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Characterisation of the passive permeability barrier of nuclear pore complexes

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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 13 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are enclosed. You will see that while referees 2 and 3 are considerably more positive regarding publication of the paper here referee 1 raises major concerns regarding the conceptual advance provided as well as regarding the overall conclusiveness of the data. Still, he/she puts forward a constructive set of comments how to improve the study. Also, referees 2 and 3 raise a number of issues that need further attention. Referees 1 and 3 also feel that the previous literature needs to be represented and discussed in a more balanced manner. We will therefore be able to consider a revised manuscript if you can address or respond to the referees' concerns in an adequate manner and to their satisfaction.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the manuscript by Mohr et al., the authors have used semi-permeabilized tissue culture cells and time resolved confocal microscopy to examine the characteristics of the NPC passive diffusion channel(s). In these studies, movement into the nucleus of a variety of different sized, presumably inert probes, was examined and the data were used to estimate the size of the passive diffusion channel(s). The authors also attempted to determine whether passive diffusion and facilitated transport occur through similar regions of the NPC. To test this hypothesis, they examined the effects of simultaneous activate transport, as well as inhibitors of facilitated transport, on the passive diffusion characteristics of the NPC. On the basis of their data, the authors speculate on the number, position, and size of the diffusion channels. Two general conclusions are drawn. First, they propose that the predominant diffusion channels through the NPC have a radius of 2.6 nm and they argue that this reflects the periodicity of FG repeats in certain nups. Second, they conclude that passive diffusion and facilitated transport occurs through the same region of the NPC.

As expected from this laboratory, the data presented in this manuscript are of high quality. I do, however, have major concerns about the degree of impact this manuscript will have on the field and, thus, its suitability for the EMBO J. In certain instances, the authors have over interpreted their data, arriving at conclusions and proposing models that are not satisfactorily supported. Specific examples are discussed below. In the absence of further experimentation that supports the authors' conclusions, the manuscript offers a limited amount of functional insight and is more appropriate for a specialized journal.

Major concerns.

1) In the Introduction and throughout the manuscript, the authors infer that, prior to this study, the accepted model for how molecules diffuse through the NPC is through peripheral channels that lie outside of the central, active transport channel. This is indeed one proposed idea. However, the authors fail to site, or satisfactorily discuss, previously published data which also supports the conclusion that the central regions of the NPC accommodate both facilitated transport and active diffusion (among these references are Feldherr et al., 1984; Felderr and Akin, 1997; Keminer and Peters, 1999). Thus, it appears that the authors are understating previous literature and inflating the significances of this study. The manuscript should be modified to remove this perception.
This study has the potential to offer greater insight into the molecular basis for the diffusion channel. In this regard, the author shows that WGA and the Impß45-462, two inhibitors of facilitated transport, also inhibit passive diffusion. This is an interesting observation, but these experiments provide only limited insight into the molecular basis for the passive diffusion channel. These inhibitors likely bind to multiple FG-nups within the NPC. Thus, it is unreasonable to conclude that these inhibitors are specifically blocking channels formed by linked FG-nups within the central transport channel. It is possible that the effects of WGA are caused by its binding to FG-nups outside the central channel (e.g. cytoplasmic or nucleoplasmic fibers), which could restrict access to the diffusion channel. In addition, these inhibitors could also block peripheral channels, either sterically or indirectly by altering the biophysical properties of the NPC framework. Considering these possibilities, it is difficult to conclude whether passive and active transport channels are one in the same or two separate entities. To address this, rather than employing these general inhibitors of transport, the authors should consider focusing on the role of specific nups in the diffusion process (e.g. through siRNA depletion or using specific antibodies) as a means of defining the specific features of the NPC that govern passive diffusion.

2) In their evaluation of the import of NTF2, the authors should consider the possibility that this molecule could enter the nucleus by both passive and active transport pathways.

3) The experiments presented in this manuscript have been performed using semi-permeabilized tissue culture cells and the authors have compared and contrasted their estimates of the size of the passive diffusion channel (~2.6 nm in radius) to measurements made in living cells. It important for the authors to consider these differences in the experimental systems, which could contribute to the observed differences in the size estimates of the passive diffusion channel. I recognize that the semi-

permeabilized cells continue to support facilitated import, but this does not exclude the possibility that the passive permeability properties of the NE are altered under these conditions. Ideally, the authors should consider testing some of their diffusion reporters by microinjection into live cells.

