How to operate a nuclear pore complex by Kap-centric control

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Nuclear pore complexes (NPCs) mediate molecular transport between the nucleus and cytoplasm in eukaryotic cells. Tethered within each NPC lie numerous intrinsically disordered proteins known as FG nucleoporins (FG Nups) that are central to this process. Over two decades of investigation has converged on a view that a barrier mechanism consisting of FG Nups rejects non-specific macromolecules while promoting the speed and selectivity of karyopherin (Kaps) receptors (and their cargoes). Yet, the number of NPCs in the cell is exceedingly small compared to the number of Kaps, so that in fact there is a high likelihood the pores are always populated by Kaps. Here, we contemplate a view where Kaps actively participate in regulating the selectivity and speed of transport through NPCs. This so-called “Kap-centric” control of the NPC accounts for Kaps as essential barrier reinforcements that play a prerequisite role in facilitating fast transport kinetics. Importantly, Kap-centric control reconciles both mechanistic and kinetic requirements of the NPC, and in so doing potentially resolves incoherent aspects of FG-centric models. On this basis, we surmise that Kaps prime the NPC for nucleocytoplasmic transport by fine-tuning the NPC microenvironment according to the functional needs of the cell.

Introduction

Eukaryotic cells maintain order and function by the exchange of macromolecules between the nucleus and cytoplasm. Every second, mixed within a rich biological milieu, essential proteins, RNAs and metabolites impinge, crowd and translocate across the nuclear envelope.¹ This process is termed nucleocytoplasmic transport, which proceeds through aqueous channels known as nuclear pore complexes (NPCs).²

The modus operandi that facilitates the selectivity and speed of signal-specific cargoes through NPCs is one that continues to fascinate and confound in equal measure. Despite bearing a 50 nm-diameter central channel, only molecules smaller than 40 kDa or 5 nm in size can passively diffuse in an unhindered manner through the NPC, whereas larger macromolecules are excluded.³ In contrast, rapid transport is accorded to nuclear transport receptors such as karyopherins (Kaps, also known as importins and exportins) that identify, bind and shuttle large specific cargoes that would otherwise be rejected by the NPC in the absence of Kaps.⁴ Kaps distinguish essential cargoes such as transcription factors from other non-essential proteins based on peptide sequences known as nuclear localization or nuclear export signals (NLS or NES).⁵,⁶ This is typified by the import receptor Kapβ1 (Impβ; 100 kDa),⁷ which binds NLS-cargoes via an adaptor Kapα (Impα; 60 kDa) to altogether form transport-competent complexes that are many times more massive than the putative 40 kDa size exclusion limit of the NPC. It is even more perplexing how large transport complexes appear to surpass passive diffusion rates when traversing the NPC.⁸

FG-centric Barriers

The functional constituents of the NPC consist of intrinsically disordered proteins that contain large numbers of phenylalanine-glycine (FG) repeats known as FG nucleoporins (FG Nups).⁹ FG...
Nups bear multiple FG-repeat motifs that are essential for transport selectivity because they exert multivalent binding interactions with Kaps.\textsuperscript{10-12} Over 200 FG Nups are physically constrained to the inner walls of the NPC by tethering domains, upon which disordered FG domains (typically 200 – 700 aa in length) emanate to explore the aqueous central channel.\textsuperscript{13} Hence, each tether point defines the relative position, surface density, and collective FG Nup morphology inside the NPC.\textsuperscript{14} On this basis, the FG Nups are reasoned to manifest barrier-like properties that guard against non-specific proteins while simultaneously providing selective access to Kaps that bind to FG-repeat.

The ongoing debate about NPC function largely stems from an inability to directly resolve FG Nup structure and dynamics inside the NPC.\textsuperscript{15} So far, FG Nup behavior has been inferred from indirect approaches such as immunolabelling transmission electron microscopy,\textsuperscript{15} various fluorescence microscopies,\textsuperscript{16,17} and most recently mechanical measurements obtained by indentation-based atomic force microscopy.\textsuperscript{18} Therefore, any information regarding the structure and function of the NPC barrier is still largely phenomenological and based in part on the following rationale:

1. The FG Nups adopt collective arrangements that exert barrier-like functionality.
2. Specific Kap-FG interactions ensure NPC transport selectivity; insufficient binding leads to barrier rejection.
3. High mobility ensues from specific Kap-FG interactions.

