

Karyopherins regulate nuclear pore complex barrier and transport function

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Nucleocytoplasmic transport is sustained by karyopherins (Kaps) and a Ran guanosine triphosphate (RanGTP) gradient that imports nuclear localization signal (NLS)-specific cargoes (NLS-cargoes) into the nucleus. However, how nuclear pore complex (NPC) barrier selectivity, Kap traffic, and NLS-cargo release are systematically linked and simultaneously regulated remains incoherent. In this study, we show that Kap α facilitates Kap β 1 turnover and occupancy at the NPC in a RanGTP-dependent manner that is directly coupled to NLS-cargo release and NPC barrier function. This is underpinned by the binding affinity of Kap β 1 to phenylalanine-glycine nucleoporins (FG Nups), which is comparable with RanGTP-Kap β 1, but stronger for Kap α -Kap β 1. On this basis, RanGTP is ineffective at releasing standalone Kap β 1 from NPCs. Depleting Kap α -Kap β 1 by RanGTP further abrogates NPC barrier function, whereas adding back Kap β 1 rescues it while Kap β 1 turnover softens it. Therefore, the FG Nups are necessary but insufficient for NPC barrier function. We conclude that Kaps constitute integral constituents of the NPC whose barrier, transport, and cargo release functionalities establish a continuum under a mechanism of Kap-centric control.

Introduction

Nuclei physically segregate transcription from the cytoplasmic translation machinery in eukaryotic cells. Hence, gene expression relies on a controlled exchange of proteins and mRNA between the nucleus and cytoplasm. This is known as nucleocytoplasmic transport (NCT; Nigg, 1997; Görlich and Kutay, 1999; Stewart, 2007; Christie et al., 2016), which is regulated by three key elements: cargo-carrying karyopherin (Kap) receptors (specifically importins and exportins), the GTPase Ran, and aqueous channels that perforate the nuclear envelope (NE), known as nuclear pore complexes (NPCs; Eibauer et al., 2015; von Appen et al., 2015). Together, they facilitate the selectivity, transport efficiency, and accumulation of diverse cargoes in the nucleus.

NPCs are permeable to small molecules, but the entry of large, nonspecific entities is hindered (Popken et al., 2015; Timney et al., 2016). Tethered inside each NPC are several highly dynamic, intrinsically disordered proteins that impede the passage of nonspecific macromolecules (Sakiyama et al., 2016). These so-called phenylalanine-glycine nucleoporins (FG Nups) also harbor numerous FG repeats that bind Kaps (Rexach and Blobel, 1995; Bayliss et al., 2000; Allen et al., 2001), which achieve selective transport within milliseconds (Kubitscheck et al., 2005; Yang and Musser, 2006; Tu et al., 2013). Out of 20 members of the Kap β family in humans, the 97-kD import

receptor karyopherin β 1 (Kap β 1 or importin- β) regulates the canonical NCT pathway of diverse cargoes that comprise classical NLSs (NLS-cargo; Kimura et al., 2017). Kap β 1 itself does not bind classical NLS-cargoes directly but recruits a 58-kD adapter known as karyopherin α (Kap α or importin- α ; Pumroy and Cingolani, 2015) that contains a C-terminal NLS-binding domain and an N-terminal autoinhibitory importin- β -binding (IBB) domain (Christie et al., 2016).

Current NPC models are FG centric and rely on the notion that the FG Nups reject nonspecific cargoes while promoting Kap-regulated transport. As a consequence, it remains a matter of debate whether the FG Nups resemble a gel-like permeability barrier (Frey and Görlich, 2007), a virtual gate (Rout et al., 2000) consisting of polymer brushes (Lim et al., 2007), or a mixture of gel and brush conformations (Yamada et al., 2010). In terms of their binding, individual FG repeats engage Kap β 1 by a rapid succession of multiple low-affinity interactions (Hough et al., 2015; Milles et al., 2015). Nevertheless, at equilibrium timescales, these manifest multivalent interactions that enhance binding affinity through avidity (Schoch et al., 2012; Kapinos et al., 2014). Approximately 1,000 selective translocation events ensue per NPC per second in both directions (Ribbeck et al., 1998), where 100 Kap β 1 molecules are estimated to occupy the pore at steady state (Paradise et al., 2007; Tokunaga et al., 2008; Lowe et al., 2015). We ourselves had shown that FG Nup layers undergo conformational changes

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Abbreviations used: CD, circular dichroism; DOL, degree of labeling; IBB, importin- β binding; ITC, isothermal titration calorimetry; MST, microscale thermophoresis; NCT, nucleocytoplasmic transport; NE, nuclear envelope; Ni-NTA, nickel-nitrilotriacetic acid; NPC, nuclear pore complex; SPR, surface plasmon resonance.

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to accommodate large numbers of Kap β 1 molecules depending on Kap β 1 concentration (Kapinos et al., 2014). At physiological Kap β 1 concentrations in particular, i.e., $\sim 10 \mu\text{M}$ (Paradise et al., 2007), we found that a pool of strongly bound Kap β 1 molecules occupies the FG Nups along with a more weakly bound pool that engages a reduced number of FG repeats. Indeed, evidence of these two pools can be found in the bimodal kinetics of exogenous Kap β 1 in digitonin-permeabilized cells (Lowe et al., 2015) and mRNA export in living cells (Grünwald and Singer, 2010). We therefore proposed a mechanism known as Kap-centric control whereby Kap β 1 occupancy in the FG Nups plays a role in regulating NPC barrier selectivity and transport speed (Lim et al., 2015).

Although NLS-cargo-Kap α -Kap β 1 complexes exhibit facilitated diffusion through the NPC (Yang et al., 2004), the release and accumulation of NLS-cargo in the nucleus is energetically activated. This is regulated by the small GTPase Ran, which has GTP- and GDP-bound forms that are asymmetrically distributed in the nucleus and cytoplasm, respectively (Görlich et al., 1996; Weis et al., 1996; Lyman et al., 2002). Importantly, RanGTP functionally releases both NLS-cargo and Kap α in the nucleus upon binding Kap β 1 (Görlich et al., 1996; Catimel et al., 2001; Stewart, 2007), after which RanGTP-Kap β 1 complexes are exported back to the cytoplasm (i.e., turned over) for eventual reuse.

Whereas the aforementioned processes define individual aspects of NCT, the manner by which NPC barrier selectivity, Kap exchange, and NLS-cargo release function simultaneously under equilibrium conditions remains incoherent. As a matter of fact, each of these processes involves Kap β 1, which binds Kap α in the cytosol to ferry NLS-cargoes, FG Nups to bypass the NPC, and RanGTP in the nucleus to deliver cargo. Hence, the binding of one partner may well impact on another to influence the entire transport continuum. One peculiarity pertains to Kap β 1 turnover, where a prevailing notion claims that RanGTP concomitantly promotes Kap β 1 dissociation from the FG Nups after cargo delivery into the nucleus (Rexach and Blobel, 1995). This is based on *in vitro* evidence showing that RanGTP abolishes Kap β 1-FG Nup interactions (or more exactly, Kap60 from Kap95 in some cases; Rexach and Blobel, 1995; Floer et al., 1997; Ben-Efraim and Gerace, 2001; Lyman et al., 2002; Lim et al., 2007). When lacking FG Nup binding, however, the as-formed RanGTP-Kap β 1 complex would itself resemble a nonspecific cargo whose export via NPCs (i.e., Kap β 1 turnover) violates NCT selectivity. To add to the confusion, recombinant Kap β 1 is typically retained at the NE of permeabilized cells, whereas Kap α and NLS-cargoes accumulate within the nucleoplasm when RanGTP is present (Görlich et al., 1995; Moroianu et al., 1995).

In this work, we have combined biophysical quantitation and functional permeabilized cell assays to explore the molecular interconnections between NPC barrier function, Kap β 1 occupancy, turnover, and NLS-cargo release. First, we used surface plasmon resonance (SPR) to measure the effect of RanGTP on the binding affinities of Kap β 1, Kap α -Kap β 1, and MG-NLS-Kap α -Kap β 1 (where MG-NLS is a 76-kD maltose-binding protein (MBP)-GFP-NLS cargo fusion protein) to the FG Nups along with associated conformational changes in FG Nup layers. Second, we analyzed the concentration dependence and stoichiometry of different Kap β 1 complexes and their combined influence on FG Nup binding. Third, we validated the respective *in situ* behaviors using digitonin-per-

meabilized cells. Our results show that Kap α -Kap β 1 exhibits a pronounced binding and occupancy at the NPCs. RanGTP triggers the release of both Kap α and NLS-cargo by converting Kap α -Kap β 1 to RanGTP-Kap β 1, which binds the FG Nups more weakly and leads to reduction in total Kap β 1 occupancy. However, RanGTP has no eluting effect on standalone Kap β 1 because RanGTP-Kap β 1 shares the same binding affinity to the FG Nups as Kap β 1. Finally, depleting Kap α -Kap β 1 by RanGTP abrogates NPC barrier function, which is rescued by adding back either Kap β 1 or Kap α -Kap β 1. Still, under active transport conditions, Kap β 1 turnover leads to a softening of the barrier. It therefore follows that Kap α mediates Kap β 1 occupancy and turnover in a RanGTP-dependent manner to impart Kap-centric control at the NPC.

Results

Binding of RanGTP-Kap β 1 to FG Nups

SPR was used to correlate *in situ* equilibrium and kinetic aspects of RanGTP-Kap β 1 binding to conformational changes in Nup214, Nup62, Nup98, and Nup153 (denoted as cNup214, cNup62, cNup98, and cNup153). As before, this uses BSA to probe FG layer height (Fig. S1; Schoch et al., 2012; Kapinos et al., 2014). Before SPR experimentation, all Kaps and transport complexes were characterized in aqueous solution using isothermal titration calorimetry (ITC), microscale thermophoresis (MST), and circular dichroism (CD; Fig. S2). This gave an equilibrium dissociation constant of $K_d = 35 \pm 12.5 \text{ nM}$ for RanGTP-Kap β 1, which compares well with previous estimates (Bednenko et al., 2003; Hahn and Schlenstedt, 2011; Lolodi et al., 2016). Thereafter, we applied RanGTP-Kap β 1 (1.5:1) ranging from 10 nM to 20 μM Kap β 1, giving RanGTP-Kap β 1 fractions of 25% up to 100% for the SPR experiments (Fig. 1 A). Thus, the FG Nups typically interacted with RanGTP-Kap β 1 in a diminishing background of free Kap β 1 (and RanGTP) with increasing Kap β 1 concentration. Upon completion of a binding sequence (Fig. 1 B), each FG Nup layer was regenerated by NaOH treatment to remove RanGTP-Kap β 1. We then measured how RanGTP binds to standalone Kap β 1-FG Nup complexes to compare the two scenarios.

RanGTP-Kap β 1-FG Nup binding is accompanied by an incremental increase in the FG Nup layer height that plateaus at the highest RanGTP-Kap β 1 concentrations (Fig. 1 C). This behavior, which differs quantitatively between FG Nups, likely originates from differences in their intrinsic properties. However, there are qualitative similarities to the binding of standalone Kap β 1 (Kapinos et al., 2014) that signifies FG Nup saturation at micromolar RanGTP-Kap β 1 concentrations. Nevertheless, there is a slight height increase for RanGTP-Kap β 1 over standalone Kap β 1 because of the larger hydrodynamic diameter of RanGTP-Kap β 1 ($9.6 \pm 1.3 \text{ nm}$) compared with standalone Kap β 1 ($6.8 \pm 1.8 \text{ nm}$; Table S1). Moreover, RanGTP binding to preformed Kap β 1-FG Nup layers elicits further increases in height, as opposed to a decrease as one would expect if RanGTP-Kap β 1 would subsequently unbind. Hence, it is evident that RanGTP does not facilitate the release of standalone Kap β 1 from the FG Nups.