4) In their discussion of the size of the passive diffusion channel, the authors argue that the maintenance of the Ran gradient across the NE necessitates a passive diffusion barrier with a size limit that would prevent Ran from diffusing out of the nucleus (i.e. within the range that they have defined). This argument is only relevant if the majority of Ran in the nucleus is free and not bound to other proteins (is there evidence for this?) and that the rate of active import of free Ran is exceeded by passive efflux. Experiments in yeast suggest that mutations that increase the size of the diffusion channel do not shutdown import (Shulga et al., 2000), suggesting that the conclusion that a passive diffusion channel with a radius larger than 2.6 nm is incompatible with the maintenance of the Ran gradient is flawed.

5) This manuscript contains a large number of grammatical errors. At times it is difficult to read. A case in point is the second paragraph of the Discussion. I suggest the authors carefully reexamine and modify the structure of the text.

Minor point
1) If the Alexa488-labelled 11 AA peptide is inert, why is it concentrating at the NE in Fig. 4B.

Referee #2 (Remarks to the Author):

Structural models of the NPC often suppose seperate diffusion and transport channels. In this study Mohr et al. challenge this view and provide evidence for a common channel. This is based on two arguments. 1) diffusion channel sizes are consistent with a mesh channel system as described by the FG-hydrogel model; 2) WGA and truncated importin-beta that have been previously thought to be specific inhibitors for transport also inhibit diffusion.

This is an interesting manuscript, but has a few problems.

1. What is the error in the measurements? For the calculation of the channel sizes and influx rates it is unclear influx in how many cells were used. Are the graphs in figure 2 and 3 and 5 based on "representative" permeabilized cells? If so how many? Are datapoints averaged or is the fit averaged? What is the error? Measurements of channel sizes and influx rates should be an average of multiple cells in several independent experiments with estimation of error included.

2. The mechanism of inhibition of imp-beta45-462 remains unexplained. A propensity to multimerize is mentioned, but even a pentamer of importin beta does not come close to the inhibitory power of imp-beta45-462 (Table 4 and Suppl. Fig. 1). The authors should admit this.

3. As far as I can see the authors cannot exclude the existence of different pools of NPCs, each with their own channel size. The authors should discuss this possibility.

4. In Fig. 2 GFP and MBP seem to be fitted to y=kt not y=a+kt

Referee #3 (Remarks to the Author):

In this manuscript, Mohr and colleagues analyze the permeability of the nuclear pore complex (NPC) for passive ("inert") cargoes, which do not contain karyophilic transport signals. Measuring the diffusion kinetics of inert cargoes of various sizes the authors conclude that the NPC contains a heterogeneous population of channels. The predominant diameter of the diffusion channels is ~2.6nm but openings for larger cargoes also exist. Translocation of small cargoes is efficiently inhibited by known blockers of active transport including WGA and the dominant importin beta fragment, Impß45-462. Based on these observations the authors argue that active, transport receptor mediated translocations and passive NPC passages occur through a common channel system.

Passive translocation through the NPC has been first studied more than 30 years ago and this study

revisits some of these original observations with a variety of carefully chosen reporters and modern quantitative methods using an established in vitro transport sytem. Overall, this paper contributes significantly to our understanding of nucleocytoplasmic transport and NPC function and should be published in EMBO J.

1. Interestingly, a single NPC channel scenario cannot explain the diffusion kinetics of larger cargoes and the authors obtained the best fit using a 3-channel model with channel diameters of 1.76, 2.63 and 4.32 nm, respectively. A priori, this is inconsistent with a single gate. However, the authors also observe that inhibitors of active transport (WGA and Impß45-462) affect passive diffusion providing an argument for a shared channel. Yet, WGA and Impß45-462 have little or no effect on the diffusion of small cargoes. Therefore, the authors cannot entirely exclude the presence of separate small diffusion channels.

2. The dogmatic style of the paper can be irritating at times. For example, 'conceptual arguments' cannot be used to exclude that certain solutions are not employed within cells. Properties evolve but there are no intelligent design principles. Furthermore, current structure models of NPCs are still very poorly resolved (above 5 nm) and it is impossible to exclude the existence of small, separate channels based on currently available structures.