Inevitably, \textit{in vitro}-derived FG Nup materials, such as supramolecular hydrogel meshworks,\textsuperscript{19,20} and molecular brushes,\textsuperscript{21,22} or their proposed combinations\textsuperscript{23} dominate how we treat the FG Nups as the key barrier components, as evident in artistic depictions of the NPC. Regardless, what is clear is that the FG Nups – being intrinsically disordered – are sensitive to experimental design \textit{in vitro}, and can adopt different morphologies at different length scales with diverse structures, characteristics and properties.\textsuperscript{24} We refer the interested reader to ref. 24 for a recent review on the matter.

Yet, insofar as such “FG-centric” models are able to rationalise mechanistic NPC function, explanations of fast transport kinetics remain vague and even paradoxical. At the structural level, each Kap\textsubscript{1} molecule consists of approximately 10 hydrophobic grooves that can all potentially bind FG-repeats.\textsuperscript{10-12} Moreover, the FG Nups contain between 5 to around 50 FG-repeats\textsuperscript{25} that can simultaneously bind individual Kaps at multiple contact points. Hence, Kap-FG Nup binding is characterized by highly multivalent interactions,\textsuperscript{26,27} which are recognized to enhance stability and specificity due to strong binding avidity.\textsuperscript{28} As raised by Tetenbaum-Novatt \textit{et al.}\textsuperscript{29} the known sub-\textmu{M} Kap\textsubscript{1}-FG domain binding affinities\textsuperscript{11,30-32} may “ensure” NPC transport selectivity but contradict the rapid ~5 ms dwell times of nucleocytoplasmic transport cargoes \textit{in vivo}\textsuperscript{33} and \textit{in vitro}.\textsuperscript{34,36} It is intriguing that even FG Nup-coated nanopores are able to recapitulate both the selectivity and speed of Kap\textsubscript{1} translocation with a dwell time of ~2.5 ms,\textsuperscript{37} in close agreement with NPC transport values. What might promote fast translocation in the NPC instead of slowing it down?

**Selectivity, Speed and Space: The Microenvironment Matters**

In fact, the NPC problem conflates specific binding, translocation speed and nanoscale spatial constraints. As the sole conduit that connects the cytoplasm and the nuclear interior, each NPC is above all a major intracellular transport hub that regulates the traffic of essential nucleocytoplasmic cargoes ranging from signal transducing proteins\textsuperscript{38} to RNAs.\textsuperscript{39} Not surprisingly, up to 100 Kaps occupy the pore at steady state at cellular Kap concentrations of ~10 \textmu{M}.\textsuperscript{40-42} With 20 different Kaps shuttling diverse cargoes through the NPC,\textsuperscript{43} it would be reasonable to expect that \textit{a priori} the pore is overcrowded and that any remaining space within is very limited. This makes it difficult to assign to each Kap the functional role of a “key” that unlocks the FG Nup “gate.” In part, this is exacerbated by promiscuous binding interactions that presumably occur simultaneously between different Kaps, transport factors and FG Nups.\textsuperscript{44} Assuming sub-\textmu{M} binding affinities were required to ensure Kap selectivity, the NPC would clog and remain spatially inaccessible under such crowding conditions. How then does specific binding lead to fast Kap translocation? More so, how does a collective FG Nup barrier fit and function in such a crowded space? How does space aid or attenuate transport?

The first clue to shed light on the problem was uncovered in 2006 when Yang and Musser found that the transport efficiency and interaction time of specific cargoes were inversely impacted by Kap\textsubscript{1} concentration.\textsuperscript{36} Using permeabilized cells, they found that low Kap concentrations (i.e., < 1 \textmu{M} Kap\textsubscript{1}) yielded long interaction times (i.e., slow) and low transport efficiency whereas high Kap\textsubscript{1} concentrations (i.e., 15 \textmu{M} Kap\textsubscript{1}) yielded short interaction times (i.e., fast) and high transport efficiency. This was later corroborated by Timney \textit{et al.} who showed that increasing amounts of Kaps \textit{in vivo} did indeed expedite rather than slow down cargo import rates.\textsuperscript{45} Now, to argue from a FG-centric point-of-view, transport selectivity and speed should follow from specific Kap-FG Nup binding as a criterion to alleviate barrier (spatial) constraints in the pore. But presuming the NPC is mostly occluded by FG Nups (arranged such as in a highly cross-linked meshwork), Kap binding and occupancy in the pore would more likely obstruct space and hinder traffic at cellular Kap concentrations. Likewise, low Kap concentrations would lead to faster translocation due to low Kap occupancy and more free space in the pore. Given that the converse is true, one has to ponder how a spacious pore delays transport, while a more crowded one expedites it. Do the FG Nups retain the same barrier conformation, identity and role in spite of these differences? As noted by Yang and Musser,\textsuperscript{36} changes in pore occupancy could provide “a general means by which cargo interaction times and transport efficiencies can be modulated.”

