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Large Cargo Transport by Nuclear Pores: Implications for the Spatial Organization of FG-Nucleoporins

Li-Chun Tu, Guo Fu, Anton Zilman and Siegfried M. Musser

Corresponding author: Siegfried M. Musser, Texas A&M Health Science Center

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Transaction Report:

Editor: Andrea Leibfried

1st Editorial Decision 01 July 2013

Thank you for submitting your manuscript entitled 'Large Cargo Transport by Nuclear Pores: Implications for the Spatial Organization of FG-Nucleoporins'. I have now received all reports on your paper. Please excuse again the delay in getting back to you, but I had involved a fourth expert to cover some of the technical aspects of the manuscript.

As you can see below, referee 1, 3 and 4 appreciate your work and mainly suggest amendments in the text to improve your manuscript and to strengthen your data and claims. Referee 2, however, raises concerns regarding the generality of your conclusions and suggests a number of experiments to further extend the findings. In particular, the referee thinks that it would be good testing whether other large cargos with different import receptors show similar effects, and to test the predictions made by the model by using cells that lack central or peripheral nucleoporins. I don't know if you have data on hand to address these issues specifically. If so it would be good to include them in a revised version. If not, let's discuss this issue further.

Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees.
I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the concerns raised at this stage.

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

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

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REFEREE COMMENTS

Referee #1

In this paper, Tu and colleagues analyze the transport of large cargos through the nuclear pore complex (NPC). As shown before by the Gorlich group, the authors confirm here that large cargos require multiple nuclear transport receptors for transport. Using single molecule experiments, they demonstrated that multiple NTRs are not needed for initial docking at the NPC but for efficient translocation (whereas efficiency is defined as the percentage of successful translocation events). The authors also develop a quantitative model to examine the role of multivalent interactions between multiple NTRs and components of the pore.
Overall Tu et al is an interesting and solid manuscript that contributes to our understanding of nuclear transport and the selective properties of the NPC. It should be published after minor modifications.
1. The material & methods section is very short, and the reader has to go back to earlier publications in order to understand the approach and the terminology that is used by the authors. They should expand the material and methods (for example, to better explain how the trajectories are aligned) and briefly introduce some of the key terminology ('transport efficiency', 'interaction frequency', etc.) in the main text. That would make the paper much more accessible to the general readership.
2. In the last sentence of the abstract, the authors talk about FG-transportin interactions without introducing "transportin". They should probably refer to FG-NTR interactions

Referee #2

In their manuscript „Large cargo transport by nuclear pores: implications for the spatial organization of FG-nucleoporins" Tu et al. introduce a modified nuclear import cargo, M9-betaGal-8C, and convincingly demonstrate that it can bind up to four molecules of transportin, depending on transportin/cargo ratios. The authors also show that import of this cargo into nuclei of permeabilized cells depends on transportin and can be stimulated by Ran, although exogenous Ran is not absolutely required for transport. The authors then use their established single-molecule techniques for the analysis of nuclear import of mono- and multivalent transport cargos. They determine import efficiencies (Fig. 2C) and interaction times (2D) of the cargo with the nuclear pore in dependence of transportin/cargo ratios. The authors find that at low transportin concentrations (i.e. when probably only a single molecule of transportin is bound to the cargo) the interaction time with the pore was significant, whereas import efficiencies were very low. Increasing the transportin/cargo ratio had only a small effect (up to two-fold) on interaction times. Import efficiencies, however, clearly increased. Theoretical (and very intuitive) considerations suggested that at the expected FG-concentrations at the nuclear pore the number of transportin molecules within an import complex should have a strong effect on the lifetime of the transportin/cargo-pore interaction. Based on this discrepancy, the authors develop a model with a low concentration of FG-repeats at the cytoplasmic and the nucleoplasmic side of the pore and a much denser distribution in the center of the pore, i.e. within the permeability barrier. Similar suggestions (based on experimental data) were published previously.