1st Revision - authors' response 05 May 2009

Point-by-point answers to the reviewers' comments.

Referee #1 (Remarks to the Author):

In the manuscript by Mohr et al., the authors have used semi-permeabilized tissue culture cells and time resolved confocal microscopy to examine the characteristics of the NPC passive diffusion channel(s). In these studies, movement into the nucleus of a variety of different sized, presumably inert probes, was examined and the data were used to estimate the size of the passive diffusion channel(s). The authors also attempted to determine whether passive diffusion and facilitated transport occur through similar regions of the NPC. To test this hypothesis, they examined the effects of simultaneous activate transport, as well as inhibitors of facilitated transport, on the passive diffusion characteristics of the NPC. On the basis of their data, the authors speculate on the number, position, and size of the diffusion channels. Two general conclusions are drawn. First, they propose that the predominant diffusion channels through the NPC have a radius of 2.6 nm and they argue that this reflects the periodicity of FG repeats in certain nups. Second, they conclude that passive diffusion and facilitated transport occurs through the same region of the NPC.

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Major concerns.

1) In the Introduction and throughout the manuscript, the authors infer that, prior to this study, the accepted model for how molecules diffuse through the NPC is through peripheral channels that lie outside of the central, active transport channel.This is indeed one proposed idea. However, the authors fail to site, or satisfactorily discuss, previously published data which also supports the conclusion that the central regions of the NPC accommodate both facilitated transport and active diffusion (among these references are Feldherr et al., 1984; Felderr and Akin, 1997; Keminer and Peters, 1999). Thus, it appears that the authors are understating previous literature and inflating the

significances of this study. The manuscript should be modified to remove this perception.

We changed the wording and supplemented the references of this paragraph. We now state: "...this 'separate channel hypothesis' is in conflict with other models of NPC function (Feldherr et al., 1984; Feldherr and Akin, 1997; Keminer and Peters, 1999) and in particular to those assuming that FG repeat domains form a common barrier for passive and facilitated exchange (Macara, 2001; Ribbeck and Görlich, 2001; Rout and Aitchison, 2001)."

Apart from that, we wish to emphasize that the issue of common or separate channels has so far not been resolved and that our study now closes this gap. Likewise, the concepts that WGA and Impß45-462 are also inhibitors for passive transport and that NPCs behave towards inert material like sieves with a heterogeneous mesh size are not only novel, but also they unite previous apparently conflicting data.

This study has the potential to offer greater insight into the molecular basis for the diffusion channel. In this regard, the author shows that WGA and the Impß45-462, two inhibitors of facilitated transport, also inhibit passive diffusion. This is an interesting observation, but these experiments provide only limited insight into the molecular basis for the passive diffusion channel. These inhibitors likely bind to multiple FG-nups within the NPC. Thus, it is unreasonable to conclude that these inhibitors are specifically blocking channels formed by linked FG-nups within the central transport channel. It is possible that the effects of WGA are caused by its binding to FG-nups outside the central channel (e.g. cytoplasmic or nucleoplasmic fibers), which could restrict access to the diffusion channel. In addition, these inhibitors could also block peripheral channels, either sterically or indirectly by altering the biophysical properties of the NPC framework. Considering these possibilities, it is difficult to conclude whether passive and active transport channels are one in the same or two separate entities.

(A) We demonstrated that not only WGA and Impß$^{45-462}$, but also functional nuclear transport receptors reduce passive fluxes through NPCs. It is our understanding that nuclear transport receptors can either traverse a common barrier for passive and facilitated passage or use dedicated "active channels", but not channels that conduct inert material only. By this definition, exclusively passive channels should not interact with a nuclear transport receptor. The most plausible explanation for the observation that passive transport is suppressed by the receptors is therefore the model that receptors and inert material follow similar paths through NPCs.

(B) The reviewer suggested that WGA or Impß$^{45-462}$ might alter the biophysical properties of the NPC framework in such a way that passive channels indirectly get blocked. This would have to involve quite exotic structural changes within NPCs. As also full-length NTRs suppress passive NPC passage, it is not plausible to us why these physiological NPC ligands should cause such exotic conformational changes within a nuclear pore complex.

(C) Impß$^{45-462}$ is derived from a bona fide transport receptor and therefore should only bind bona fide importin ß binding sites. The mutant should therefore only inhibit passage along the path of facilitated transport.

