact between Ran / Gsp1-GTP and Blm10.

p. 20-21 discussion
Our assumption that Blm10 acts as a CP-specific nuclear import receptor is further supported by structural similarities between Blm10 and β karyopherins, although a β karyopherin-typical CRIME motif was not identified in Blm10. The heterogeneity of Ran binding sites within β karyopherins underscores the difficulty in identifying new members of the karyopherin superfamily from the databases. The degree of sequence similarity between the putative Ran binding sites of the entire superfamily is only about 10%, and there are no invariant residues that can be identified as a core Ran binding motif (Macara, 2001). However, a stretch of acidic amino acid residues and scattered tryptophans that are similarly arranged as in the Ran binding site of β karyopherins (Vetter et al, 1999) are present in the C-terminus of Blm10. This acidic patch of amino acids is accessible in a groove between the CP and Blm10. Whether this groove between Blm10 and CP serves as an initial docking site for Ran-GTP, remains to be analysed by future X-ray studies. So far, we can only envision a model based on the structures in the PDB databases (Blm10-CP-Blm10, 1VSY; karyopherin β bound to Ran-GTP, 2bku). By comparing the overall structures of Blm10-CP-Blm10 and Ran-GTP that was extracted from the karyopherin β bound to Ran-GTP one can imagine that Ran-GTP fits into the groove between Blm10 and the CP (Fig. 6). Ran-GTP’s interaction with the acidic domain at the Blm10 C-terminus may induce conformational changes that weaken the interaction of the Blm10’s penultimate tyrosine with the CP.

Few proteins with karyopherin-like HEAT repeats are reported to translocate cargoes without the aid of additional karyopherins arising the question whether it is a property of HEAT repeat proteins to interact with nuclear pore complexes (Macara, 2001). At least HEAT repeat proteins such as Blm10 would reduce the reliance on single karyopherins with regard to the mass of CP and other proteins that must be imported into the nucleus upon the exit of quiescence.
Model of CP import through nuclear pores. (A) Natively disordered nuclear pore proteins build the entropic barrier that can be passed by macromolecules, if they are bound to carriers such as β karyopherins. During cell proliferation inactive CP precursor complexes are imported by the classical NLS receptor karyopherin αβ (Lehmann et al, 2002). Upon the exit of quiescence CP precursor complexes are not available and Blm10 escorts mature CP into the nucleus. (B) Cartoon for the interaction of Ran-GTP with Blm10-bound CP. The structure of Blm10-CP-Blm10 ((Sadre-Bazzaz et al, 2010), PDB 1YSY) reveals a dome-like fold of Blm10 that covers the CP α ring except for a unique groove at the C-terminal region of Blm10. Here, a stretch of acidic amino acid residues resides that resembles major contact sites of Ran-GTP to β karyopherins (depicted in yellow according to the alignment of yeast karyopherin β and human transportin). Ran-GTP was extracted from the structure of yeast karyopherin β bound to Ran-GTP ((Lee et al, 2005), PDB 2bku) and oriented into the groove of Blm10’s C-terminal region. Blm10 is depicted in red, CP α rings in blue, CP β rings in cyan and Ran-GTP in green.

We leave it to the reviewers’ and editor’s decision to show Fig. 6:
The cartoon in Fig. 6B was drawn according to the PDB structures. The model below based on the PDB structure is still fictive and should not be shown in the paper, if the editor and reviewer 3# agree with us.

Page 7: "...resembles the structure of karyopherin β." The references cited at this point are based on predictions from sequence. The crystal structure of Blm10-CP-Blm10 is available (see above) and should be used to compare with the available structures of karyopherins.

On p. 7 we changed this sentence by:

The X-ray structure of Blm10-CP-Blm10 revealed a superhelical dome-like structure of Blm10 on top of the CP a ring that appeared somewhat at odds with Blm10’s characterization as a proteasome activator and seemed more consistent with possible functions as an adaptor, assembly factor, or inhibitor (Sadre-Bazzaz et al, 2010). Blm10 belongs to the family of HEAT-repeat proteins such as karyopherins (Kajava et al, 2004), although the shared structural features do not necessarily mean that HEAT-repeat proteins function as karyopherins. However, the arrangement of Blm10’s α-helices in a toroid fold is similar to the solenoid fold of karyopherin b (Glickman & Raveh, 2005; Huber & Groll, 2012).

3) RanGTP binding:

The directionality of nucleo-cytoplasmic transport by karyopherins relies on RanGTP association either to import (Ran binding promotes import-cargo dissociation) or to export (Ran binding in a ternary complex promotes export-complexes formation).