Influence of RanGTP on Kap α -Kap β 1-FG Nup binding

Next, we sought to determine how Kap α -Kap β 1 complexes interact with the FG Nups and how RanGTP might affect their

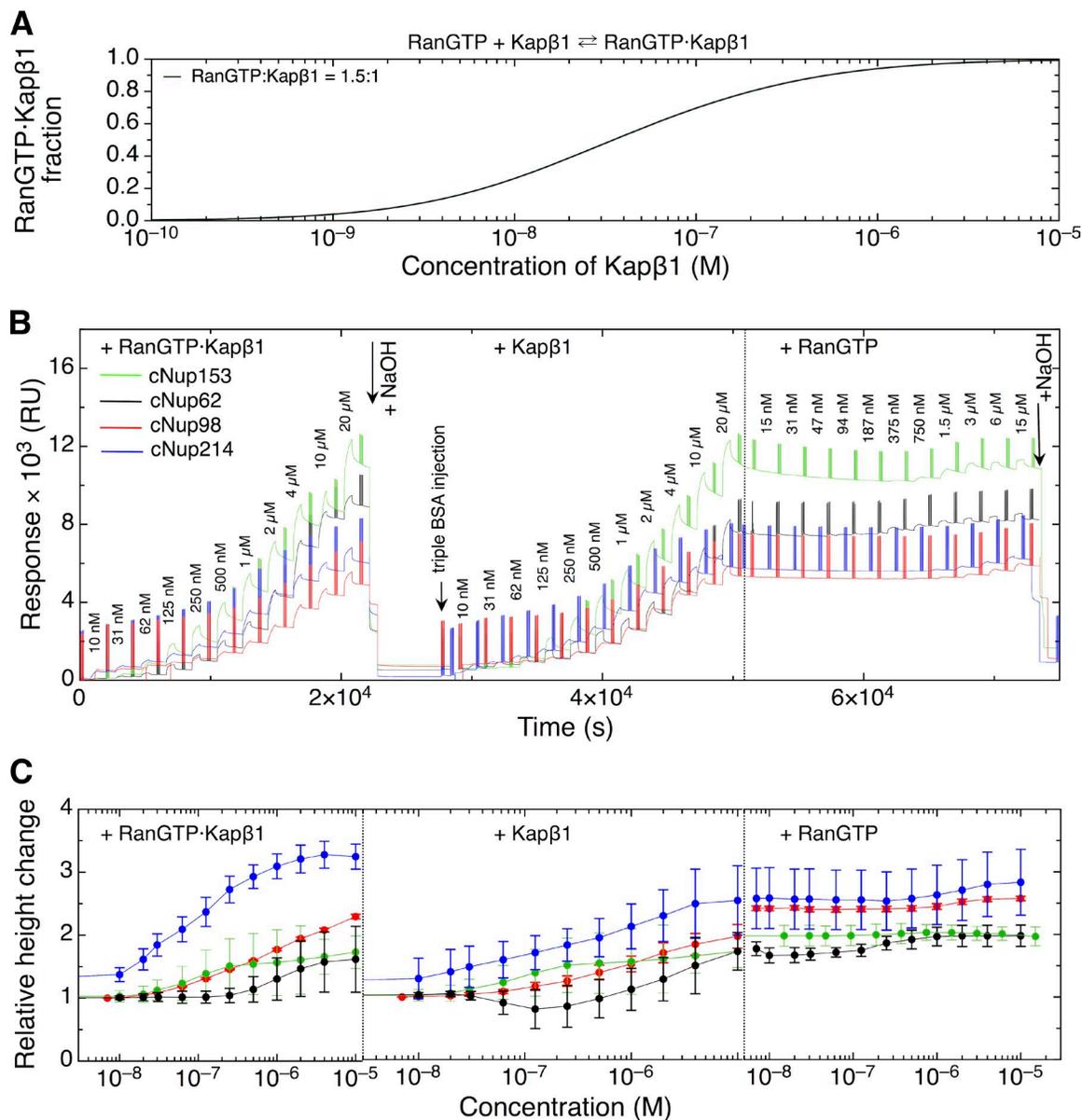


Figure 1. **RanGTP does not dissociate standalone Kap β 1 from the FG Nups.** (A) Calculated solution binding isotherm for RanGTP/Kap β 1 at a ratio of 1.5:1 with $K_d = 35$ nM. (B) SPR response curves for RanGTP·Kap β 1 binding to different FG Nups followed by injections of Kap β 1 and RanGTP after a NaOH regeneration step. Vertical signals correspond to triple BSA injections that are used to measure FG Nup layer height. RU, resonance units. (C) Corresponding height changes to the FG Nup layer with respect to B. $n = 10$ per FG Nup. Error bars denote standard deviation.

binding. As before, ITC determination gave an equilibrium dissociation constant of $K_d = 210 \pm 77$ nM for Kap α -Kap β 1 binding (Fig. S2 B), in good agreement with previous values (Falces et al., 2010). At a mixing ratio of 1.5:1, $\sim 40\%$ Kap α -Kap β 1 was expected to form at 100 nM Kap β 1, and this reaches $\sim 80\%$ at 1 μ M and 95% at 10 μ M Kap β 1 (Fig. 2 A). Therefore, in our SPR assay, Kap α -Kap β 1 complexes interacted with the FG Nups in a background of free Kap α and Kap β 1, which diminished as Kap α -Kap β 1 concentration increased.

After reaching 20 μ M Kap α -Kap β 1, up to 15 μ M RanGTP was added to test its efficacy to dissociate Kap α . In marked contrast to Fig. 1 C, RanGTP led to reductions in both the Kap α -Kap β 1-FG Nup binding response and layer height (Fig. 2, B and C), except for cNup98, potentially because of its lower capacity to bind Kap β 1 than the other FG Nups (Ka-

pinos et al., 2014). We attribute this decrease to the release of Kap α after the conversion of Kap α -Kap β 1 to RanGTP·Kap β 1. This is reasonable given that the exchange of Kap α (58 kD) to RanGTP (26 kD) leads to a reduction in total FG Nup-bound mass (Fig. 2 C). Likewise, we observed similar behavior when MG-NLS-Kap α -Kap β 1 was bound to cNup153, indicating that RanGTP·Kap β 1 binding triggered the release of MG-NLS and Kap α from the FG Nup layer (Fig. S3).

Separately, we found that RanGTP·Kap β 1 exhibited promiscuous binding interactions (Wagner et al., 2015) with pre-formed Kap α -Kap β 1-FG Nup layers, which led to an increase in both the SPR signal and layer height (Fig. S3, D and E). Hence, this indicates that RanGTP·Kap β 1 and Kap α -Kap β 1 can simultaneously bind and coexist within the FG Nups without directly interacting with one another.

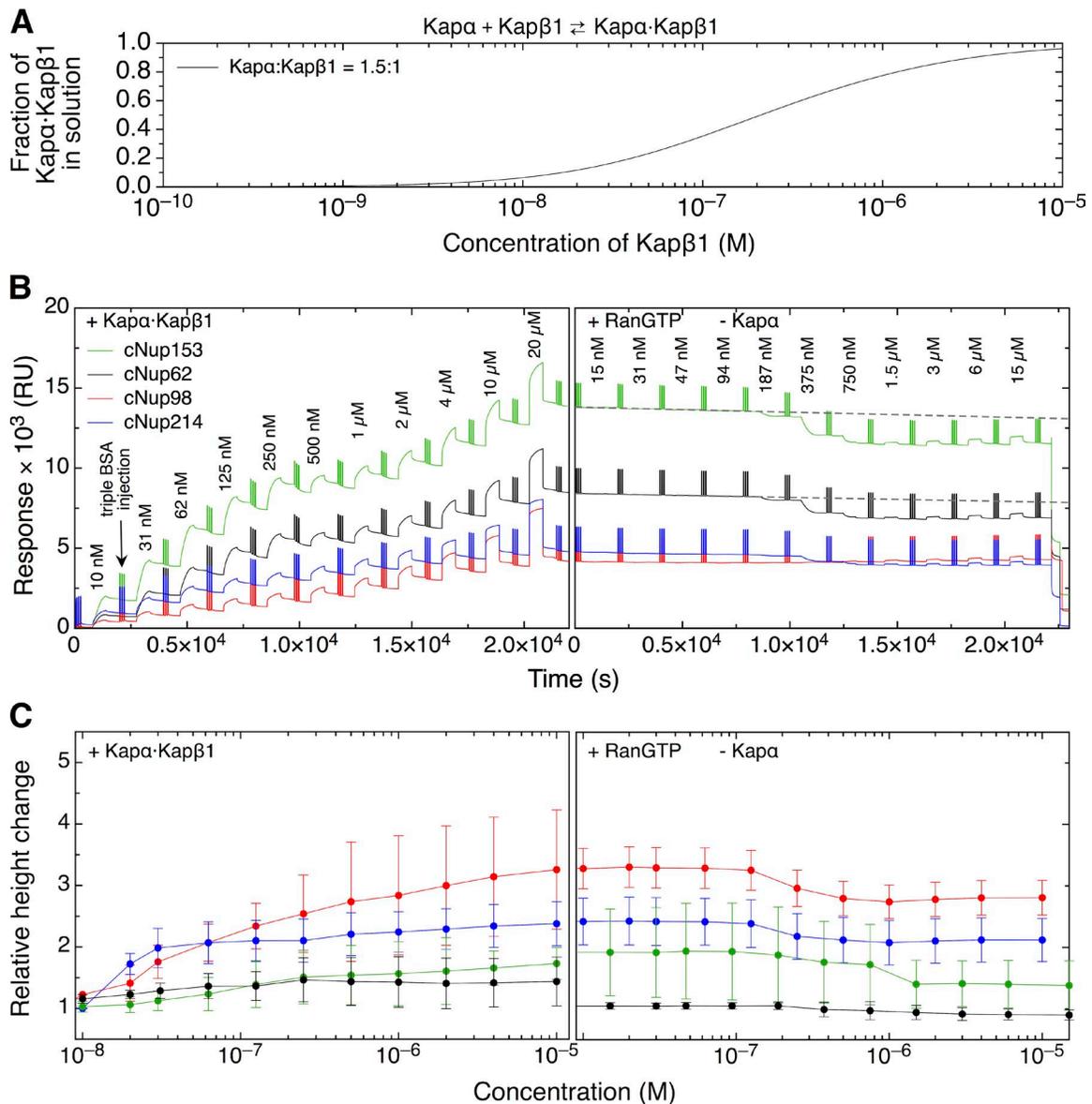


Figure 2. **RanGTP dissociates Kap α from FG Nup-bound Kap α -Kap β 1.** (A) Solution binding isotherm calculated for Kap α /Kap β 1 at a ratio of 1.5:1 with $K_d = 210$ nM. (B) SPR response curves for Kap α -Kap β 1 binding to different FG Nups show that Kap α is eluted by RanGTP. Vertical signals correspond to triple BSA injections that are used to measure FG Nup layer height. RU, resonance units. (C) Corresponding height changes to the FG Nup layer with respect to B. $n = 10$ per FG Nup. Error bars denote standard deviation.

Comparing Kap β 1, Kap α -Kap β 1, MG-NLS-Kap α -Kap β 1, and RanGTP-Kap β 1 binding to FG Nups

Fig. 3 A summarizes the affinities associated with the binding of each transport complex to the FG Nups as determined from Langmuir isotherm analysis (Fig. S4 A). Indeed, all four entities exhibit two-phase binding interactions with the FG Nups with distinct affinities at the ~ 0.1 μ M (strong) and ~ 10 μ M (weak) ranges. These two binding regimes are also distinguishable by their kinetic behavior (Fig. S4 B), where (a) strong binding (lower K_d) is associated with fast on-rates (k_{on}) and slow off-rates (k_{off}), thereby suggesting that uptake into unsaturated FG Nup layers is rapid and stable; and (b) weak binding (higher K_d) of near-saturated FG Nup layers comes from a reduction in k_{on} and increase in k_{off} being a characteristic of more transient interactions.

Interestingly, Kap α -Kap β 1 has a 10-fold higher affinity (lower K_d) in the strong binding phase than Kap β 1 and RanGTP-Kap β 1, which are overall similar. This indicates that Kap α -Kap β 1 complexes are more stable when binding FG Nups than Kap β 1 or RanGTP-Kap β 1. However, this is unexpected because Kap α itself does not bind the FG Nups (Fig. S4 C) with the exception of cNup153, which binds Kap α weakly, i.e., $K_d = 1.3 \pm 0.1$ μ M because of an NLS-like sequence at its C terminus (Makise et al., 2012; Ogawa et al., 2012). Nevertheless, Kap α -Kap β 1 complexes seem to be less flexible than free Kap β 1 molecules, and this might serve to stabilize the binding of Kap α -Kap β 1 to the FG Nups (Cingolani et al., 2000; Tauchert et al., 2016). Still, MG-NLS-Kap α -Kap β 1-FG Nup binding is not measurably stronger than Kap α -Kap β 1 (Fig. 3 and Fig. S4 D), which suggests that MG-NLS does not influence their binding.