Impß$^{45-462}$ does not block docking of NLS·importinα·importinß beta complexes to NPCs (see Figure 7 in Kutay (1997), EMBO 16: 1153). We therefore regard it as unlikely that it targets the cytoplasmic NPC-filaments to a significant extend. The same applies to WGA (see Newmeyer & Forbes (1988), Cell 52: 641).

(D) In the fully defined FG hydrogel system, we could recapitulate all relevant features of passive and facilitated NPC passage (see accompanying manuscript). The FG hydrogels allowed facilitated receptor-mediated cargo influx and suppressed passive entry. However, just as NPCs, they were not absolutely tight towards inert material, but permitted a significant entry of GFP-sized molecules. In the hydrogel system, we could also reproduce that both, NTRs and Impß$^{45-462}$, suppress passive influx. These were the most stringent plausibility tests and a proof of principle for the common barrier scenario.

In the hydrogel system, we can exclude the possibility that nucleoporins that normally build the framework of NPCs contributed passive diffusion channels.

To address this, rather than employing these general inhibitors of transport, the authors should consider focusing on the role of specific nups in the diffusion process (e.g. through siRNA depletion or using specific antibodies) as a means of defining the specific features of the NPC that govern passive diffusion.

(A) It was our aim to probe the properties of a native and fully functional permeability barrier. The question as to what forms this barrier has been addressed in the accompanying manuscript. There we report that the permeability barrier of NPCs can be surprisingly well reconstituted as an FG hydrogel and that structural nucleoporins are not required for forming such NPC-like barrier.

(B) We have general doubts that RNAi experiments are appropriate to address the question of separate or common channels. Most of the mass of an NPC is made up of the rigid scaffold of structural nucleoporins. It is generally accepted that movement of material through NPCs is restricted to some kind of channels, which implies that the rigid body of an NPC is a tight barrier. It is self-evident that the removal of individual or several nucleoporins from NPCs can generate voids and thus new channels. Accordingly, even if the depletion of a given Nup increased passive fluxes through NPCs, one could still not unambiguously conclude that the removed Nup would also form passive diffusion channels in an intact pore complex.

The suggestion for RNAi experiments is also too vague to be translated into a meaningful experiment. Which nucleoporins should be depleted? Which phenotypes should we probe for and at which stage of the depletion should the permeabilised cells be prepared? NPCs and nuclear transport are essential; depletions with drastic effects on either passive or facilitated transport will therefore result in dead cells before these can be permeabilised. How should we distinguish between defects in NPC assembly and a specific defect in passive or facilitated transport?

(C) The suggested antibody experiment is also too vague to be implemented: What should the binding of an antibody to a specific nucleoporin tell us about passive, active or common channels? Does the reviewer expect that antibodies can be found that affect only passive or only facilitated transport? How many and which epitopes should be tested? How should such experiment be interpreted? Antibody inhibition experiments are notorious for giving false-positive results. How should we exclude that steric hindrance accounts for any of the observed effects? What would be a meaningful specificity
control?

(D) FG repeats are by far the most promising NPC modules for forming the permeability barrier. Antibody inhibition experiments are definitively not a promising approach to test this. An NPC contains ≈ 5000 repeat units. Neutralising them would require 2500 IgG molecules or an antibody mass of 400 MDa per NPC (which has a mass of only 100 MDa). It is obvious that this antibody mass cannot fit into the central NPC channel and that such massive protein accumulation would have drastic non-specific effects.

2) In their evaluation of the import of NTF2, the authors should consider the possibility that this molecule could enter the nucleus by both passive and active transport pathways.

We considered this possibility. However, as passive passage of the similarly sized GFP is 100-times slower than that of NTF2, the passive contribution will not significantly change the overall rate of NTF2 passage through NPCs.

3) The experiments presented in this manuscript have been performed using semi-permeabilized tissue culture cells and the authors have compared and contrasted their estimates of the size of the passive diffusion channel (~2.6 nm in radius) to measurements made in living cells. It important for the authors to consider these differences in the experimental systems, which could contribute to the observed differences in the size estimates of the passive diffusion channel. I recognize that the semipermeabilized cells continue to support facilitated import, but this does not exclude the possibility that the passive permeability properties of the NE are altered under these conditions. Ideally, the authors should consider testing some of their diffusion reporters by microinjection into live cells.