If Blm10 binds to RanGTP and not to RanGDP, its localization is
affected by perturbing the Ran cycle and RanGTP addition does not stabilize putative transport receptor (Blm1)-cargo dissociation, it is unclear whether Blm10 might function as an export or as an import factor.

In vivo we observe the dissociation of Blm10-CP by Ran-GTP arguing for Blm10 as an import factor (Fig. 4E). Also nuclear import of CP is delayed in the absence of Blm10. If Blm10 were an export factor, CP would remain nuclear in blm10null. Nuclear export of CP into the cytoplasm is clearly not affected by the absence of Blm10.

The experiment performed to test dissociation of Blm10-CP complex by RanGTP is critical to provide important information on the putative function of Blm10 as a nuclear import receptor, namely on the directionality and on cargo release/association (Page 16, bottom; Suppl. Information Page 4). It should be shown and discussed.

p17: We did the experiment again and show it in Fig. 4G.

The gel in Fig. 4D provides nice information on mapping the Ran binding region of Blm10. However, the gel/western windows are cut very close to the bands and it is difficult to judge size/specificity. The experiment should be shown as in Fig. 4C.

We now show the full blot in Fig. 4C.

4) Over-expression of Blm10 causes sequestration of CP into PSG:

The authors should discuss in more depth how this observation could be reconciled with Blm10 function in nuclear import.

Comparable with quiescence, over-expression of Blm10 results in the RP-CP dissociation and cell cycle arrest similar to quiescence (p. 10).

As mentioned in our response to reviewer #2 we performed a synthetic genetic screen and identified Blm10 and in addition about 30 genes involved in PSG formation. Kinases and phosphatases seem to affect CP and Blm10 movements but the underlying mechanism is not yet understood. We are right at the beginning to understand PSG formation.

Minor issues:

- The construct Blm10ΔC1804-2143 should be labeled consistently: it is also mentioned as Blm10-C1804-2143 or Blm10C.
I still think it is better to leave it as it is. Blm10C is an abbreviation.

- Fig S2: a decrease of RP-CP in the last lane, lower panel is apparent. One would expect an increase of particle formation over time. Can the authors comment?

In blm10null it takes a few hours, until RP-CP is fully assembled in the nucleus. We think that the CP is assembled from newly synthesized precursor complexes in blm10null (Fig. S3B).

Again, we want to thank you for your helpful comments.

We would be very happy about a positive answer.

Sincerely yours,

Cordula Enenkel
Thank you for submitting a new version of your manuscript (previously EMBOJ-2012-83615) on Blm10 and nuclear import of proteasomes for our consideration. To assess the conclusiveness and decisiveness of the revised data especially on Blm10 as dedicated nuclear import facilitator, I once more consulted with the original referee 3 as an expert in nucleocytoplasmic transport. I am afraid to say that this referee, whose comments are copied below, still feels that the presented data are unable to strongly support the potentially far-reaching key conclusions of this study. In particular, the reviewer remains unconvinced by the functional, mechanistic and structural evidence for Blm10 as an importin-like factor. In light of these criticisms, which mirror our own concerns from assessing the responses in your letter, I see little choice but to return the manuscript to you with the final decision that we cannot offer to publish it in The EMBO Journal. I am sorry that we still cannot be more positive, but hope that you will soon receive more encouraging news elsewhere.

REFEREE REPORTS:

Referee #3:

In this study Weberuss et al. investigate the function of the proteasome activator Blm10 using a combination of yeast genetics, fluorescence and biochemistry. The authors show that in blm10 mutants proteasome core particles (CPs) are diffusely localized during quiescence. This suggests no sequestration in cytoplasmic PSG granules of CPs in the absence of Blm10. Induction of Blm10 overexpression in blm10 cells results in relocalization of Blm10 signal to PSGs. Import assays in Xenopus nuclei with labeled proteasome particles show a two-fold increase of CP nuclear import in presence of Blm10 as compared with CPs alone. Localization studies in yeast strains impaired in the Ran cycle show abnormal redistribution of CP upon induction of cell proliferation. Finally, binding assays with purified proteins show association of Blm10 with RanGTP and with the nucleoporin Nup53.

These results are interpreted as requirement of Blm10 to localize CPs to PSGs in quiescence and to import mature CPs to the nucleus during cell proliferation.