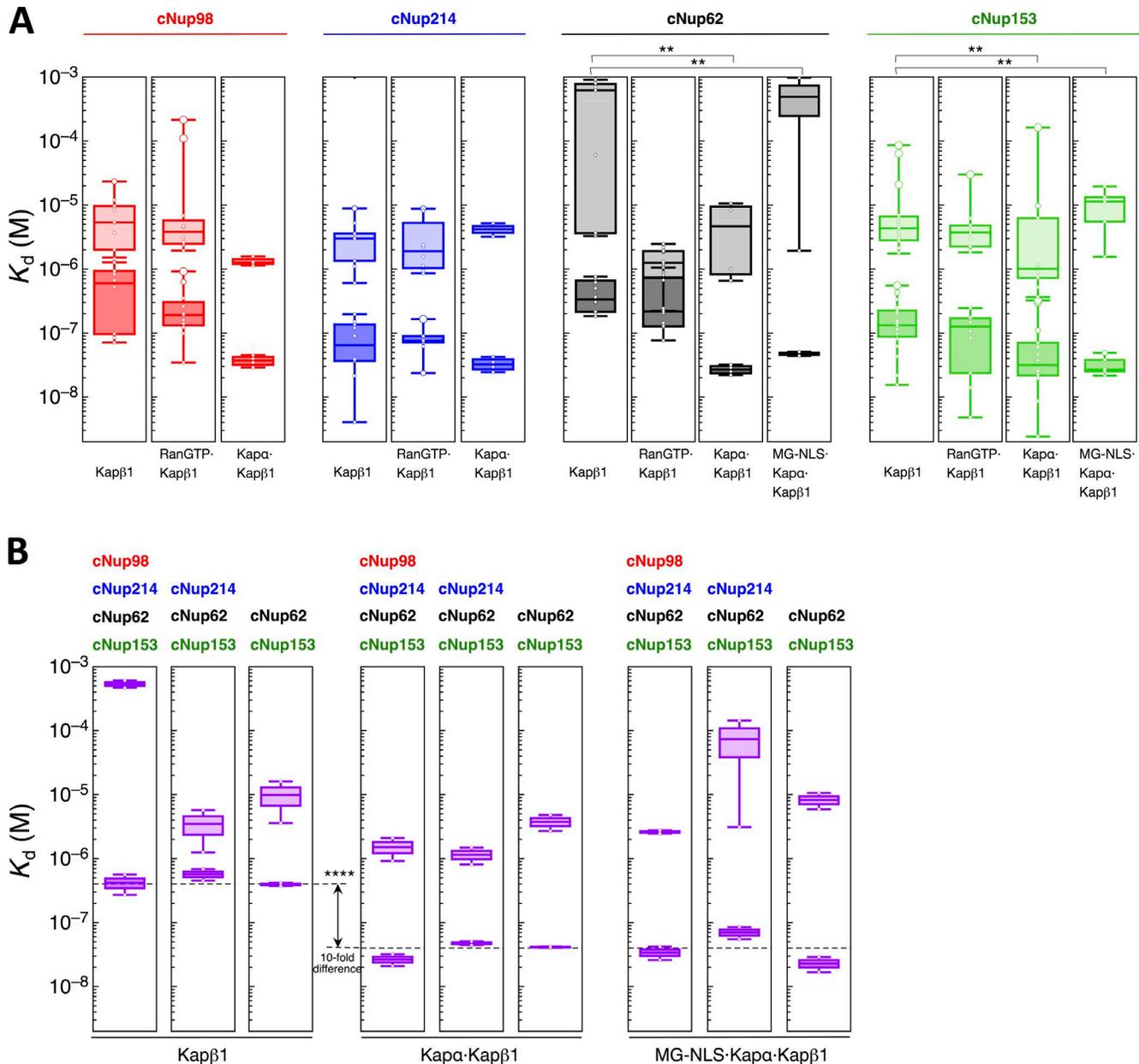


Figure 3. **FG Nups bind Kap α -Kap β 1 more strongly than Kap β 1 and RanGTP-Kap β 1, which are similar.** (A) FG Nup binding of Kap β 1 and related transport complexes are characterized by two distinct equilibrium dissociation constants at $\sim 0.1 \mu\text{M}$ (strong) and $\sim 10 \mu\text{M}$ (weak). In all cases, Kap α switches the Kap β 1 complex to a quantitatively higher binding affinity (lower K_d). RanGTP lowers the binding state to a value that is comparable with standalone Kap β 1. MG-NLS cargo does not significantly affect Kap α -Kap β 1 binding to Nup153 and Nup62. **, $P < 0.01$; Student's t test (see Table S3). (B) Kap α -Kap β 1 and MG-NLS-Kap α -Kap β 1 have a quantitatively higher binding affinity (lower K_d) than Kap β 1 when binding mixed FG Nups. To aid comparison, dashed lines at K_d values of 40 and 400 nM show that Kap α -Kap β 1 or MG-NLS-Kap α -Kap β 1 binds the FG Nups 10-fold stronger than standalone Kap β 1. ****, $P < 0.0001$; Student's t test. Box plots denote the median, first, and third quartiles. Error bars denote standard deviation, including outliers.

To more closely mimic binding in the NPC, we also tested mixed layers comprised of different FG Nup combinations. As before, we observed a similar ~ 10 -fold higher affinity of Kap α -Kap β 1 and MG-NLS-Kap α -Kap β 1 over standalone Kap β 1, regardless of the FG Nup layer composition (Fig. 3 B). Based on these results, we hypothesized that Kap α release is essential for Kap β 1 turnover because Kap β 1-FG Nup affinity is most effectively reduced when RanGTP replaces Kap α to bind Kap β 1 (i.e., $\text{RanGTP-Kap}\beta 1 > K_d > \text{Kap}\alpha\text{-Kap}\beta 1$). Importantly, this provides further evidence that RanGTP does not facilitate the release of standalone Kap β 1 from the FG Nups (i.e., $\text{RanGTP-Kap}\beta 1 \approx K_d \approx \text{Kap}\beta 1$).

Equilibrium analysis of RanGTP-Kap β 1 dissociation from the FG Nups

RanGTP concomitantly liberates Kap α and NLS-cargoes upon binding Kap β 1. However, it remains unclear to what extent RanGTP-Kap β 1 dissociates from the FG Nup layer. A technical limitation of SPR is that the relative amount of different Kap β 1 complexes that bind the FG Nups is not obvious. To be precise, the overall binding response at equilibrium (R_{eq}) is proportional to the amounts of standalone Kap β 1, Kap α -Kap β 1, and MG-NLS-Kap α -Kap β 1 complexes that coexist in solution and their respective affinities to the FG Nups (Fig. S5 A). This is further determined by their respective binding iso-

therms that vary as a function of their molar concentrations and ratio (Sun et al., 2013).

Three parallel reactions then proceed in the presence of RanGTP (Fig. S5, B–D). First, RanGTP binds standalone Kap β 1, which has a minimal impact on Kap β 1–FG Nup binding. Second, RanGTP converts Kap α –Kap β 1 to RanGTP–Kap β 1, which facilitates Kap β 1 dissociation and concomitantly elutes Kap α . Third, RanGTP elutes Kap α and MG-NLS cargo by converting MG-NLS–Kap α –Kap β 1 to RanGTP–Kap β 1, which likewise promotes Kap β 1 dissociation from the FG Nups. To correlate these behaviors, we tested the effect of 5 μ M RanGTP on increasing ratios of MG-NLS/Kap α /Kap β 1 (ranging from 1:1:4 to 10:10:1) against cNup153 (Fig. S5 E). Moreover, this was repeated at three specific Kap β 1 concentrations ($C_{\text{Kap}\beta 1}$): 10, 100, and 1,000 nM (Fig. S5, E–G). We note that the change in the normalized equilibrium binding response (ΔR_{norm}) at each Kap β 1 concentration would then depend solely on the ratio between different Kap β 1 complexes because the absolute number of Kap β 1 molecules remains the same.

Overall, RanGTP elicited the largest reduction of total FG Nup-bound mass at 100 nM Kap β 1, which diminishes below \sim 10 nM and above 1 μ M Kap β 1. This nonmonotonic behavior agrees qualitatively with equilibrium calculations (Fig. 4 and Fig. S5 G), which explain that (a) the number of Kap β 1–FG binding complexes is small at 10 nM Kap β 1; (b) total Kap β 1 reduction at the FG Nups is greatest at 100 nM, being close to the affinity (K_d) of RanGTP–Kap β 1, Kap α –Kap β 1, and MG-NLS–Kap α –Kap β 1; and (c) reduction is diminished at 1 μ M Kap β 1 because of a saturation of Kap β 1 complexes. Moreover, the reduction in binding response (ΔR_{norm}) scales with the amount of eluted Kap α and MG-NLS as well as RanGTP–Kap β 1 dissociation ($\Delta \text{Kap}\beta 1$) as defined by MG-NLS/Kap α /Kap β 1 ratios. Hence, Kap β 1 dissociation from the FG Nups is directly coupled to Kap α /NLS-cargo release by the action of RanGTP.

RanGTP facilitates Kap β 1 turnover at NPCs by eluting Kap α

With the biophysical insights noted above, we sought to confirm whether Kap α did indeed play a role in facilitating the release of Kap β 1 from functional NPCs in a series of digitonin-permeabilized cell assays (Fig. 5 A). We had previously shown that a pool of endogenous Kap β 1 (endoKap β 1) persisted at the NE for a prolonged duration after permeabilization (Lim et al., 2015). Upon confirming that endogenous Kap α (endoKap α) colocalizes with endoKap β 1 at the NE, we used Ran mix, which reinstates the soluble transport machinery to power NCT in permeabilized cells (Görlich et al., 1995). This consisted of 5 μ M RanGDP, 4 μ M NTF2, and an energy-regenerating system (2 mM GTP, 0.1 mM ATP, 4 mM creatine phosphate, and 20 U/ml creatine kinase) in transport buffer. After Ran mix treatment, immunofluorescence assays showed that endoKap β 1 was significantly depleted at the NE (Fig. 5 B). Importantly, this ensured that the NPCs were as close as possible to a ground state, i.e., vacant, to minimize interference with the binding and retention of its exogenous counterparts. We then incubated the permeabilized cells in exogenous Kap β 1 (i.e., Kap β 1–Alexa Fluor 568; hereafter denoted as exoKap β 1) followed by a second Ran mix treatment to evaluate exoKap β 1 turnover. Interestingly, standalone exoKap β 1 was poorly displaced from the NPCs (Fig. 5 C), thereby corroborating our biophysical analyses showing that RanGTP does not facilitate the turnover of standalone Kap β 1 (Fig. 1).

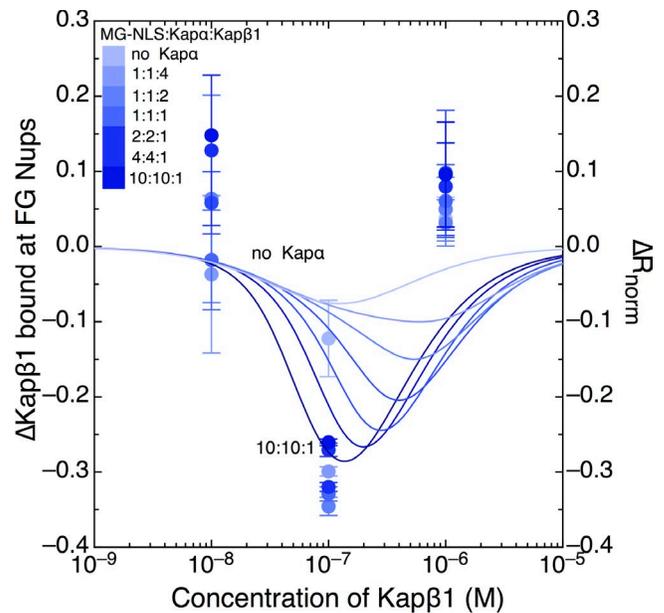


Figure 4. FG Nup binding efficiency depends on MG-NLS/Kap α /Kap β 1 ratio, concentration, and RanGTP. Relative decrease of FG Nup-bound Kap β 1 due to RanGTP at different MG-NLS/Kap α /Kap β 1 ratios. A maximal reduction of Kap β 1 occurs when MG-NLS/Kap α /Kap β 1 = 10:10:1. Varying MG-NLS/Kap α /Kap β 1 ratios at constant $C_{\text{Kap}\beta 1}$ = 10, 100, and 1,000 nM enables comparisons between experiment (ΔR_{norm} ; colored dots) and equilibrium calculations ($\Delta \text{Kap}\beta 1$; colored lines). For more information, see Fig. S5.

Thereafter, we wanted to verify that exoKap α was required for facilitating exoKap β 1 turnover at NPCs using RanGTP. We then incubated endoKap-depleted permeabilized cells in 10:1 ratios of exoKap α (i.e., Kap α –Alexa Fluor 488) and exoKap β 1 for $C_{\text{Kap}\beta 1}$ = 10 nM and 100 nM and a 4:1 ratio for $C_{\text{Kap}\beta 1}$ = 1 μ M, with a calculated coupling efficiency of 30, 80, and 95%, respectively, to remain consistent with our biophysical analyses. Before Ran mix treatment, exoKap β 1 was distinctly localized to the NE, whereas exoKap α was located both at the NE and within the nucleus as observed previously (Fig. 5 D; Görlich et al., 1995). This can be attributed to the slow natural dissociation of exoKap α from exoKap β 1 in the absence of RanGTP (Catimel et al., 2001). After Ran mix treatment, however, both exoKap α and exoKap β 1 reduced in a nonmonotonic manner (as predicted by our equilibrium calculations) with a maximum reduction of \sim 50% at $C_{\text{Kap}\beta 1}$ = 100 nM (Fig. 5, E and F). This therefore confirms that Kap α facilitates Kap β 1 turnover in a RanGTP-dependent manner.