We performed a microinjection experiment into nuclei of Xenopus oocytes and followed the efflux of

thioredoxin and MBP simultaneously. The result was that MBP crossed NPCs ≈ 450-times more slowly than thioredoxin. This ratio is pretty close to the numbers obtained in the permeabilised cell system, where MBP passed NPCs 100-times more slowly than thioredoxin. The small difference can easily be attributed to the fact that oocytes are extremely specialised cells and that they still contain endogenous nuclear transport receptors (which tighten the passive diffusion barrier).

4) In their discussion of the size of the passive diffusion channel, the authors argue that the maintenance of the Ran gradient across the NE necessitates a passive diffusion barrier with a size limit that would prevent Ran from diffusing out of the nucleus (i.e. within the range that they have defined). This argument is only relevant if the majority of Ran in the nucleus is free and not bound to other proteins (is there evidence for this?)

Several years ago, we studied by computer simulation what would happen if efflux of RanGTP from nuclei were not retarded by NPCs and the nuclear envelope (see Görlich et al., 2003, EMBO 22: 1088). Within an intact nuclear membrane and intact NPCs, a nuclear : cytoplasmic RanGTP ratio of ≈ 1000 : 1 was obtained. Without a nuclear envelope, this ratio would drop to ≈ 2 : 1, whereby the nuclear concentration would still be sufficient for dissociating importin·cargo complexes (≈ 1μ M). The cytoplasmic concentration (≈0.5 μM), however, would already be sufficient to prevent formation of importin·cargo complexes. We therefore regard our it as solid that free RanGTP needs to be constrained to the inside of nuclei and that its dissipation into the cytoplasm has to be suppressed. The reviewer now suggests that binding of nuclear RanGTP to some other protein could delay efflux from nuclei and that this would allow a higher passive exclusion limit of NPCs. Such binding partner should be at least as abundant as Ran. In addition, it should bind the GTP-bound form and yet not interfere with binding to importins or exportins. In the literature, we could not find evidence for a protein that would meet theses criteria. Furthermore, over the past 15 years, others and we looked hard to identify the complete set of cellular interaction partners of RanGTP and we regard it as unlikely that others and we could possibly have missed such an abundant RanGTP binder.

…and that the rate of active import of free Ran is exceeded by passive efflux.

This is definitively not the case. NTF2 (the mediator of Ran import) accelerates NPC-passage of Ran about 15-fold (Görlich et al. (2003), EMBO 22:1088). The fact that NTF2 is essential for viability in yeast (Corbett & Silver (1996), J Biol Chem. 271:18477) suggests that the passive influx of Ran into nuclei is too slow to sufficiently sustain Ran-driven nuclear transport. Conversely, this suggests that NPCs considerably suppress passive efflux of Ran.

Previously, we computer-simulated the situation where the permeability of NPCs for RanGTP is increased (causing proportionally increased cytoplasmic levels) and found that this clearly has a negative impact on importin-mediated protein transport (see e.g. the first three rows in Table IV, Görlich et al. 2003, EMBO 22:1088). We regard these results as solid.

Experiments in yeast suggest that mutations that increase the size of the diffusion channel do not shutdown import (Shulga et al., 2000), suggesting that the conclusion that a passive diffusion channel with a radius larger than 2.6 nm is incompatible with the maintenance of the Ran gradient is flawed.

(A) see previous point above.

(B) The half-time for equilibrating GFP between nucleus and cytoplasm is ≈ 5 minutes in permeabilised HeLa cells (strain CCL2) and ≈ 3 minutes in yeast cells (a rough estimate from Shulga et al., 2000). Since the ratio of NPCs per nucleus to nuclear volume is also similar between the two organisms, we can conclude that the passage rate of GFP through NPCs and hence also the sieving properties of NPCs appear evolutionary pretty conserved (the difference being within a 10% deviation of the apparent passive channel radius). We find it perfectly reasonable to assume that this conservation might be the result of evolutionary pressure. This assumption was not phrased as experimental result, but was clearly indicated as a speculation in the Discussion and we feel that this is justified and appropriate.

(C) NPCs of nup170Δ or nup188Δ S. cerevisiae strains allow higher passive fluxes than wild type

cells (Shulga et al., 2000). In the case of nup170Δ, the competitive fitness has been measured and was significantly reduced as compared to the corresponding wild type strain (see: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=NUP170). These data suggest that selective pressure would eliminate mutants displaying such an increased passive exclusion limit within a few days of competitive growth. This is very much what we discussed in the manuscript.