Major concerns are:

Based from the fluorescence images, the effect of Blm10 deletion on CP nuclear import is not clear-cut:

-In blm10 CP localization cells appears to be largely diffused in the cytoplasm but also nuclear in quiescence (Fig. 1B). This is at odds with the conclusion that Blm10 is required to import CPs.

- In the absence of Blm10 the described two hours delay in import of CPs in the nucleus is difficult to assess (Fig. 3A). There is so much diffused signal as compared to the wt that the only way to compare the two situations would be to measure the ratio nucleus/cytoplasm signal. In the mutant a nuclear signal appears to be present although is not easily distinguishable due to the increased cytoplasmic signal.

The functional and structural similarity with importins is unclear:

- If Blm10 acts as an import facilitator (similarly to an importin), the expected efficiency of import would be higher than the two-fold increase in CP accumulation observed (Fig. 3 B, D).

- The experiment of cargo dissociation by RanGTP is puzzling (fig. 4G): Blm10-CP-Blm10 seems to co-precipitate better in presence of RanGTP - unexpected for an import factor - and there seems to be RanGTP signal in both lanes where RanGTP was not included.
-Blm10 consists of HEAT repeats similarly to importin-like nuclear transport receptors. However, the architecture of Blm10 is very different from these proteins since the RanGTP binding site is mapped at a short region at the very C-terminal end of the protein in contrast to all importins the recognize RanGTP at the N-terminus.

It was nice to talk to you at the CSH meeting of the Ubiquitin Family and to hear that we are allowed to submit our manuscript “Blm10 facilitates nuclear import of proteasome core particles” for reconsideration. We hope that our new data are convincing.

We addressed the 3rd reviewer’s comments as following.

-In blm10 CP localization cells appears to be largely diffused in the cytoplasm but also nuclear in quiescence (Fig. 1B). This is at odds with the conclusion that Blm10 is required to import CPs.

We thank the reviewer for focusing our attention to the residual amount of nuclear localization of CP under these conditions. We think that CP staining of the NE / ER may arise from CP involved in ERAD and we have included this in the text (page 9, line 15).

- In the absence of Blm10 the described two hours delay in import of CPs in the nucleus is difficult to assess (Fig. 3A). There is so much diffused signal as compared to the wt that the only way to compare the two situations would be to measure the ratio nucleus/cytoplasm signal.

We followed the suggestion of the reviewer and quantified the ratio of nuclear / cytoplasmic pixel intensity and included it in Fig. 3A).

The functional and structural similarity with importins is unclear:
- If Blm10 acts as an import facilitator (similarly to an importin), the expected efficiency of import would be higher than the two-fold increase in CP accumulation observed (Fig. 3 B, D).

A larger efficiency of import is described in the literature for the classical 60 kDa NLS-BSA cargo and importin ab. However, a large cargo like the 750 kDa CP appears to be transported with reduced efficiency.

- The experiment of cargo dissociation by RanGTP is puzzling (fig. 4G): Blm10-CP-Blm10 seems to co-precipitate better in presence of RanGTP - unexpected for an import factor - and there seems to be RanGTP signal in both lanes where RanGTP was not included.

To address this critique, the old Fig. 4G was replaced by the new Fig. 4F showing that Blm10-CP is dissociated by Ran/Gsp1-GTP. Blm10 and Gsp1-GTP co-migrate in native PAGE suggesting complex formation in solution. In the old Fig. 4G thrombin-cleaved GST-Gsp1 was used which did not yield functional Gsp1. We realized this problem when Gsp1 did not bind our control protein importin . In the new Fig. 4F we used His-tagged Gsp1 which binds importin .

We agree that Blm10 does not contain the typical importin-like Ran-binding domain within the N-terminal arch of the protein. We clarified this in the text:

Search for primary sequence similarities between karyopherins and Blm10 did not reveal structural determinants for Ran-GTP binding within the N-terminal arch of Blm10, that allow to assign Blm10 as a new classical member of the karyopherin family (Kutay et al, 1997; Vetter et al, 1999). However, the C-terminal part of Blm10, the most conserved region of the protein, shows consecutive HEAT modules stacking together in a parallel fashion with a slight clock-wise twist, comparable with the arrangement in the three dimensional structure of the Ran-binding arch of karyopherins (Cook et al, 2007). See page 15.
Only the Xray structure of the Blm10-Gsp1-GTP complex will provide a final proof and clear answer to this question, but we believe that Xray structure analysis is beyond the scope of the present manuscript.