The few experimental data presented in this manuscript largely confirm previous results (e.g. "transportin-mediated import does not require Ran" - Englmeier et al., 1999; Ribbeck et al., 1999, Lyman et al., 2002 or "multiple NTR molecules are required for efficient transport of a large cargo" - Ribbeck and Gorlich, 2002). The single molecule experiments (essentially the results described in Fig. 2C and D) largely fit to those "old" data (and the difference in interaction times in 1x and 4x NTR-cargo complexes could be significant). One problem here is that it is very hard to control the stoichiometry of the transport complexes in the milieu of the permeabilized cells, in particular within the "crowded" NPC. Would other large cargos with different import receptors show similar effects? Predictions made by the model are not further tested (e.g. using cells that lack central or peripheral nucleoporins). Furthermore, the model does not explain the RanGTP-requirement for import of certain large cargos (Lyman et al., 2002).

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Together, we are left with a powerful experimental approach and a quantitative model that does not allow discriminating between the current models in the field that address the molecular underpinnings of the permeability barrier (e.g. hydrogel vs. virtual gate). The manuscript (and the theoretical considerations) might be better suited to publication in a more specialized journal, other than the EMBO J.

Minor points
The assays using permeabilized cells should be described in more detail. Was Ran preloaded with GTP? Or do the authors expect that GDP-GTP exchange occurs in the nuclei of permeabilized cells? Were pre-assembled transportin-cargo complexes used for the in vitro assay? A more detailed description of the methods in the main text would be very helpful. Define "import efficiencies".

Fig. 2: B: Is the difference +/- Ran significant? Why would RanGTP lead to an increased interaction time? p-values must be presented for important measurements in A-E.

p.8. The authors mention that they pre-treated "permeabilized cells with RanGTP to wash away any NTRs bound at cytoplasmic locations". This may have just the opposite effect, as RanGTP targets importin beta to RanBP2.

Fig. S2: check labeling (E537Q, W999L should be enzymatically inactive, not "+")

Fig. S3D: mention total number of analyzed protein complexes.

Referee #3

Nuclear pore complexes (NPCs) are the gatekeepers of the nucleus allowing the free passage of small substances but requiring selective transport mechanisms for large cargos (i.e. most proteins and mRNA-protein complexes). Whereas the overall transport mechanisms like the function of nuclear transport receptors and the role of the small GTPase is well understood the actual passage of the cargo though the NPC is highly controversial. A specific feature of NPC proteins (nucleoporins) is critical for this, a high number of phenylalanine-glycine (FG) repeats, which form both a barrier for inert molecules and interact with transport receptors. Proposed models disagree whether the FG repeats interact or rather repel each other and what the effect of transport receptor binding to FG repeats is. Precise analysis of transport of cargo-transport receptor complexes though the pore by high resolution measurements is mandatory to resolve this issue.
Tu et al describe in their manuscript „Large Cargo Transport by Nuclear Pores: Implications for the Spatial Organization of FG-Nucleoporins" single molecule measurements of NPC passage of a large transport cargo by high resolution microscopy. Their finding indicates that for a large transport cargo with multiple binding sites for transport receptors interaction with one transport receptor is sufficient for NPC binding but it needs multiple interactions to allow efficient passage through the pore. This idea of multivalent interactions increasing the affinity between transport receptors and the NPC (i.e. FG repeat nucleoporins) is important for large import complexes such as the one studied here but most likely also for export of the huge ribosomal subunits and other RNA-protein complexes. The authors present a model of the NPC where the density of FG repeats in the centre of the pore and the peripheries differs and provide by theoretical analysis how this could contribute to the transport capability and features of the NPC.
The experimental part is of high quality and the model suggested interesting and stimulating. I am sure this work interests a broad readership and therefore I suggest that it is published in the EMBO Journal.

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Referee #4

The role of FG-nups in nuclear pore passage is somewhat puzzling because transient interactions between NTRs and FG groups are needed for passage, but at the same time the strength of these interactions must be weak enough to facilitate efficient passage of NTRs through the NPC. The authors take an innovative, in vivo approach to observe transport dynamics with fluorescence microscopy and single particle tracking across the nuclear envelope. Based on their data a quantitative model is presented which accounts for efficient transport of NTRs in dependence of multiple FG-NTR binding sites, and suggests that the spatial configuration of the FG network is integral to achieving selective transport through NPCs. The authors may want to consider the following questions and comments during the editorial process.