(D) The results presented by Shulga et al. (2000) suggest that an NLS-GFP reporter is almost exclusively nuclear in wild type cells, but shows a significant cytoplasmic signal in a nup170Δ or nup188Δ mutant background. Nevertheless, the reporter in the mutant strains is still predominantly nuclear, i.e. mutant NPCs suppress passive efflux still reasonably well. The defect is therefore relatively mild. Furthermore, the NLS reporter should respond more sensitively than the Ran gradient to an increased passive diffusion limit, because its nuclear accumulation is not only limited by passive efflux, but it also requires two nuclear RanGTP molecules per active import event (one for dissociation of importin α from ß and one for exporting importin α back to the cytoplasm). If the nuclear to cytoplasmic ratio of NLS-GFP is changed in the mutants by a factor of 5 (that is what Figure 2 in Shulga et al. (2000) suggests), then the nucleo-cytoplasmic RanGTP gradient should be diminished by a smaller factor. This reduction appears to lower the fitness of the yeast cells, but still supports life. All this is fully consistent with our discussion.

5) This manuscript contains a large number of grammatical errors. At times it is difficult to read. A case in point is the second paragraph of the Discussion. I suggest the authors carefully reexamine and modify the structure of the text.

The manuscript has been thoroughly re-checked for grammar and readability.

Minor point
1) If the Alexa488-labelled 11 AA peptide is inert, why is it concentrating at the NE in Fig. 4B.

It is true that the Alexa488-labelled peptide bound weakly to the cytoplasmic remnants of the permeabilised cells. However, this was not an NPC staining, but coincides with the rER. Since only a tiny fraction of the peptide was trapped there, it did not significantly change the free concentration and therefore did not interfere with the measurement.

Referee #2 (Remarks to the Author):

Structural models of the NPC often suppose seperate diffusion and transport channels. In this study Mohr et al. challenge this view and provide evidence for a common channel. This is based on two arguments. 1) diffusion channel sizes are consistent with a mesh channel system as described by the FG-hydrogel model; 2) WGA and truncated importin-beta that have been previously thought to be specific inhibitors for transport also inhibit diffusion.

This is an interesting manuscript, but has a few problems.

1. What is the error in the measurements? For the calculation of the channel sizes and influx rates it is unclear influx in how many cells were used. Are the graphs in Figure 2 and 3 and 5 based on "representative" permeabilized cells? If so how many? Are datapoints averaged or is the fit averaged? What is the error? Measurements of channel sizes and influx rates should be an average of multiple cells in several independent experiments with estimation of error included.

We agree that measurements of channel sizes and influx rates should represent an average of multiple cells in several independent experiments and also include error estimates. Therefore, Table I shows the standard deviations for the flux ratios qi between individual experiments, which was larger than any of the corresponding "intra-experiment" standard deviations, i.e. the standard deviation between individual cells of an individual measurement. For each probe, at least 3 independent experiments (generally 3 to 9) were performed. We monitored at least 2 cells (generally 2 to 10) per experiment and a total of at least 21 (generally 21-43) for each probe. The number of cells that could be watched simultaneously depended on the scan rate and was lower for very fast scans.

Inhibition experiments as shown in Figure 5 were repeated at least once (generally 2 to 7

independent experiments) with a total of at least 9 cells (generally 9 to 43 cells). Nuclei treated with inhibitors (WGA or Impß$^{45-462)}$ showed less variability than untreated nuclei. The deviation in the "inhibition factors" is therefore dominated by the variability of untreated nuclei.

The Figures 1 and 4, showing microscopic data, document one representative experiment each. Figures 2 and 5 show quantifications of individual experiments, whereby data had been averaged for all (undamaged) cells in the respective experiments. Tables and Figure 3 show averages of all relevant experiments.

2. The mechanism of inhibition of imp-beta45-462 remains unexplained. A propensity to multimerize is mentioned, but even a pentamer of importin beta does not come close to the inhibitory power of imp-beta45-462 (Table 4 and Suppl. Fig. 1). The authors should admit this.