Thank you for your patience while the resubmission of your previously reviewed manuscript was now assessed by a trusted arbitrating referee, who has looked at the revised manuscript as well as at the last comments of the original referee and your response to them. While the arbitrating referee agrees that some of the concerns raised during the previous review were indeed well taken, he/she however feels that especially in light of your most recent RanGTP effect data, there is now sufficiently strong support for the main conclusions on Blm10 being an import factor for core particles. Furthermore, he/she does not consider the Ran binding site mapping to the Blm10 C-terminal region a critical issue in light of structural considerations, and feels that various reasons (such as presence of endogenous Blm10 in extracts, or the large size of core particles) could well explain the lack of higher-fold stimulation of CP import by exogenous Blm10.

In summary, given these latest comments, I am pleased to inform you that we should now be able to accept this manuscript for publication in The EMBO Journal, following some remaining minor revisions as follows:

- the arbitrating referee suggests to remove the less convincing and largely correlation-based bleomycin data in Figure 5 (you may at least want to deemphasize them and move them to the supplementary information, or only mention these implications briefly in the discussion section)

- please revisit the presentation and explanation of the model in Figure 6, as pointed out by the referee, panel B is currently not very convincing nor compelling, and panel A would be far more informative by depicting classical import of immature proteasome complexes during log phase, their accumulation in cytoplasmic granules during stationary phase, and the Blm10-mediated reimport of mature core particles upon re-entry into growth phase.

- the referee also requests more informative descriptions in the method section and figure legends, e.g. re. His-Blm10 purification (from yeast? via Ni-NTA or other means? which buffers?), Xenopus nuclear import assays (presence or prior removal of frog proteins?), etc.

- finally, please provide source data files for the various electrophoretic gels and blots, in order to make the primary data more accessible and to allow readers to judge the full gels from which the minimized panels were derived. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I am therefore returning the manuscript to you at this stage for a final round of minor modification, hoping that once we will have received your final version we should then be able to swiftly proceed with its formal acceptance and production. Please do not hesitate to get back to me should you have any additional questions.

We are very glad that you consider accepting our manuscript with minor modifications. We want to express our gratitude especially to the arbitrating referee who reviewed our manuscript again and judged our responses to our previous referees.
Below you will find our response to the arbitrating referee's comments (in blue):

The arbitrating referee suggests to remove Figure 5 (you may at least want to deemphasize them and move them to the supplementary information, or only mention these implications briefly in the discussion section).

We moved Fig. 5 to the supplementary part (new Fig. S6) and mentioned the results briefly at the end of the discussion.

Please revisit the presentation and explanation of the model in Figure 6, as pointed out by the referee, panel B is currently not very convincing nor compelling, and panel A would be far more informative by depicting classical import of immature proteasome complexes during log phase, their accumulation in cytoplasmic granules during stationary phase, and the Blm10-mediated reimport of mature core particles upon re-entry into growth phase.

We are grateful for the suggestion. We removed panel B from the old Fig. 6. Nuclear import of the CP is now well documented in the new Fig. 5 (previous Fig 6.) showing cells grown to log and stat phase.

The referee also requests more informative descriptions in the method section and figure legends, e.g. re. His-Blm10 purification (from yeast? via Ni-NTA or other means? which buffers?), Xenopus nuclear import assays (presence or prior removal of frog proteins?)

In the method section we now mention the details of Blm10 purification (p. 24/25):
His12-tagged versions of full length Blm10 were purified ….The released His-tagged Blm10 is transferred into PBSKMT containing 5 mM b-mercaptoethanol. Blm10 was used directly after purification for solution binding studies and reconstitution assays.

Nuclei were assembled from fractionated Xenopus egg extract as previously described (Savulescu et al. 2011). The egg extract presumably contained putative amphibian orthologs of Blm10, which could not be removed due to the lack of characterized antibodies. Oregon Green-labelled CP was introduced into the reconstitution reactions after nuclear assembly was completed and nuclear import was assessed by epifluorescence or confocal microscopy as mentioned on p. 23.

Despite the fact that my laboratory was relocated first within Berlin and later to Toronto, I was able to collect almost all source file data for the various electrophoretic gels and blots from which figure panels were derived.
I only missed the original blots for Blm10 in Fig. 1CD. However, I did these experiments several times and could replace the missing Blm10 blots by equivalent blots, which I brought to Toronto with my lab books.

Finally I would like to thank you again for reconsidering our work.

Blm10 is a protein quite difficult to work with and it took us some time to gather all the experience for the work described in the manuscript. We would be happy if our efforts will be acknowledged by publication of the manuscript.