Kap β 1 depletion abrogates NPC barrier function

Next, we asked whether the retained pool of endoKap α –endoKap β 1 might play a role in reinforcing the FG Nup barrier, as we had hypothesized previously (Lim et al., 2015). Separately, we applied 1 μ M MG (MBP–GFP) nonspecific cargoes that lacked the NLS and 1 μ M MG-NLS specific cargoes to test for nuclear leak-in (Fig. 6 A). Indeed, both cargoes did not permeate into the nucleus when endoKap α –endoKap β 1 was present (Fig. 6 B). Surprisingly, however, both cargoes readily entered into the nucleus upon depleting the NE of endoKap α –endoKap β 1 by Ran mix (Fig. 6 C). Moreover, adding back only 100 nM exoKap β 1 sufficiently restored

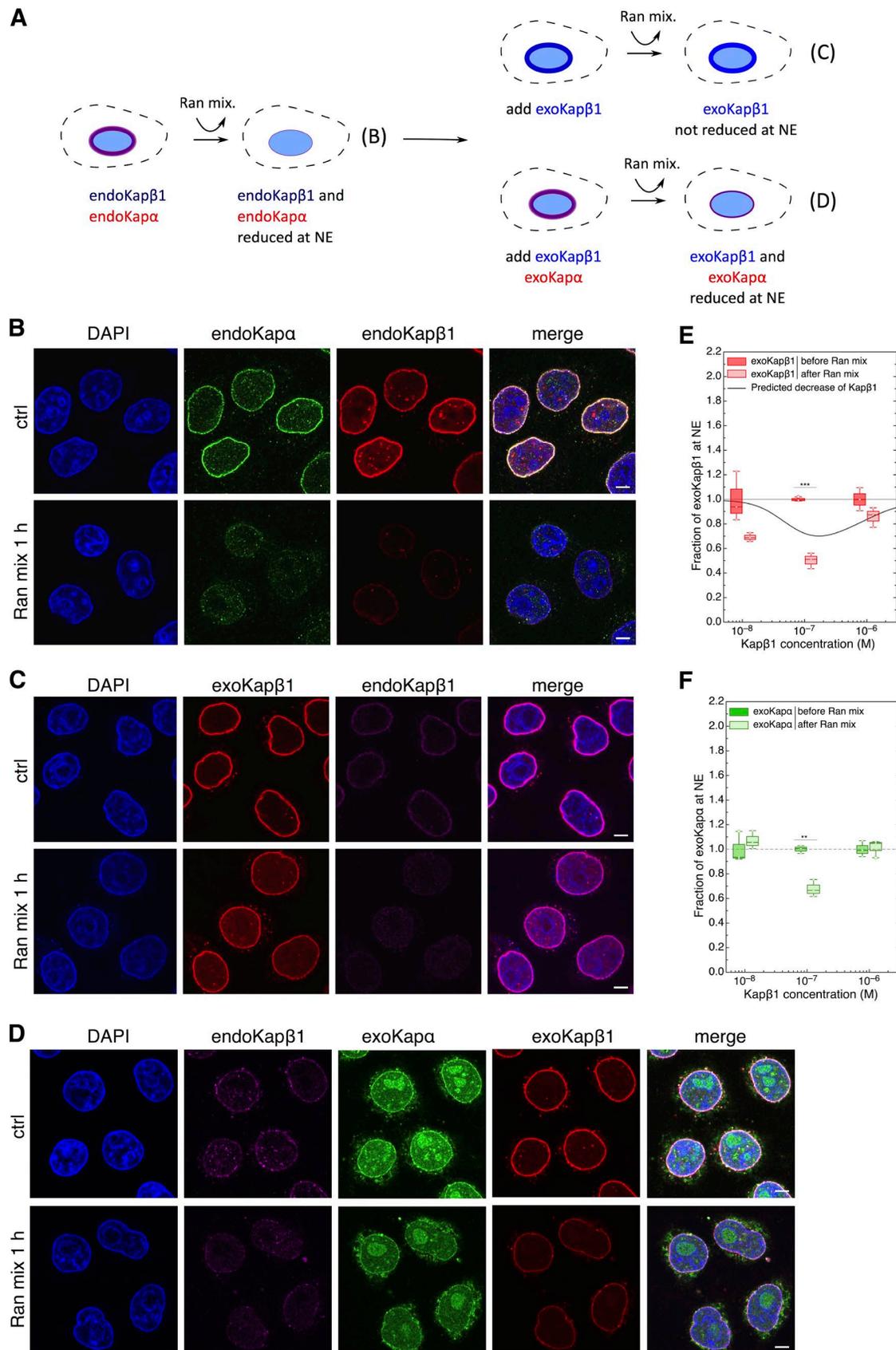


Figure 5. **Kap α facilitates the RanGTP-mediated release of Kap β 1.** (A) Cartoon illustration of the experiment. (B) Immunofluorescence reveals that endoKap α and endoKap β 1 are retained and colocalize at the NE after permeabilization. Ran mix treatment effectively leads to a reduction in both endogenous pools. (C) ExoKap β 1 is not reduced by Ran mix after repopulating endoKap-reduced NPCs with 100 nM exoKap β 1. (D) Ran mix effectively reduces

the NPC barrier to prevent the passive transport of both cargoes into the nucleus (Fig. 6, D–F). Hence, this proves that Kap β 1 occupancy regulates NPC barrier function by reinforcing the FG Nups against the passive transport of macromolecular cargoes.

Kap β 1 turnover at NPCs is coupled to NLS-cargo release

Then, we sought to correlate the active transport of MG-NLS into the nucleus with the RanGTP-dependent reduction of exo Kap β 1 and release of exoKap α from NPCs (Fig. 7 A). A key objective was to elicit comparisons between permeabilized cell assays and the equilibrium analyses (Fig. 4 and Fig. S5). As mentioned above, we incubated endoKap-depleted permeabilized cells with 10:1 ratios of exoKap α and exoKap β 1 for $C_{\text{Kap}\beta 1} = 10 \text{ nM}$ and 100 nM and a 2:1 ratio for $C_{\text{Kap}\beta 1} = 1 \mu\text{M}$, supplemented with $1 \mu\text{M}$ MG-NLS. The MG-NLS concentration was fixed to facilitate direct comparisons with respect to their nuclear uptake between experiments. Consequently, Ran mix affected exoKap β 1 in the same nonmonotonic manner as when MG-NLS was absent (Fig. 5 E), leading to a maximum reduction of 41% at 100 nM exoKap β 1 (Fig. 7, B and C). Even so, Ran mix treatment did not completely dissociate exoKap α from the NE (Fig. 7 D). This may be explained by complex formation with its export receptor, cellular apoptosis susceptibility protein (CAS) and RanGTP (Kutay et al., 1997a) at the NPC.

In parallel, the passive transport of MG-NLS into the nucleus increased with exoKap α /exoKap β 1 concentration before Ran mix treatment, which is consistent with the findings of Yang and Musser (2006). After this, Ran mix facilitated an active nuclear uptake of MG-NLS (Fig. 7 E). However, this had a rather low significant difference, likely because of dilution inside the nucleus. In any case, MG-NLS signal increase is more apparent at 100 nM exoKap β 1, but less so at $1 \mu\text{M}$ exoKap β 1, because of the use of a 2:1 ratio of exoKap α /exoKap β 1 in the latter experiment. Still, neither exoKap β 1 reduction nor an active uptake of MG-NLS proceeded in control experiments that excluded exo Kap α (Fig. 7, F and G). Hence, Kap β 1 turnover at NPCs is facilitated by Kap α and is directly coupled to NLS-cargo release.

Kap β 1 turnover softens the NPC barrier

Finally, we wanted to ascertain the fate of nonspecific cargoes under the same exogenous conditions. As before with only exo Kap β 1 at the NE (Fig. 6 D), passive nuclear entry of MG was forbidden in permeabilized cells pretreated with exoKap α -exo Kap β 1. However, in the presence of Ran mix, we observed a marginal uptake of MG correlated to exoKap β 1 reduction at the NE (Fig. 8), though not to the same extent as when the NPCs were vacant (Fig. 6 C). This indicates that the NPC barrier is less rigid under active transport conditions, which is consistent with in vivo observations of nonspecific transport (Popken et al., 2015; Timney et al., 2016).

Discussion

Kap α facilitates Kap β 1 turnover to regulate NPC function

Kap α plays a key role in unifying selective barrier, transport, and cargo release functionalities at the NPC to regulate NCT. We anticipate that mainly NLS-cargo-Kap α -Kap β 1 complexes populate the NPCs in vivo rather than their standalone equivalents. Beyond its role as an adapter for NLS-cargo, Kap α appears functionally important for switching on the high-affinity state of Kap β 1 toward the FG Nups to promote NLS-cargo import. RanGTP then downgrades this complex to a state of lower affinity to facilitate the turnover of RanGTP-Kap β 1 from the NPC. Interestingly, the softening of the NPC barrier (Fig. 8, B and C; Popken et al., 2015; Timney et al., 2016) that follows might place an upper limit on space constraints within the pore to maintain transport speed and accessibility. Still, because of their lack of cross-reactivity, both NLS-cargo-Kap α -Kap β 1 and RanGTP-Kap β 1 can co-exist in a dynamic equilibrium at the NPC to maintain nucleocytoplasmic transport.

Kap β 1-FG Nup binding affinity depends on Kap α and RanGTP

At the molecular level, MG-NLS-Kap α -Kap β 1, Kap α -Kap β 1, RanGTP-Kap β 1, and standalone Kap β 1 can all bind FG Nups, though MG-NLS-Kap α -Kap β 1 and Kap α -Kap β 1 exhibit a higher affinity for the FG Nups than RanGTP-Kap β 1 and standalone Kap β 1, which are similar (Fig. 3). This is consistent with the increased binding of IBB-Kap95 to the FG Nups over standalone Kap95 and RanGTP-Kap95 (Eisele et al., 2010). However, it disagrees with the notion that RanGTP either completely (Delphin et al., 1997; Floer et al., 1997; Ben-Efraim and Gerace, 2001) or partially (Allen et al., 2001; Pyhtila and Rexach, 2003) diminishes FG repeat interactions with standalone Kap β 1 to facilitate its release from NPCs. Previously, we had reported that Kap β 1 binding induced a conformational compaction in cNup153 that was reversible with excess RanGTP (Lim et al., 2007). Given our present insights, RanGTP likely facilitated Kap β 1 release as a result of the extremely low binding efficiency of Kap β 1 to cNup153 at the sub-nanomolar concentrations used.

Structural evidence for the enhanced binding of Kap α -Kap β 1

From a structural perspective, Kap β 1 is a flexible α -helical solenoid consisting of 19 tandem HEAT repeats (Cingolani et al., 1999, 2000; Bayliss et al., 2000; Fukuhara et al., 2004; Lee et al., 2005; Zachariae and Grubmüller, 2008; Yoshimura et al., 2014). Not surprisingly, the Kap β 1 structure is sensitive to solvent conditions (Forwood et al., 2008, 2010; Halder et al., 2015) and Kap α /IBB (Cingolani et al., 1999, 2000; Lee et al., 2005). Because of their overlapping binding sites on Kap β 1, RanGTP is known to trigger structural rearrangements within Kap β 1 to release Kap α /IBB (Conti et al., 2006), but how it modulates the affinity of Kap β 1 toward the FG Nups

exoKap β 1 after repopulating endoKap-reduced NPCs with exoKap α -exoKap β 1 (Kap α /Kap β 1 = 10:1; $C_{\text{Kap}\beta 1} = 100 \text{ nM}$). (E) From D, exoKap β 1 retention at the NE after Ran mix (light red) shows qualitative agreement with equilibrium calculations (solid line) as a function of exoKap β 1 concentration. To aid comparison, these values were normalized by preRan mix values (dark red). (F) From D, exoKap α retention at NE before (dark green) and after Ran mix (light green). **, $P < 0.01$; ***, $P < 0.001$. Student's t test (see Table S3). Box plots denote the median, first, and third quartiles. Error bars denote standard deviation, including outliers. Bars, $5 \mu\text{m}$. In C and D, an endogenous protein-specific antibody that does not cross react with exoKap β 1 was used to immunostain for endoKap β 1. See Materials and methods for details.