We now include data demonstrating that the importin beta mutant indeed forms oligomers and binds FG repeats stronger than wild type importin beta. However, we do agree with the reviewer that the oligomerisation is not yet the complete explanation. We expanded the Discussion accordingly.

3. As far as I can see the authors cannot exclude the existence of different pools of NPCs, each with their own channel size. The authors should discuss this possibility.

Agreed. In fact, the low occurrence of larger 4.3 nm channels (≈1 per 10 NPCs) clearly suggests heterogeneity between individual NPCs. The Discussion now includes the possibility that this heterogeneity might not only originate from spontaneous opening of inter-repeat contacts, but also from damaged NPCs or NPCs in statu nascendi*.*

4. In Fig. 2 GFP and MBP seem to be fitted to y=kt not y=a+kt

The first case is a special case of y=a+kt with a=0. All GFP and MBP curves have been fitted with y=a+kt, because "a" allows for an offset in time (the first frame hardly ever coincides exactly with the exact time of addition of the fluorescent substrate) and for an offset in the fluorescent signal.

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Thank you!

1. Interestingly, a single NPC channel scenario cannot explain the diffusion kinetics of larger cargoes and the authors obtained the best fit using a 3-channel model with channel diameters of 1.76, 2.63 and 4.32 nm, respectively. A priori, this is inconsistent with a single gate. However, the authors also observe that inhibitors of active transport (WGA and Imp□45-462) affect passive diffusion providing an argument for a shared channel. Yet, WGA and Imp□45-462 have little or no effect on the diffusion of small cargoes. Therefore, the authors cannot entirely exclude the presence of separate small diffusion channels.

We agree. In fact, already in the previous version we stated that hydrated protein structures cannot generate a water-tight barrier (in a different context though). To discuss this issue further, we included the following text:

"Besides this main barrier, additional channels might exist within the rigid framework of NPCs. However, to be consistent with our data, these channels should be rather narrow and contribute only little to the transport of macromolecules. Their conductance for ions and metabolites should also be small compared to the enormous capacity expected for a main barrier based on FG repeats."

2. The dogmatic style of the paper can be irritating at times. For example, 'conceptual arguments' cannot be used to exclude that certain solutions are not employed within cells. Properties evolve but there are no intelligent design principles. Furthermore, current structure models of NPCs are still very poorly resolved (above 5 nm) and it is impossible to exclude the existence of small, separate channels based on currently available structures.

We are definitively no proponents of "intelligent design" and therefore changed the text to avoid such perception. Nevertheless, we regard the actual argument of the "offending paragraph" as valid: A strict "separate channel scenario" should assume a central channel of ultimate selectivity, i.e., a channel that allows only receptor-mediated passage, but completely blocks inert material. It is hard to envision what a proteinaceous barrier, which is absolutely tight against passive passage of even ions and that at the same time allows facilitated passage of ribosomes, could possibly look like. It is even harder to envision, which selective pressure could have enforced the evolution of such a super-tight structure, if additional passive diffusion channels must in turn bypass its tightness.

2nd Editorial Decision 05 June 2009

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that they are now all supporting publication of your paper here. Referee 1 still has a number of specific points in his/her comments to the authors (see below) that I would like to transmit to you for your information.

You will receive a formal acceptance letter early next week.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORT

Referee #1 (Remarks to the Author):

Several improvements have been made to the manuscript and reasonable responses have been provided to many of the points raised in the previous review. Some points, however, remain contentious. Among them is whether the manuscript provides significant molecular insight into the position and composition of the diffusion channels through the NPC. As this was not an overriding issue amongst all the Reviewers, I will not pursue this point. I would suggest, however, that the use of antibodies (more specifically Fab fragments) directed against FG-Nups positioned within the central channel of the NPC (e.g. Nup62) would, in fact, provide greater molecular insight into the specific role of the central channel components in passive diffusion through the NPC. Antibody tools can serve as a much more specific set of tools than WGA or the Imp 45-462 mutant, which binds many FG-nups.

Finally, the portion of my inquiry questioning the authors' idea that the size of the diffusion channel assists in restricting the efflux of Ran out of the nucleus seems to have been misunderstood. I recognize that NTF2 plays a significant role in the accumulation of Ran in the nucleus. For this reason, I suggest that this would effectively lead to the accumulation of Ran in the nucleus, even if the size of the diffusion channel was significantly larger than that predicted by the authors.