1. What is the role of water in FG-NTR interactions? If, as suggested in the last paragraph, the authors were to investigate the role of weak charge interactions, the entropic and enthalpic costs of displacing water molecules will likely become very important. The authors may want to consider including a discussion of the retention of water in the FG network and displacement of water molecules in the center of the NPC may affect the thermodynamics of NTR-FG interactions.

2. Figures:
a. In the Materials and Methods section (page 20) the authors state, "All errors are reported as 68% confidence intervals (standard deviations)." This statement presumably only applies to the data in Figure 1B?
b. In Figure 2, the authors do not comment on the sources of error. Are the error bars in Figures 2A-E calculated from multiple measurements? In addition, in Figure 2 the y-axis is labeled "Import Efficiency (%)". Does this percentage refer to the total number of observed events? If so, why do less than 25% of species observed pass through the nuclear pore? If not, the authors may want to specifically comment on this in the text or in the figure legend.

3. Grammar and language:
a. Page 21: Four reference titles contain the symbol "®" instead of the Greek symbol.
b. The terms "significant" and "substantial" were used at least ten and five times, respectively. While these terms help the reader to infer that the evidence to support a given claim is strong or important, they do not provide a quantitative description of evidence except in very few cases (i.e., in reference to statistical significance). The authors should consider presenting their conclusions using more specific and exact terms to describe their results and to place them in the context of existing literature.

1st Revision - authors' response 02 September 2013

Response to Reviewer #1:

1. The material & methods section is very short, and the reader has to go back to earlier publications in order to understand the approach and the terminology that is used by the authors. They should expand the material and methods (for example, to better explain how the trajectories are aligned) and briefly introduce some of the key terminology ('transport efficiency', 'interaction frequency', etc.) in the main text. That would make the paper much more accessible to the general readership.

Response: We thank the Reviewer for these helpful and constructive suggestions. We have significantly expanded the Materials and Methods section (pp. 21-23) and added a paragraph in the main text (pp. 6-7) to introduce the key terminology and enhance the article's accessibility to the general readership. We now consistently use 'import efficiency' instead of 'transport efficiency'.

2. In the last sentence of the abstract, the authors talk about FG-transportin interactions without introducing "transportin". They should probably refer to FG-NTR interactions.

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Response: We agree with the Reviewer's comments. "FG-NTR" is now used in the abstract rather than "FG-transportin".

Response to Reviewer #2:

- Would other large cargos with different import receptors show similar effects?

Response: This is an excellent question and is a major focus of our current research program. Unfortunately, it is not so easy to test similar cargos. We only seek to use well-characterized proteins, and slight, seemingly innocuous modifications can have substantial, unexpected effects. For example, the initial M9-βGal construct was too heavily labeled with fluorescent dyes (as discussed in the Supplementary Information). In addition, we tried to purify another large cargo (IBB-βGal) that binds to a different NTR (Importin β), but it formed aggregates during purification. We do indeed expect that similar cargos that bind to different NTRs will behave differently, and are actively working on this. However, substantial additional effort is required to experimentally test this.

- Predictions made by the model are not further tested (e.g. using cells that lack central or peripheral nucleoporins).

Response: These are great suggestions, but not easy to implement, since very few cell lines with the appropriate nucleoporin/depletions/deletions exist for mammalian cells. Also, since the proper characterization of such cell lines in our hands and to our standards, and the single molecule experiments themselves, remain timeconsuming, we believe that these experiments lie outside the scope of the present work and are best left for a future manuscript.

- Furthermore, the model does not explain the RanGTP-requirement for import of certain large cargos (Lyman et al., 2002).