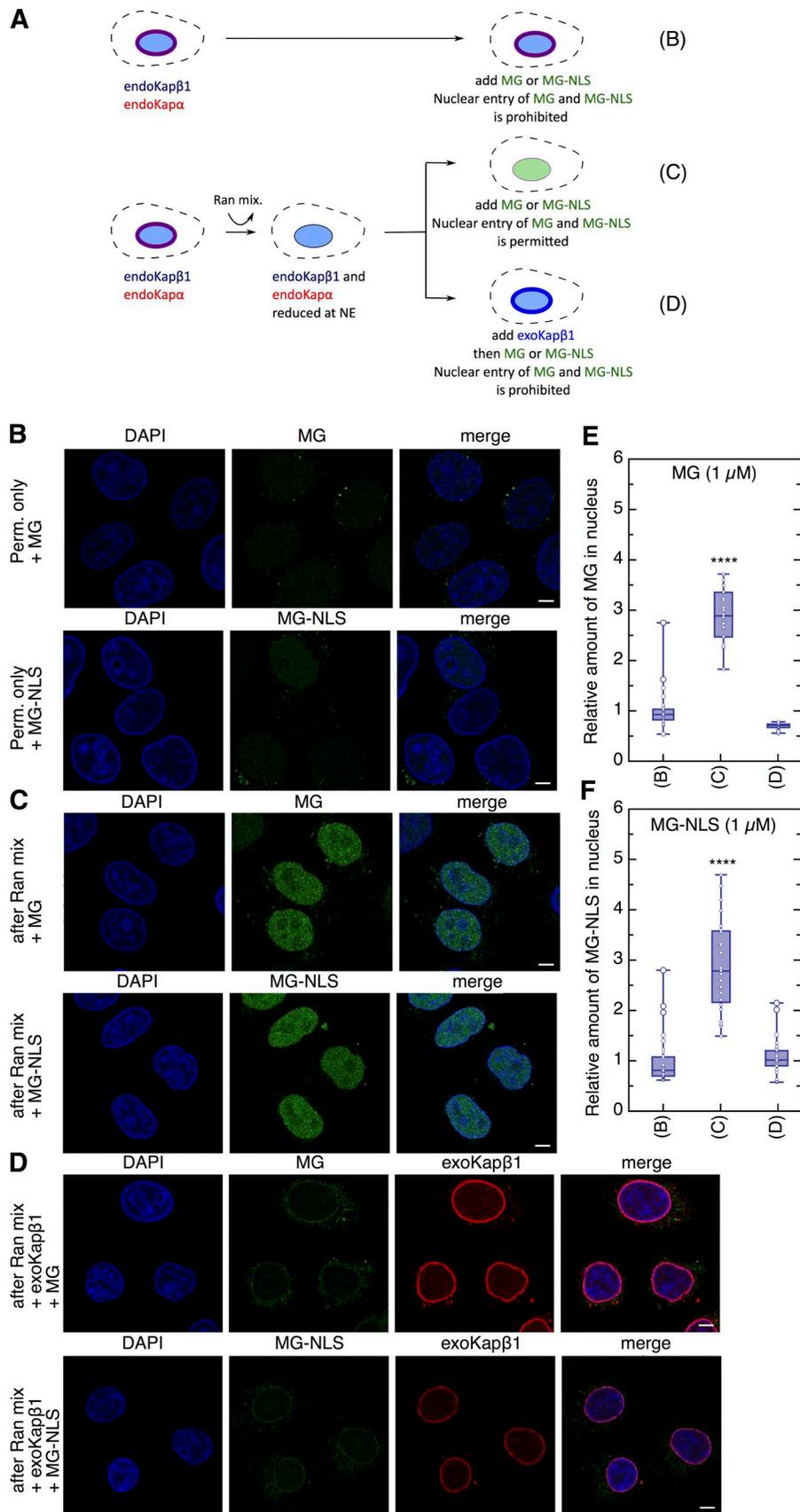


Figure 6. FG Nups are necessary but insufficient for NPC barrier function. (A) Cartoon illustration of all experiments. (B) Nuclear entry of MG and MG-NLS is prohibited in the presence of endoKap α and endoKap β 1 immediately after digitonin permeabilization. (C) Depleting endoKap α and endoKap β 1 by Ran mix abrogates NPC barrier function and facilitates the passive entry of MG and MG-NLS into the nucleus. (D) Adding back 100 nM exoKap β 1 sufficiently rescues NPC barrier function to prohibit MG and MG-NLS from entering the nucleus. (E) Fluorescence quantitation of MG in the nucleus at each of the above conditions. (F) Fluorescence quantitation of MG-NLS in the nucleus at each of the above conditions. $n = 3$ per experimental condition with a total of at least 26 cells each. ****, $P < 0.0001$; Student's t test. Box plots denote the median, first, and third quartiles. Error bars denote standard deviation, including outliers. Bars, 5 μ m.

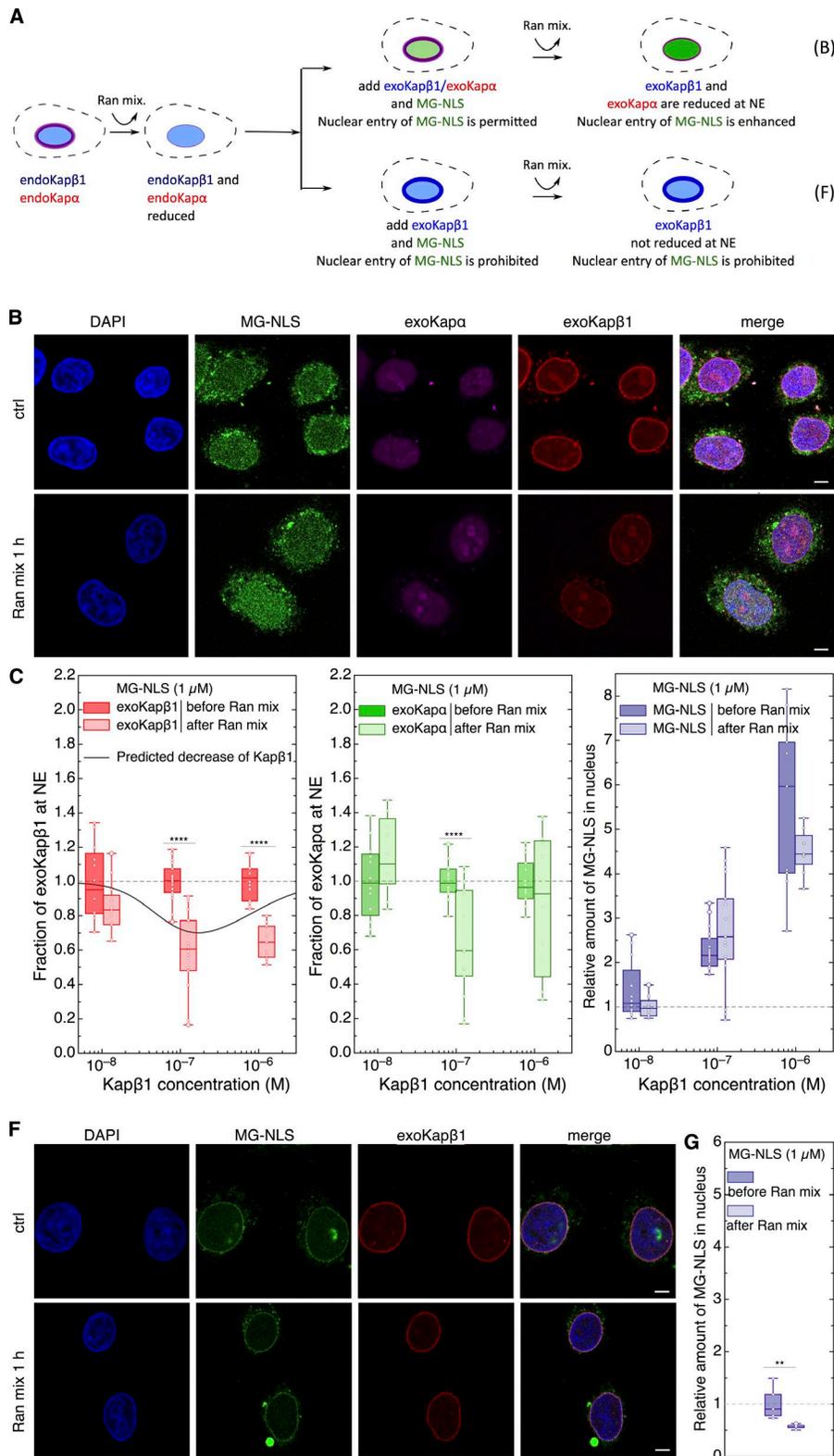


Figure 7. Kapβ1 turnover at NPCs is directly coupled to NLS-cargo release. (A) Cartoon illustration of all experiments. (B) Ran mix effectively reduces exoKapβ1 after repopulating endoKap-reduced NPCs with MG-NLS-exoKapα-exoKapβ1. (C) ExoKapβ1 retention at the NE after Ran mix (light red) shows qualitative agreement with equilibrium calculations (solid line) as a function of exoKapβ1 concentration. To aid comparison, these values were normalized by preRan mix values (dark red). (D) ExoKapα retention at NE before (dark green) and after Ran mix (light green). (E) Comparison of passive (dark purple) and Ran mix-activated (light purple) accumulation of MG-NLS in the nucleus. (F) Neither passive nor Ran mix-activated nuclear entry of MG-NLS was permitted in NPCs repopulated with exoKapβ1 (no exoKapα). ExoKapβ1 does not turn over under these conditions. (G) Fluorescence quantitation of nuclear MG-NLS before (dark purple) and after Ran mix (light purple) from F. $n = 3$ per experimental condition with a total of 10–12 cells each. **, $P < 0.01$; ****, $P < 0.0001$; Student's t test. Box plots denote the median, first, and third quartiles. Error bars denote standard deviation, including outliers. Bars, 5 μm.

remains unclear. Previous studies show that Kapα-Kapβ1 is more structurally compact than RanGTP-Kapβ1 (Cingolani et al., 1999; Fukuhara et al., 2004; Lee et al., 2005), and this might stabilize FG repeat binding on the outer Kapβ1 surface (Bayliss et al., 2000; Bednenko et al., 2003; Isgro and Schulten, 2005). It may be that the flexibility of RanGTP-Kapβ1 or standalone Kapβ1 incurs a higher entropic penalty for FG

repeat binding. In any case, there is no obvious structural evidence that argues against the accessibility of FG repeats to bind RanGTP-Kapβ1. Furthermore, NLS-cargo does not impact on Kapβ1-FG Nup binding because Kapα mediates their interaction. However, it is possible that very large NLS-cargoes may limit the occupancy of the transport complex within the FG Nups (Vovk et al., 2016).

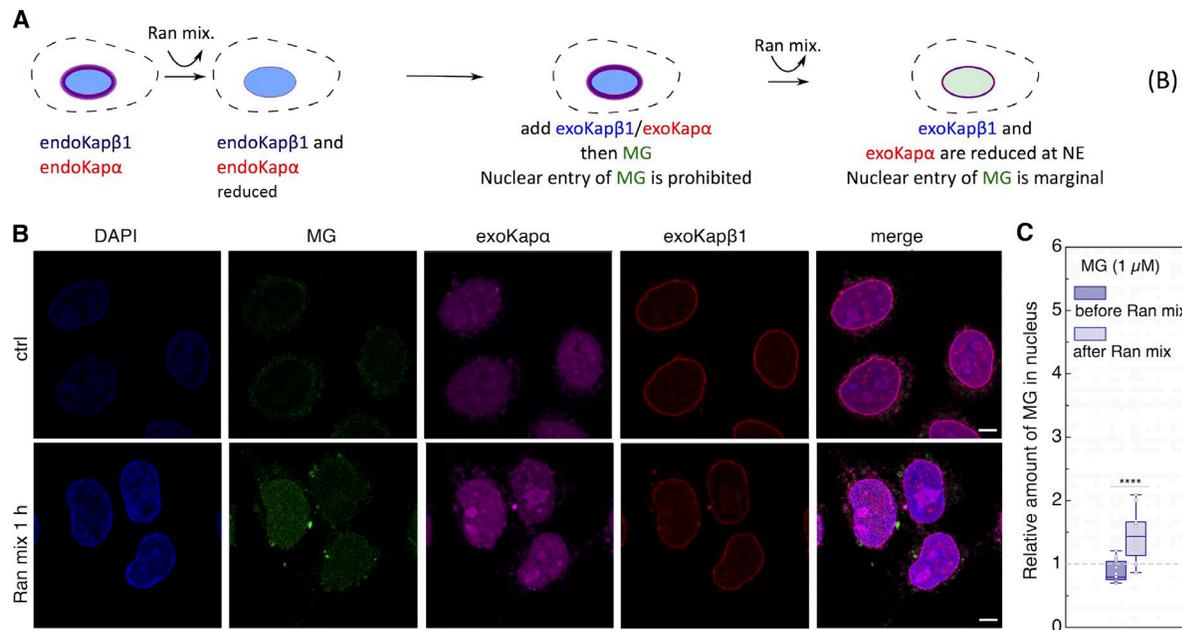


Figure 8. Kap α -facilitated turnover of Kap β 1 softens the NPC transport barrier against nonspecific cargoes. (A) Cartoon illustration of the experiment. (B) Kap β 1 turnover is coupled to a softening of the NPC transport barrier with Ran mix-activated transport. (C) Fluorescence quantitation after Ran mix shows that MG entry into the nucleus is marginally increased. $n = 3$ per experimental condition with a total of at least 13 cells being analyzed each. ****, $P < 0.0001$; Student's t test. Box plots denote the median, first, and third quartiles. Error bars denote standard deviation, including outliers. Bars, 5 μ m.