A second subsequent clue lay in the observation that Kap-FG binding caused
a collapse of FG Nup brushes, particularly at the low Kap concentrations tested, because this showed that FG Nup conformations changed with Kap binding. A third ensued from results showing that the presence of Kaps helped to tighten the barrier against non-specific entities in artificial NPCs and FG Nup gels. Fourth, even non-specific molecules could modulate and weaken Kap-FG Nup binding. Together these intersecting lines of evidence reveal the inherent sensitivity of the FG Nups to microenvironmental factors that can impact on NPC selectivity and transport kinetics.

**Biophysical Evidence of Kap-centric Control**

To investigate the molecular basis of these effects, we found it important to reproduce some basic features of the NPC microenvironment at the biophysical level, such as (i) constraining the FG Nups to a surface via a covalent tether, (ii) reproducing FG-repeat density within such a FG layer, and (iii) studying the behavior of FG layers under physiological Kap concentrations. This “peeling open” of the pore was achieved using a surface plasmon resonance (SPR) technique that we developed to correlate free equilibrium and kinetic aspects of Kapβ1-FG Nup binding to changes in FG layer formation and Kapβ1 occupancy. Briefly, this used BSA molecules as innate molecular probes that could “feel” the physical barrier properties of a surface-tethered molecular layer by not binding the FG Nups.

A common outcome we found was an increase in layer height with decreasing next-neighbor grafting distance (increased lateral crowding). This indicated molecular brush formation, which underscores the role of surface tethering as a major determinant of FG Nup morphology. We stress that this does not preclude intra-/inter-FG Nup cohesion, but merely explains that the FG Nups are extended and oriented in a net perpendicular direction under such surface constraints. At low Kapβ1 concentrations, we found that Kapβ1 molecules exhibited slow kinetic off rates due to strong Kap-FG Nup binding ($K_D \leq 1 \mu M$) that correlated with FG layer collapse. Stepwise increases in Kapβ1 concentrations then led to a gradual re-extension of the FG layer. This so-called “self-healing” can be attributed to an increase in population (and volume) of bound Kapβ1 within the FG layers, as predicted by Zilman theory. It should be noted that this was true for Nup153, Nup214, and Nup62 and its yeast ortholog Nsp1, with the exception of Nup98, which exhibited pronounced cohesion based on its poor extensibility and low capacity for incorporating Kapβ1.

Going further, we observed a “pile-up” of Kapβ1 due to limited penetration into preoccupied FG layers at physiological Kapβ1 concentrations ($\sim 10 \mu M$), which correlated with weak binding ($K_D \geq 10 \mu M$) and fast kinetic off rates. This indicates that the occupancy of bound Kapβ1 modulates subsequent kinetic behavior in a differential manner, being attributed to how many available FG-repeats each incoming Kapβ1 molecule can access and bind to. Hence, fast transport emerges from a reduction of avidity due to a saturation of the FG Nup layer with pre-bound Kapβ1. Interestingly, this impacts on other transport factors, such as, the Ran importer nuclear transport factor 2 (NTF2), which also exhibited weakened binding and fast off-rates in the presence of bound Kapβ1. Thus, Kapβ1 preloading may also reinforce the NPC permeability barrier against more hydrophobic, non-specific cargoes.

To further validate these effects, we studied the diffusive motion of Kapβ1-functionalized colloidal particles (i.e. Kap-probes) on FG layers in the presence of soluble Kapβ1. There, we found that the Kap-probes remained stuck to the FG layer (i.e., slow) at low Kapβ1 concentrations but exhibited unhindered 2-dimensional diffusion on the layer (i.e., fast) at physiological Kapβ1 concentrations. In contrast, non-specific control probes exhibited 3-dimensional diffusion that only transiently impinged the FG layer but did not bind. Evidently, the increase in Kapβ1 occupancy could diminish multivalent interactions between the Kap-probe and the FG layer until binding was sufficiently balanced to maintain selectivity, but weak enough to facilitate 2-dimensional diffusion. By analogy, this is reminiscent of a “dirty velcro effect” where Kap-probe adhesion to the FG layer is reduced as more soluble Kapβ1 molecules occupy it. It is further noteworthy that this provided the physical proof-of-principle that corroborates the “reduction of dimensionality” (ROD) hypothesis of Adam and Delbruck, later adapted by Peters for the NPC. Basically, ROD explains that the selectivity and efficiency of biomolecular transport must involve facilitated diffusional processes that are confined to one- or 2-dimensions. [Note: For an in-depth explanation of ROD, see Berg and von Hippel.] Based on this evidence, we concluded that FG Nup conformation, Kap occupancy and transport kinetics are closely interrelated.