Response: As shown in the Supplementary Information (Fig. S2), we found that wild-type M9-βGal (used in the Lyman et al. paper) entered nuclei in the absence of NTRs. As we show, this could be due either to heavy dye labeling or the ability of the cargo to bind to sugars. We therefore conclude that the results obtained by Lyman et al. using this cargo, including the RanGTP requirement for import, should be considered with caution. Note that the effects of RanGTP are strongest when the cargo complex interacts strongly with the central permeability barrier (*Eb* < 0; see Figs. 6 and S5-S6), the situation promoted by heavy dye labeling and the sugar affinity of βGal.

Minor Points:
- The assays using permeabilized cells should be described in more detail.

Response: A section titled "Permeabilized Cell Assay" was added to the Materials and Methods Section.

- Was Ran preloaded with GTP? Or do the authors expect that GDP-GTP exchange occurs in the nuclei of permeabilized cells?

Response: RanGDP and GTP were mixed and added simultaneously. GDP/GTP exchange is slow in the absence of RanGEF, which remains bound to the chromatin in permeabilized cells. Thus, we expect that RanGDP is converted to RanGTP after transport into nuclei by NTF2 (also added). This approach was used in all of our earlier work, as is now described in the new "Permeabilized Cell Assay" section.

- Were pre-assembled transportin-cargo complexes used for the in vitro assay? A more detailed description of the methods in the main text would be very helpful. Define "import efficiencies".

Response: Pre-assembled transportin-cargo complexes were indeed used for the *in vitro* import assay. The cargo and transportin were incubated on ice for at least 20 min before adding to the premeabilized cells. This is now clarified in footnote 5 of Table S1 and in the

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main text (p. 7). Import efficiency is now defined in the main text (p. 6).

- Fig. 2B: Is the difference +/- Ran significant? Why would RanGTP lead to an increased interaction time? p-values must be presented for important measurements in A-E.

Response: We thank the reviewer for these important points. Following the Reviewer's suggestion, we used a two-tailed Welch's *t* test to check the statistical significance of the observed differences under +/- Ran conditions. As now explained on p. 23 of the Materials and Methods section and in the Figure 2 caption, statistically significant differences (p-value 0.05) are now explicitly shown in Figs. 2A-E. According to this statistical test, the differences in import efficiency for M9-βGal in the presence and absence of RanGTP are not significant (Fig. 2B). We therefore deleted the discussion that presumed a significant effect of RanGTP on M9-βGal transport (p. 7). These changes do not affect any major conclusions of the paper.

- p.8. The authors mention that they pre-treated "permeabilized cells with RanGTP to wash away any NTRs bound at cytoplasmic locations". This may have just the opposite effect, as RanGTP targets importin beta to RanBP2.

Response: The Reviewer is correct that RanGTP increases the affinity of importin beta for RanBP2, and this could reduce the binding of cargo complexes to the cytoplasmic face of the NPC. However, as explained in the paragraph preceding the quoted statement, very little M9-βGal reached the nuclear envelope in our single molecule experiments at very low transportin concentrations, i.e., M9-βGal molecules had difficulty finding their way through the permeabilized cells to reach the NPCs. One possibility is that the cargo interacted with NTRs that were somehow located far away from the nuclear envelope. In single molecule experiments, such rare events are important. We reasoned that RanGTP might remove such cytoplasmically bound NTRs. However, an increase in access to the nuclear periphery was not seen. We have clarified this statement by indicating that the intent was to release NTRs at cytoplasmic locations far away from the nuclear envelope (p. 8).

- Fig. S2: check labeling (E537Q, W999L should be enzymatically inactive, not "+")

Response: Many thanks to the Reviewer for catching this error. The labeling in Fig. S2 has been clarified.

- Fig. S3D: mention total number of analyzed protein complexes.

Response: As suggested, the total number of analyzed protein complexes is now indicated in the Fig. S3D caption.

Response to Reviewer #3:

Reviewer #3 had no suggested corrections.

Response to Reviewer #4:

1. What is the role of water in FG-NTR interactions? If, as suggested in the last paragraph, the authors were to investigate the role of weak charge interactions, the entropic and enthalpic costs of displacing water molecules will likely become very important. The authors may want to consider including a discussion of the retention of water in the FG network and displacement of water molecules in the center of the NPC may affect the thermodynamics of NTR-FG interactions.