Quantitative analysis of Kap β 1 turnover in permeabilized cells

Quantitative analysis reveals that the occupancy of each Kap β 1 complex at the NPC depends on its binding affinity with the FG Nups and the extent to which Kap β 1 is depleted by RanGTP (Fig. 4). In turn, the concentration of each molecular partner and its binding isotherm with Kap β 1 will determine the relative amounts of Kap β 1 complexes (Fig. S5). To further validate this, permeabilized cell assays show that RanGTP reduces both Kap α -Kap β 1 (Fig. 5, D and E) and MG-NLS-Kap α -Kap β 1 (Fig. 7, B and C) in a nonmonotonic manner that depends on the concentration of Kap β 1.

Kap-centric versus FG-centric control

Kap-centric control departs from prevailing FG-centric views of the NPC (Fig. 9), which have attempted to explain the form and function of the NPC selective barrier in terms of FG Nup behavior (Rout et al., 2000; Frey and Görlich, 2007; Lim et al., 2007; Yamada et al., 2010). Key evidence for Kap-centric control lies with the NE retention of an endogenous pool of Kap α -Kap β 1 in permeabilized cells (Fig. 5 B), which had not been accounted for previously. This likely represents the physiological steady-state population of endoKap α -Kap β 1 at the NPC that reinforces the NPC barrier (Fig. 6 B). Interestingly, the unobstructed permeation of MG and MG-NLS into the nucleus (Fig. 6 C) was contingent on the removal of endoKap α -Kap β 1 by Ran mix (Fig. 5 B). Still more remarkable is that adding back exoKap β 1 restored barrier function against these substrates (Fig. 6, D–F). Thus, the FG Nups are necessary (to bind Kaps) but insufficient for establishing NPC barrier function. This might explain why FG repeat deletions did not have any significant impact on NPC permeability (Strawn et al., 2004).

As we have shown, the process of selective cargo transport is facilitated in a Kap concentration-dependent manner that determines the overall occupancy of Kap-cargo complexes in

the NPC. This is most likely facilitated by the highly flexible and dynamic FG Nups (Sakiyama et al., 2016) that would be able to respond and adapt to local changes in the pore. We postulate that strongly bound Kaps saturate the FG Nups to allow a weakly bound pool to pass (Kapinos et al., 2014), which is consistent with Yang and Musser (2006), who showed that higher Kap concentrations promoted higher transport efficiencies and faster transport times. Indeed, Fig. 7 E shows that the passive entry of MG-NLS into the nucleus increases with increasing exoKap β 1 (and exoKap α) concentrations. In marked contrast, any passive uptake of nonspecific MG cargoes remains blocked (Fig. 8 B; compare with Fig. 6, C and E). Hence, Kap-centric control encapsulates both NPC barrier and selective transport characteristics.

Although RanGTP binds Kap β 1 to release Kap α and NLS-cargoes from the NPC, it is less clear how RanGTP-Kap β 1 returns to the cytoplasm. If RanGTP also dissociates Kap β 1 from the FG Nups (Rexach and Blobel, 1995), RanGTP-Kap β 1 would itself resemble a large nonspecific cargo in the absence of FG Nup binding that contradicts the selective transport criteria. To retain its specificity to the FG Nups, we find that the action of RanGTP subtly switches the high-affinity binding state of MG-NLS-Kap α -Kap β 1 to the lower binding state of RanGTP-Kap β 1 to enable Kap β 1 turnover. Importantly, this is directly correlated to the concomitant release of Kap α (Fig. 5, D and F; and Fig. 7 D) and the nuclear accumulation of MG-NLS (Fig. 7, B–E). On this basis, RanGTP-Kap β 1 efflux is still specific in nature.

Implications of Kap-centric control

A key attribute of Kap-centric control may be to regulate the Ran gradient (Görlich et al., 2003; Riddick and Macara, 2005; Kopito and Elbaum, 2009). Because of its higher affinity for Kap β 1, RanGTP likely outcompetes Kap α for Kap β 1 within the nucleus. This prevents it from traversing further into the NPC

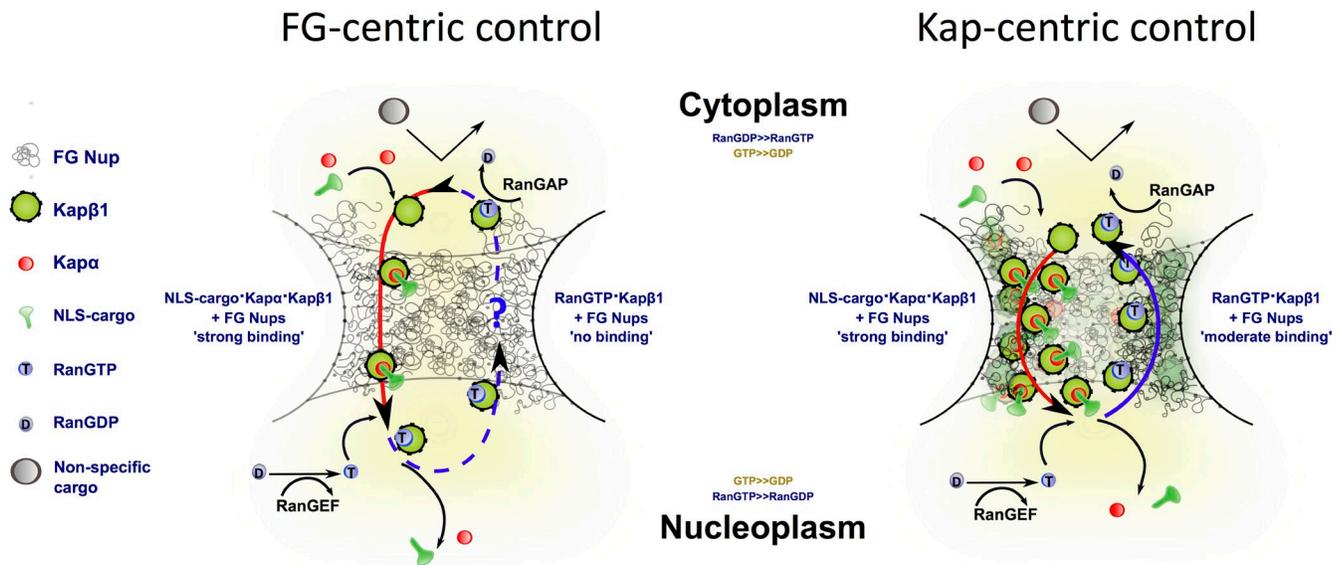


Figure 9. **FG-centric versus Kap-centric NPC transport models.** (Left) FG-centric models explain that an FG Nup barrier regulates selective transport through the NPC without invoking Kap occupancy. (Right) Kap-centric control argues that NPC barrier and transport function is regulated by Kaps in the pore. This is mediated by Kap α , which promotes NLS-cargo·Kap α ·Kap β 1 import by switching the transport complex into a high-affinity FG Nup binding state. Upon reaching the nucleus, RanGTP switches Kap β 1 back to its lower affinity state while concomitantly releasing NLS-cargo and Kap α . At steady state, RanGTP·Kap β 1 export is sustained because it does not cross react with Kap α ·Kap β 1 within the NPC. The model is constructed according to the equilibrium dissociation constants summarized in Table S4.

on its own, which might explain why RanGTP·Kap β 1 complex formation only occurs at the nuclear basket (Lowe et al., 2015). Upon reaching the cytoplasmic periphery, the chemical potential established by RanGAP (RanGTPase-activating protein) to hydrolyze GTP into GDP (Bischoff et al., 1994) followed by the release of RanGDP from Kap β 1 is what sustains RanGTP·Kap β 1 export (Moroianu and Blobel, 1995). To further underscore the importance of Kap α , we postulate that Kap α ·Kap β 1 also helps to outcompete and release RanGTP·Kap β 1 from the FG Nups there. Then, after GTP hydrolysis, the higher affinity of RanGDP toward NTF2 in comparison with Kap β 1 (Chaillan-Huntington et al., 2000; Forwood et al., 2008; Lonhienne et al., 2009) (Table S4) ensures that predominantly RanGDP·NTF2 complexes form in the cytoplasm for import into the nucleus.

Still, 19 other members consisting of importins and exportins exist in the Kap β family that bind signal-specific cargoes directly (Chook and Süel, 2011; Kimura et al., 2017). Although it remains to be ascertained if and how they contribute to Kap-centric control, IBB-cargo such as SREBP2 (Lee et al., 2003) that binds Kap β 1 directly may provide a good starting point to explore the role of Kap α -independent pathways.

Conclusion

Our work reveals how NPC function is controlled by karyopherins that shuttle cargoes between the nucleus and the cytoplasm. This disagrees with the view that the FG Nups regulate the shuttling of karyopherins and their cargoes. To operate the NPC, Kap α mediates Kap β 1 turnover and occupancy in a RanGTP-dependent manner that simultaneously regulates NLS-cargo release and NPC barrier function. Thus, a deregulation of Kap-centric control could lead to a malfunction in NCT and disease (Kau et al., 2004).

Materials and methods

FG Nup expression and purification

Cysteine-tagged FG domains of four human nucleoporins, Nup62, Nup214, Nup98, and Nup153, were cloned, expressed, and purified as described previously (Kapinos et al., 2014). All proteins were dialyzed into the appropriate buffer (see below) before experimentation. The concentration of these proteins was determined using UV measurements or Bradford assay.

WT Ran and RanQ69L expression, purification, and loading with GTP or GDP

A plasmid (pQE32) with a full-length human RanQ69L construct (the nonhydrolyzing mutant of Ran) was a gift from U. Kutay (ETH Zurich, Zurich, Switzerland) (Kutay et al., 1997b). A WT Ran construct was derived from the aforementioned plasmid using site-directed mutagenesis (primers 5'-GTATGGGACACAGCCGGCCAGGAGAAA TTCGGTGGACTG-3' and 5'-CAGTCCACCGAATTTCTCCTGGCC GGCTGTGTCCCATAC-3'). His-tagged Ran WT and RanQ69L full-length proteins were induced by 0.5 M IPTG and expressed in BL21 competent cells at 24°C overnight. The cells were lysed for 1 h at 4°C using the following buffer: 50 mM HEPES-KOH, pH 7, 100 mM NaCl, 5 mM DTT, 5 mM MgCl₂, and 20 mM imidazole with addition of 40 μ l DNase (10 mg/ml), Pefobloc, and lysozyme. Finally, Ran WT and RanQ69L were purified using a nickel–nitrilotriacetic acid (Ni-NTA) column (Roche) in an imidazole gradient (10–500 mM). The purified protein was dialyzed into 10 mM HEPES buffer, pH 7.2, with 100 mM NaCl. Then, Ran WT and RanQ69L were incubated for 30 min at 4°C with 10 mM EDTA and 1 mM GTP or GDP nucleotides. Consequently, 25 mM of excess MgCl₂ was added to ensure MgCl₂ and GTP (or GDP) binding to nucleotide-free Ran. Finally, Ran WT and RanQ69L loaded with GTP was dialyzed into PBS buffer, pH 7.2 (GIBCO by Life Sciences), in the presence of 1 mM MgCl₂ and isolated using an Äkta Purifier on a column (Superdex 200 HiLoad 16/60; GE

Healthcare). Protein purity was analyzed by 12% PAGE at 0.1% SDS (Fig. S2), and Ran WT and RanQ69L concentrations were determined by absorption measurements at 280 nm and correcting it for the GTP or GDP absorption within this region.

Kap β 1 and Kap α expression and purification

Full-length human Kap β 1 was cloned, expressed, and purified as described previously (Kapinos et al., 2014). The plasmid (pQE70) containing a full-length *Xenopus laevis* Kap α construct was a gift from U. Kutay. A full-length human Kap α construct (Addgene template pCMVTNT-T7-KPNA2; plasmid 26678) was cloned into the same pQE70 vector using EcoRI–BamII restriction enzymes. Both constructs have a His₆ tag at its C terminus with a short linker (–GSRSHHHHHH) that does not affect the complex formation of this protein with Kap β 1. Kap α was subsequently purified using an Ni-NTA column (Roche). Finally, Kap α monomers were separated and isolated using a Superdex 200 column, and the collected fractions were stored at –80°C. The final purity of the His₆-tagged Kap α and Kap β 1 was analyzed by 12% PAGE at 0.1% SDS (Fig. S2), and their concentration was determined by absorption measurements at 280 nm.

MG and MG-NLS expression and purification

Sequences of MG-NLS and MG (maltose-binding protein modified at its C terminus with GFP with and without an NLS sequence, respectively) were cloned into pPEP-TEV vector using SacI–KpnI restriction enzymes. The template plasmid containing MG sequence was a gift from L.M. Veenhoff (University Medical Center Groningen, Groningen, Netherlands). The expression and purification of these constructs were done as described for Kap β 1. The N-terminal His₆-tagged proteins were purified using a Ni-NTA column (Roche) and then were separated and isolated using a Superdex 200 column, and the collected fractions were stored at –80°C. The quality and quantity of MG-NLS or MG were verified using 12% SDS-PAGE.