**Cellular Evidence of Kap-centric Control**

Populating the NPC with strongly bound Kaps may serve to reinforce barrier functionality while providing for a finely tuned microenvironment that facilitates transport selectivity and speed. Indeed, immunostaining HeLa cells that were first fixed and then digitonin-permeabilized reveals the unperturbed steady-state cellular localization of endogenous Kapβ1. As before, this yields a distinct nuclear rim staining as shown in Figure 1. To further test for retention strength, we find that a similar staining of endogenous Kapβ1 persists in cells that were first permeabilized then incubated in buffer for prolonged periods of up to 5 hours prior to fixation. Here, the lack of any quantifiable attenuation in the fluorescence signal after prolonged exposure to such severe dilution conditions indicates that the NPC-bound fraction of Kapβ1 is extremely stable and long-lived. This is consistent with the findings of Lowe et al who showed that exogenous Kapβ1 exhibits at least two kinetically distinct pools in digitonin-permeabilized cells, one being stably bound to the NPC for tens of minutes, and another that is expedited in the presence of RanGTP. Similar bimodal kinetics has also been reported for GFP-Kapβ1 in permeabilized cells and mRNA export in living cells. Interestingly, it was found that additions of exogenous Kapβ1, as well as other receptors, further
reduced NPC permeability against passive cargoes, thereby recapitulating the role of Kaps as bona fide constituents of the NPC. Moreover, Yang and Musser, as well as Timney et al showed in vitro and in vivo respectively, that increasing amounts of Kaps expedited cargo import rates, rather than slowing it down. Taken together, this evidence indicates that a pool of strongly bound Kaps that occupy the NPC at physiological concentrations facilitates the speed and selectivity of transport. As a consequence, we reason that a lack of Kap retention rather than a lack of FG repeats leads to appreciable leakiness.

Implications of Kap-centric Control

But how can selective transport proceed more rapidly than passive diffusion? A parsimonious interpretation of Kap-centric control suggests that Kaps are weakly bound and more labile in regions of low FG-repeat availability. This may be a narrow corridor along the NPC central axis that is demarcated by strongly bound Kaps that occupy high FG-repeat density regions surrounding the pore wall. Indeed, such a picture is consistent with the preferred localization of Kapβ1 in the NPC as observed in single molecule fluorescence experiments, as well as structural changes inside the central channel as revealed by cryo-electron tomography. Fast transport kinetics would then emerge from a culmination of 3 effects along this corridor: (i) reduced Kap-FG repeat binding, (ii) ROD, and (iii) spatial confinement.

To illustrate this, we consider the 4 different transport scenarios shown in Figure 2. First, small molecules that traverse the NPC corridor by passive diffusion do so in a 3-dimensional random walk, leading to

Figure 1. The NPC-bound fraction of endogenous Kapβ1 is stable and long-lived. (A) Row 1: HeLa cells were fixed and digitonin-permeabilized as described in ref. 56. As a control, anti-Kapβ1 (Abcam) staining reveals the steady state distribution of endogenous Kapβ1, a fraction of which is localized at the nuclear envelope. This further co-localizes with anti-Nup153 (Sigma) staining, thereby signifying the presence of Kapβ1 at the NPCs. Row 2 to 4: To test for the retention of the NPC-bound pool of endogenous Kapβ1, cells were first digitonin-permeabilized then incubated in PBS buffer for 15 mins, 1 hr and 5 hr respectively, followed by fixation. The persistence of anti-Kapβ1 staining at the nuclear envelope up to 5h incubation indicates that the NPC-bound fraction of Kapβ1 is very stable and long-lived. Scale bar, 5 μm. (B) The immunofluorescent staining of Kapβ1 localized at the nuclear envelope was calibrated against Inspeck® Red calibration beads (Life Technologies) to exclude intensity variations between measurements. The data shown corresponds to Rows 1 to 4 in (A). Final intensity ratios are normalized to the control sample (Row 1) to facilitate comparisons between experiments. Student’s t-test analysis (p > 0.05) shows a negligible difference among these experimental outcomes. All images were obtained using a point scanning laser confocal microscope (LSM700, Zeiss AG).