Response: The enthalpic and entropic effects of water displacement arising from FG-NTR interactions can indeed be very important within the small confines of the FG-network. We have added text addressing this issue in the last paragraph of the discussion (p. 18).

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2. Figures:
a. In the Materials and Methods section (page 20) the authors state, "All errors are reported as 68% confidence intervals (standard deviations)."
This statement presumably only applies to the data in Figure 1B?
b. In Figure 2, the authors do not comment on the sources of error. Are the error bars in Figures 2A-E calculated from multiple measurements?

Response: We have included a new Error Analysis section in the Materials and Methods that describes how our error bars were calculated (p. 23).

- In addition, in Figure 2 the y-axis is labeled "Import Efficiency (%)". Does this percentage refer to the total number of observed events? If so, why do less than 25% of species observed pass through the nuclear pore? If not, the authors may want to specifically comment on this in the text or in the figure legend.

Response: Import efficiency is now clearly defined in the main text (p. 6). In some instances (at low transportin:cargo ratios), the import efficiency is indeed very low, i.e., very few M9-βGal molecules that bind to/interact with an NPC actually go through that NPC (instead, they abort transport, and return to the cytoplasm). These low transport efficiencies are explained by the large cargo size (Fig. 2B), and by the low number of bound NTRs. The latter issue is a major point of the paper, and is extensively discussed. We hope that our clarification of the definition of import efficiency clears up this central issue for this Reviewer.

3. Grammar and language:
a. Page 21: Four reference titles contain the symbol "®" instead of the Greek symbol.

Response: The Greek symbol beta (β) was lost during file conversion to the pdf. This is probably an operating system error (Mac vs. PC). We have confirmed that the symbols are correct in the submitted Word file (Word 2008 for Mac).

- The terms "significant" and "substantial" were used at least ten and five times, respectively. While these terms help the reader to infer that the evidence to support a given claim is strong or important, they do not provide a quantitative description of evidence except in very few cases (i.e., in reference to statistical significance). The authors should consider presenting their conclusions using more specific and exact terms to describe their results and to place them in the context of existing literature.

Response: We agree that our use of "significant" and "substantial" was imprecise. Reviewer #2 also had similar concerns and requested the use of statistical tests, which we have now included (see response to Reviewer #2). We have modified the manuscript text in various locations (pp. 10, 12, and 17) to more accurately describe comparisons of experimental results and theoretical predictions. In addition, we have clarified the Discussion in numerous places. We hope that our more explicit discussion and the use of statistical tests (described in the Materials and Methods section – p. 23) satisfy this Reviewer.

Overall, the manuscript has been improved significantly by directly addressing the criticisms and comments of the Reviewers. We are grateful for the thoughtfulness and time expended by the Reviewers in critiquing this manuscript. Lastly, we thank you for your efforts in handling this manuscript. We hope that the Reviewers and you find the current version of this manuscript suitable for publication in the *EMBO Journal*.

2nd Editorial Decision 01 October 2013

As you can see in the referees' comments below, your revised version has addressed all of their concerns except for the generality of the mechanism you describe. We do not insist on testing other large cargo or pores as this would have exceeded a 3-month revision time. Therefore, I am pleased to accept the manuscript for publication here.

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Please see below for important information on how to proceed. Make sure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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REFEREE COMMENTS

Referee #1:
The authors have addressed my concerns and I recommend publication.

Referee #2:
In the revised version of their manuscript "Large Cargo Transport by Nuclear Pores: Implications for the Spatial Organization of FG-Nucleoporins", the authors address all the minor points that were originally raised by me (and also those that were raised by the other reviewers.) They now include a statistical analysis of the data presented in Fig. 2 and clarify several issues in the text. The major criticism could not be addressed and the authors conclude that appropriate "experiments lie outside the scope of the present work and are best left for a future manuscript".

Referee #4:
The authors appropriately addressed all raised questions and comments in the revision, I have no further requests for revision.

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