Dynamic light scattering

The hydrodynamic radii of the purified proteins were measured by dynamic light scattering (Zetasizer Nano) as previously described (Kapinos et al., 2014). See Table S1 for details.

ITC

The equilibrium binding constants of RanGTP·Kap β 1 and Kap α ·Kap β 1 were measured using a microcalorimeter (VP-ITC; MicroCal, LLC; Fig. S2). All measurements were done at 25°C in a buffer of 20 mM Tris-HCL, pH 7.5, 200 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine, and 1 mM MgCl₂. To measure RanGTP·Kap β 1 complex formation, 21 μ M RanGTP was titrated into 3 μ M Kap β 1 in the measurement cell (30 injections). To measure Kap α ·Kap β 1 complex formation, 49 μ M Kap α was titrated into 6 μ M Kap β 1 in the measurement cell (30 injections).

MST

The equilibrium dissociation constant of Alexa Fluor 488–Kap α ·Kap β 1 was measured in PBS, pH 7.2, at 25°C using a microscale electrophoresis instrument (Monolith NT.115; Nano Temper Technologies; Fig. S2 D). Kap β 1 was mixed with the Alexa Fluor 488–Kap α and placed into capillaries (16 samples; 50 nM Alexa Fluor 488–labeled Kap α mixed with 11.4 nM to 5 μ M Kap β 1). The equilibrium dissociation constant of MG-NLS with the Kap α ·Kap β 1 complex was measured in the same manner (Fig. S2). Kap α ·Kap β 1 was mixed with MG-NLS and placed into capillaries (16 samples; 50 nM MG-NLS mixed with 0.06 nM to 4 μ M Kap β 1 and 0.12 nM to 8 μ M Kap α).

CD

The CD spectra of 5 μ M Kap α , 5 μ M Kap β 1, and 2.5 μ M Kap α ·Kap β 1 complexes were measured in 10-mm quartz cuvettes using a CD spectrometer (Chirascan; Applied Photophysics; Fig. S2) in PBS, pH 7.2.

SPR measurements

All SPR measurements were performed at 25°C in PBS, pH 7.2 (GIBCO by Life Technologies), with 1 mM MgCl₂ in a four flow cell instrument (Biacore T100; GE Healthcare) as described previously (Schoch et al., 2012; Kapinos et al., 2014). In brief, C₁₇H₃₆O₄S (hydroxyl-terminated tri[ethylene glycol] undecane thiol, HS-[CH₂]-[OCH₂CH₂]₃-OH, abbreviated as PUT; Nanoscience) and the cysteine-modified FG Nup domains were semicovalently grafted onto a gold sensor surface via thiol binding in cell 1 (reference) and cell 2 (sample; Fig. S1). 1% (wt/vol) BSA (Sigma-Aldrich) solution was prepared in PBS, pH 7.2. Before experimentation, the proteins were dialyzed into PBS, pH 7.2. Experiments with mixed layers were performed by premixing different FG Nup domains in equimolar ratios before being grafted onto the gold sensor surface. All protein and reagent solutions were centrifuged for 15 min at 16,000 g to remove particles and bubbles. Buffer solutions were filtered (0.22 μ m) and degassed before use. We note that both RanGTP ($n = 4$) and RanQ69L-GTP ($n = 12$) were used in these experiments. However, both variants gave similar results and are henceforth referred to as RanGTP for brevity.

Kinetic analysis of multivalent interactions

All kinetic analyses were carried out as described previously (Kapinos et al., 2014; Wagner et al., 2015). In brief, a set of 36 \times 36 ($k_{on,i}$, $k_{off,i}$) pairs was populated, and their fractional abundance was depicted as color intensity in k_{on} versus K_d and k_{off} versus K_d . Each interaction map averaged over \sim 10 individual sensograms. Calculations and visualizations were generated using Matlab (MathWorks) and Python.

Permeabilized cell assays

HeLa cells were washed with transport buffer and then permeabilized with 40 μ g/ml digitonin in transport buffer for 5 min as described previously (Adam et al., 1990). After permeabilization, the cells were washed with PBS three times for 5 min each and then incubated with Ran mix for 1 h (2 mM GTP, 0.1 mM ATP, 4 mM creatine phosphate, 20 U/ml creatine kinase, 5 μ M RanGDP, 4 μ M NTF2, and 1 mM DTT; Lowe et al., 2015). For endogenous Kap α and Kap β 1 detection, cells were fixed with 2% PFA for 15 min and stained with anti-Kap α (Santa Cruz), anti-Kap β 1 (3E9; Abcam), and DAPI (Sigma-Aldrich; Fig. 5 B). For the exogenous Kap repopulation assay, exoKap α was conjugated with Alexa Fluor 488 (Thermo Fisher; degree of labeling [DOL] 1.5) or Alexa Fluor 647 (DOL 1.4), whereas exoKap β 1 was conjugated with Alexa Fluor 568 (DOL 2.89). Either exoKap β 1 or exoKap α ·Kap β 1 (preincubated for 30 min at RT) was applied for 1 h at the concentrations specified in the main text. Cells were subsequently treated with transport buffer or Ran mix for 1 h. Subsequently, the cells were fixed and stained with anti-Kap β 1 for endoKap β 1, whereas exoKap β 1 or exoKap α was detected by Alexa Fluor labeling (Fig. 5, C and D). In this regard, anti-Kap β 1 (3E9) recognizes only endoKap β 1 but not exoKap β 1 according to product-specific information. All other experiments involving MG, MG-NLS, and/or exogenous Kaps were conducted by separating a respective batch of cells into parallel sets after the first Ran mix treatment. This was to facilitate (a) immunostaining of endoKap α and endoKap β 1 in the absence of exoKaps and (b) detection of exoKap α and/or exoKap β 1, MG, and MG-NLS after subsequent Ran mix treatments (i.e., not requiring endoKap staining). To investigate NPC barrier function, the nuclear accumulation of 1 μ M MG or 1 μ M MG-NLS was first measured in permeabilized cells or Ran mix–treated cells after 1-h

incubation. The cells were then treated with 100 nM exoKap β 1 together with MG-NLS or 100 nM exoKap β 1 followed by MG for another 1 h (Fig. 6 B–D). To test for Kap β 1 turnover, MG-NLS coincubated with either exoKap α -Kap β 1 or exoKap β 1 alone was introduced to Ran mix-treated permeabilized cells followed by a second step (Fig. 7). In comparison, Ran mix-treated permeabilized cells were sequentially treated with exoKap α -Kap β 1 and then MG or MG and Ran mix together to check whether the NPC barrier softened during Kap β 1 turnover (Fig. 8). In all cases, cells were then fixed and stained with DAPI.

Fluorescence image analysis

Fluorescence images were obtained at RT with Zen 2010 software using an LSM700 upright confocal microscope (Zeiss) with an oil-immersed 63 \times /1.4 NA PLAN APO objective and two photomultiplier tube detectors (Hamamatsu). Nuclear rim staining quantification was performed using ImageJ software (National Institutes of Health). Raw data for both DAPI and Kap β 1 channels were first duplicated. The nuclear rim of each permeabilized cell was then defined as a region of interest by converting the DAPI channel into a binary image, followed by the processes of (a) filling holes (to fill up the whole nucleus), (b) outlining (to obtain the nuclear rim outline), and (3) dilating (to generate an \sim 700-nm width for the nuclear rim). This region of interest was then applied to measure the mean fluorescence intensity of endoKap β 1 or exoKap β 1 in the Kap β 1 channel as well as in the Kap α channel. The intensity of Ran mix-treated samples was normalized to transport buffer-treated control samples. The mean fluorescence intensity of MG-NLS or MG was measured from the nuclear region defined by the DAPI channel. Similarly, the intensity was normalized to the control samples. Analyzed cell numbers are specified in respective figure legends.

Online supplemental material

Fig. S1 explains the SPR experimental procedure. Fig. S2 presents a summary of ITC, MST, SDS-PAGE, and CD characterization of Kap β 1 and its related complexes in solution. Fig. S3 shows SPR measurements that compare and contrast the effect of RanGTP on the binding of MG-NLS-Kap α -Kap β 1 and standalone Kap β 1 complexes to cNup153, as well as the promiscuous FG Nup binding of RanGTP-Kap β 1 and Kap α -Kap β 1. Fig. S4 shows equilibrium Langmuir isotherm and kinetic analysis of standalone Kap β 1, RanGTP-Kap β 1, Kap α -Kap β 1, and MG-NLS-Kap α -Kap β 1 binding to cNup153, cNup62, cNup214, and cNup98, respectively. Fig. S5 summarizes the quantitative binding analysis by equilibrium calculations and SPR at different mixing ratios and concentrations. Table S1 shows the hydrodynamic diameters of the relevant proteins and transport complexes as determined by dynamic light scattering. Table S2 summarizes Student's *t* test results that validate the significance of the observed differences indicated in the box plots of Fig. 3. Table S3 summarizes Student's *t* test results that validate the significance of the observed differences indicated in the box plots of Figs. 5, 6, 7, and 8. Table S4 provides a comprehensive summary of all complex interactions and their equilibrium dissociation constants.

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The authors declare no competing financial interests.

Author contributions: L.E. Kapinos, B. Huang, and R.Y.H. Lim conceived and designed the study. L.E. Kapinos performed all bio-

physical experiments and quantitative analysis. B. Huang carried out all permeabilized cell assays, including confocal microscopy and image analysis. C. Rencurel cloned, expressed, and purified proteins. R.Y.H. Lim wrote the paper together with L.E. Kapinos and B. Huang. All authors discussed the results and commented on the manuscript.

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References

- Adam, S.A., R.S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807–816. <http://dx.doi.org/10.1083/jcb.111.3.807>
- Allen, N.P., L. Huang, A. Burlingame, and M. Rexach. 2001. Proteomic analysis of nucleoporin interacting proteins. *J. Biol. Chem.* 276:29268–29274. <http://dx.doi.org/10.1074/jbc.M102629200>
- Bayliss, R., T. Littlewood, and M. Stewart. 2000. Structural basis for the interaction between FxFG nucleoporin repeats and importin- β in nuclear trafficking. *Cell.* 102:99–108. [http://dx.doi.org/10.1016/S0092-8674\(00\)00014-3](http://dx.doi.org/10.1016/S0092-8674(00)00014-3)
- Bednenko, J., G. Cingolani, and L. Gerace. 2003. Importin β contains a COOH-terminal nucleoporin binding region important for nuclear transport. *J. Cell Biol.* 162:391–401. <http://dx.doi.org/10.1083/jcb.200303085>
- Ben-Efraim, I., and L. Gerace. 2001. Gradient of increasing affinity of importin β for nucleoporins along the pathway of nuclear import. *J. Cell Biol.* 152:411–417. <http://dx.doi.org/10.1083/jcb.152.2.411>
- Bischoff, F.R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl. 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. *Proc. Natl. Acad. Sci. USA.* 91:2587–2591. <http://dx.doi.org/10.1073/pnas.91.7.2587>
- Catimel, B., T. Teh, M.R. Fontes, I.G. Jennings, D.A. Jans, G.J. Howlett, E.C. Nice, and B. Kobe. 2001. Biophysical characterization of interactions involving importin- α during nuclear import. *J. Biol. Chem.* 276:34189–34198. <http://dx.doi.org/10.1074/jbc.M103531200>
- Chaillan-Huntington, C., C.V. Braslavsky, J. Kuhlmann, and M. Stewart. 2000. Dissecting the interactions between NTF2, RanGDP, and the nucleoporin XFXFG repeats. *J. Biol. Chem.* 275:5874–5879. <http://dx.doi.org/10.1074/jbc.275.8.5874>
- Chook, Y.M., and K.E. Süel. 2011. Nuclear import by karyopherin- β s: recognition and inhibition. *Biochim. Biophys. Acta.* 1813:1593–1606. <http://dx.doi.org/10.1016/j.bbamcr.2010.10.014>
- Christie, M., C.W. Chang, G. Róna, K.M. Smith, A.G. Stewart, A.A. Takeda, M.R. Fontes, M. Stewart, B.G. Vértessy, J.K. Forwood, and B. Kobe. 2016. Structural biology and regulation of protein import into the nucleus. *J. Mol. Biol.* 428:2060–2090. <http://dx.doi.org/10.1016/j.jmb.2015.10.023>
- Cingolani, G., C. Petosa, K. Weis, and C.W. Müller. 1999. Structure of importin- β bound to the IBB domain of importin- α . *Nature.* 399:221–229. <http://dx.doi.org/10.1038/20367>
- Cingolani, G., H.A. Lashuel, L. Gerace, and C.W. Müller. 2000. Nuclear import factors importin α and importin β undergo mutually induced conformational changes upon association. *FEBS Lett.* 484:291–298. [http://dx.doi.org/10.1016/S0014-5793\(00\)02154-2](http://dx.doi.org/10.1016/S0014-5793(00)02154-2)
- Conti, E., C.W. Müller, and M. Stewart. 2006. Karyopherin flexibility in nucleocytoplasmic transport. *Curr. Opin. Struct. Biol.* 16:237–244. <http://dx.doi.org/10.1016/j.sbi.2006.03.010>
- Delphin, C., T. Guan, F. Melchior, and L. Gerace. 1997. RanGTP targets p97 to RanBP2, a filamentous protein localized at the cytoplasmic periphery of the nuclear pore complex. *Mol. Biol. Cell.* 8:2379–2390. <http://dx.doi.org/10.1091/mbc.8.12.2379>
- Eibauer, M., M. Pellanda, Y. Turgay, A. Dubrovsky, A. Wild, and O. Medalia. 2015. Structure and gating of the nuclear pore complex. *Nat. Commun.* 6:7532. <http://dx.doi.org/10.1038/ncomms8532>
- Eisele, N.B., S. Frey, J. Piehler, D. Görlich, and R.P. Richter. 2010. Ultrathin nucleoporin phenylalanine-glycine repeat films and their interaction with nuclear transport receptors. *EMBO Rep.* 11:366–372. <http://dx.doi.org/10.1038/embor.2010.34>
- Falces, J., I. Arregi, P.V. Konarev, M.A. Urbaneja, D.I. Svergun, S.G. Taneva, and S. Bañuelos. 2010. Recognition of nucleoplasmin by its nuclear transport receptor importin α/β : insights into a complete import complex. *Biochemistry.* 49:9756–9769. <http://dx.doi.org/10.1021/bi101179g>