Figure 2. Hypothetical translocation scenarios in the NPC. Left to right: Small passive molecules that diffuse into the NPC exhibit long 3D search trajectories (and therefore long dwell times) due to a lack of FG-Nup binding. The dirty velcro effect reduces the travel distance of Kaps by promoting 2D diffusion along the luminal surface of the translocation corridor. This reduction of dimensionality leads to shorter dwell times and rapid translocation rates. Large non-specific molecules experience hindered diffusion and have a lower probability to enter the pore. Increased pore confinement and the dirty velcro effect expedite the translocation of large cargos with multiple Kaps due to reduced search trajectories that stem from 1D diffusion. Note: For clarity of illustration, other diffusing entities have been omitted from each drawing. However, overlaying these scenarios does reveal how different spatio-temporal routes might co-exist in the pore. C = cytoplasm, N = nucleus.
comparably long “search” trajectories and passage times before exiting the NPC. Notably, collisions with other diffusing entities in the NPC corridor (omitted for clarity of illustration) such as dynamically fluctuating FG Nups, Kaps, as well as both specific and non-specific cargoes are likely to further delay passage. In comparison, weakly bound Kaps might exhibit a 2-dimensional random walk along the luminal “surface” of the translocation corridor owing to the dirty velcro effect with brief excursions into the luminal space.\(^52\) This could in fact expedite transport because the temporal requirement of a 2-dimensional search process for finding the exit of a spatially constrained pore can be considerably less than in 3 dimensions. According to ROD theory,\(^3,5,55\) the mean diffusion time \(t\) for a particle (assuming a constant diffusion coefficient) is related to

\[
\tau_{3D} \sim L/b \text{ in three dimensions,}\quad (1)
\]

\[
\tau_{2D} \sim \ln(L/b) \text{ in two dimensions,}\quad (2)
\]

where \(b\) is the radius of a small target (e.g., pore exit) and \(L\) is the radius of a larger diffusional space (e.g., pore). Likewise, NTF2\(^6,64\) as well as non-specific molecules that exert sufficient FG-repeat binding\(^6,63,64\) might exhibit varying degrees of ROD-like translocation.

In the absence of FG-repeat binding, large non-specific molecules would have a lower probability to enter the pore due to hindered diffusion near the Kap-centric barrier. Yet, this raises fundamental questions as to how very large receptor-cargo complexes traverse the NPC corridor upon entering the pore. More specifically, how can sufficient space be created to accommodate large cargoes in the NPC? One possible explanation could be that the NPC structurally dilates with increasing Kap occupancy.\(^65\) Regardless, it is important to bear in mind that the barrier is not a solid obstruction but rather consists of dynamic interactions between diffusing Kaps and fluctuating FG Nups. Being highly flexible, KapB1 may even adopt slightly different conformations depending on the NPC microenvironment.\(^66\) Nevertheless, from the perspective of Kap-centric control a displacement of preloaded Kaps from the FG Nups would result in the effective retraction/reduction of the NPC barrier. We speculate that the presence of multiple Kaps on large cargoes\(^67,68\) (or receptors that shuttle messenger ribonucleic proteins\(^57,60\)) may be able to displace preloaded Kaps because of increased binding avidity with the FG Nups. Indeed, the presence of multiple Kaps has also shown to improve the transport efficiency of large cargoes.\(^58\) Finally, the increased confinement within the pore may promote relatively fast transport via quasi one-dimensional diffusion.\(^67\) All in all these scenarios are consistent with the notion that passage through the NPC is not a rate-limiting step\(^57\) – except when long-lived Kaps are depleted from the pore.

### Conclusion

Kap-centric control describes a view where NPC transport selectivity and speed are regulated by the occupancy of Kaps in the pore. This departs from FG-centric views in that the FG Nups might function as malleable molecular “receptacles” that accumulate Kaps at NPCs. This alludes to how Kaps regulate and reinforce the FG Nups in a concentration dependent manner to bring about NPC barrier function. Importantly, Kap-centric control provides the missing link in FG-centric views by reconciling mechanistic and kinetic requirements of the NPC. Not only does this impact on the binding and transport kinetics of subsequent Kaps, but also the diffusion volume inside the NPC, which together determine transport rates. Moreover, multivalent interactions with the FG Nups confer onto Kaps the ability to establish a continuum of different kinetic behaviors. Therefore, under certain cellular cues different Kaps may access different temporal pathways rather than only spatial ones.\(^70\) For instance, the activation of signaling pathways\(^38\) can cause a regulation or deregulation of certain Kaps (leading to perturbations in their expression levels i.e., concentrations) with physiological or pathological consequences such as cancer.\(^71,72\) Thus, Kap-centric control may serve to regulate nucleocytoplasmic transport by fine-tuning the NPC microenvironment according to the functional needs of the cell.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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