- Floer, M., G. Blobel, and M. Rexach. 1997. Disassembly of RanGTP-karyopherin β complex, an intermediate in nuclear protein import. *J. Biol. Chem.* 272:19538–19546. <http://dx.doi.org/10.1074/jbc.272.31.19538>
- Forwood, J.K., T.G. Lonhienne, M. Marfori, G. Robin, W. Meng, G. Guncar, S.M. Liu, M. Stewart, B.J. Carroll, and B. Kobe. 2008. Kap95p binding induces the switch loops of RanGDP to adopt the GTP-bound conformation: implications for nuclear import complex assembly dynamics. *J. Mol. Biol.* 383:772–782. <http://dx.doi.org/10.1016/j.jmb.2008.07.090>
- Forwood, J.K., A. Lange, U. Zachariae, M. Marfori, C. Preast, H. Grubmüller, M. Stewart, A.H. Corbett, and B. Kobe. 2010. Quantitative structural analysis of importin- β flexibility: paradigm for solenoid protein structures. *Structure*. 18:1171–1183. <http://dx.doi.org/10.1016/j.str.2010.06.015>
- Frey, S., and D. Görlich. 2007. A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes. *Cell*. 130:512–523. <http://dx.doi.org/10.1016/j.cell.2007.06.024>
- Fukuhara, N., E. Fernandez, J. Ebert, E. Conti, and D. Svergun. 2004. Conformational variability of nucleocytoplasmic transport factors. *J. Biol. Chem.* 279:2176–2181. <http://dx.doi.org/10.1074/jbc.M309112200>
- Görlich, D., and U. Kutay. 1999. Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* 15:607–660. <http://dx.doi.org/10.1146/annurev.cellbio.15.1.607>
- Görlich, D., F. Vogel, A.D. Mills, E. Hartmann, and R.A. Laskey. 1995. Distinct functions for the two importin subunits in nuclear protein import. *Nature*. 377:246–248. <http://dx.doi.org/10.1038/377246a0>
- Görlich, D., N. Panté, U. Kutay, U. Aebi, and F.R. Bischoff. 1996. Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.* 15:5584–5594.
- Görlich, D., M.J. Seewald, and K. Ribbeck. 2003. Characterization of Ran-driven cargo transport and the RanGTPase system by kinetic measurements and computer simulation. *EMBO J.* 22:1088–1100. <http://dx.doi.org/10.1093/emboj/cdg113>
- Grünwald, D., and R.H. Singer. 2010. In vivo imaging of labelled endogenous β -actin mRNA during nucleocytoplasmic transport. *Nature*. 467:604–607. <http://dx.doi.org/10.1038/nature09438>
- Hahn, S., and G. Schlenstedt. 2011. Importin β -type nuclear transport receptors have distinct binding affinities for Ran-GTP. *Biochem. Biophys. Res. Commun.* 406:383–388. <http://dx.doi.org/10.1016/j.bbrc.2011.02.051>
- Halder, K., N. Dölker, Q. Van, I. Gregor, A. Dickmanns, I. Baade, R.H. Kehlenbach, R. Ficner, J. Enderlein, H. Grubmüller, and H. Neumann. 2015. MD simulations and FRET reveal an environment-sensitive conformational plasticity of importin- β . *Biophys. J.* 109:277–286. <http://dx.doi.org/10.1016/j.bpj.2015.06.014>
- Hough, L.E., K. Dutta, S. Sparks, D.B. Temel, A. Kamal, J. Tetenbaum-Novatt, M.P. Rout, and D. Cowburn. 2015. The molecular mechanism of nuclear transport revealed by atomic-scale measurements. *eLife*. 4:e10027. <http://dx.doi.org/10.7554/eLife.10027>
- Isgro, T.A., and K. Schulten. 2005. Binding dynamics of isolated nucleoporin repeat regions to importin- β . *Structure*. 13:1869–1879. <http://dx.doi.org/10.1016/j.str.2005.09.007>
- Kapinos, L.E., R.L. Schoch, R.S. Wagner, K.D. Schleicher, and R.Y.H. Lim. 2014. Karyopherin-centric control of nuclear pores based on molecular occupancy and kinetic analysis of multivalent binding with FG nucleoporins. *Biophys. J.* 106:1751–1762. <http://dx.doi.org/10.1016/j.bpj.2014.02.021>
- Kau, T.R., J.C. Way, and P.A. Silver. 2004. Nuclear transport and cancer: from mechanism to intervention. *Nat. Rev. Cancer*. 4:106–117. <http://dx.doi.org/10.1038/nrc1274>
- Kimura, M., Y. Morinaka, K. Imai, S. Kose, P. Horton, and N. Imamoto. 2017. Extensive cargo identification reveals distinct biological roles of the 12 importin pathways. *eLife*. 6:e21184. <http://dx.doi.org/10.7554/eLife.21184>
- Kopito, R.B., and M. Elbaum. 2009. Nucleocytoplasmic transport: a thermodynamic mechanism. *HFSP J.* 3:130–141. <http://dx.doi.org/10.2976/1.3080807>
- Kubitscheck, U., D. Grünwald, A. Hoekstra, D. Rohleder, T. Kues, J.P. Siebrasse, and R. Peters. 2005. Nuclear transport of single molecules: dwell times at the nuclear pore complex. *J. Cell Biol.* 168:233–243. <http://dx.doi.org/10.1083/jcb.200411005>
- Kutay, U., F.R. Bischoff, S. Kostka, R. Kraft, and D. Görlich. 1997a. Export of importin α from the nucleus is mediated by a specific nuclear transport factor. *Cell*. 90:1061–1071. [http://dx.doi.org/10.1016/S0092-8674\(00\)80372-4](http://dx.doi.org/10.1016/S0092-8674(00)80372-4)
- Kutay, U., E. Izaurralde, F.R. Bischoff, I.W. Mattaj, and D. Görlich. 1997b. Dominant-negative mutants of importin- β block multiple pathways of import and export through the nuclear pore complex. *EMBO J.* 16:1153–1163. <http://dx.doi.org/10.1093/emboj/16.6.1153>
- Lee, S.J., T. Sekimoto, E. Yamashita, E. Nagoshi, A. Nakagawa, N. Imamoto, M. Yoshimura, H. Sakai, K.T. Chong, T. Tsukihara, and Y. Yoneda. 2003. The structure of importin- β bound to SREBP-2: nuclear import of a transcription factor. *Science*. 302:1571–1575. <http://dx.doi.org/10.1126/science.1088372>
- Lee, S.J., Y. Matsuura, S.M. Liu, and M. Stewart. 2005. Structural basis for nuclear import complex dissociation by RanGTP. *Nature*. 435:693–696. <http://dx.doi.org/10.1038/nature03578>
- Lim, R.Y.H., B. Fahrenkrog, J. Köser, K. Schwarz-Herion, J. Deng, and U. Aebi. 2007. Nanomechanical basis of selective gating by the nuclear pore complex. *Science*. 318:640–643. <http://dx.doi.org/10.1126/science.1145980>
- Lim, R.Y.H., B. Huang, and L.E. Kapinos. 2015. How to operate a nuclear pore complex by Kap-centric control. *Nucleus*. 6:366–372. <http://dx.doi.org/10.1080/19491034.2015.1090061>
- Lolodi, O., H. Yamazaki, S. Otsuka, M. Kumeta, and S.H. Yoshimura. 2016. Dissecting in vivo steady-state dynamics of karyopherin-dependent nuclear transport. *Mol. Biol. Cell*. 27:167–176. <http://dx.doi.org/10.1091/mbc.E15-08-0601>
- Lonhienne, T.G., J.K. Forwood, M. Marfori, G. Robin, B. Kobe, and B.J. Carroll. 2009. Importin- β is a GDP-to-GTP exchange factor of Ran: implications for the mechanism of nuclear import. *J. Biol. Chem.* 284:22549–22558. <http://dx.doi.org/10.1074/jbc.M109.019935>
- Lowe, A.R., J.H. Tang, J. Yassif, M. Graf, W.Y. Huang, J.T. Groves, K. Weis, and J.T. Liphardt. 2015. Importin- β modulates the permeability of the nuclear pore complex in a Ran-dependent manner. *eLife*. 4. <http://dx.doi.org/10.7554/eLife.04052>
- Lyman, S.K., T. Guan, J. Bednenko, H. Wodrich, and L. Gerace. 2002. Influence of cargo size on Ran and energy requirements for nuclear protein import. *J. Cell Biol.* 159:55–67. <http://dx.doi.org/10.1083/jcb.200204163>
- Makise, M., D.R. Mackay, S. Elgort, S.S. Shankaran, S.A. Adam, and K.S. Ullman. 2012. The Nup153-Nup50 protein interface and its role in nuclear import. *J. Biol. Chem.* 287:38515–38522. <http://dx.doi.org/10.1074/jbc.M112.378893>
- Milles, S., D. Mercadante, I.V. Aramburu, M.R. Jensen, N. Banterle, C. Koehler, S. Tyagi, J. Clarke, S.L. Shammass, M. Blackledge, et al. 2015. Plasticity of an ultrafast interaction between nucleoporins and nuclear transport receptors. *Cell*. 163:734–745. <http://dx.doi.org/10.1016/j.cell.2015.09.047>
- Moroianu, J., and G. Blobel. 1995. Protein export from the nucleus requires the GTPase Ran and GTP hydrolysis. *Proc. Natl. Acad. Sci. USA*. 92:4318–4322. <http://dx.doi.org/10.1073/pnas.92.10.4318>
- Moroianu, J., G. Blobel, and A. Radu. 1995. Previously identified protein of uncertain function is karyopherin alpha and together with karyopherin beta docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA*. 92:2008–2011. <http://dx.doi.org/10.1073/pnas.92.6.2008>
- Nigg, E.A. 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature*. 386:779–787. <http://dx.doi.org/10.1038/386779a0>
- Ogawa, Y., Y. Miyamoto, M. Oka, and Y. Yoneda. 2012. The interaction between importin- α and Nup153 promotes importin- α/β -mediated nuclear import. *Traffic*. 13:934–946. <http://dx.doi.org/10.1111/j.1600-0854.2012.01367.x>
- Paradise, A., M.K. Levin, G. Korza, and J.H. Carson. 2007. Significant proportions of nuclear transport proteins with reduced intracellular mobilities resolved by fluorescence correlation spectroscopy. *J. Mol. Biol.* 365:50–65. <http://dx.doi.org/10.1016/j.jmb.2006.09.089>
- Popken, P., A. Ghavami, P.R. Onck, B. Poolman, and L.M. Veenhoff. 2015. Size-dependent leak of soluble and membrane proteins through the yeast nuclear pore complex. *Mol. Biol. Cell*. 26:1386–1394. <http://dx.doi.org/10.1091/mbc.E14-07-1175>
- Pumroy, R.A., and G. Cingolani. 2015. Diversification of importin- α isoforms in cellular trafficking and disease states. *Biochem. J.* 466:13–28. <http://dx.doi.org/10.1042/BJ20141186>
- Pyhtila, B., and M. Rexach. 2003. A gradient of affinity for the karyopherin Kap95p along the yeast nuclear pore complex. *J. Biol. Chem.* 278:42699–42709. <http://dx.doi.org/10.1074/jbc.M307135200>
- Rexach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell*. 83:683–692. [http://dx.doi.org/10.1016/0092-8674\(95\)90181-7](http://dx.doi.org/10.1016/0092-8674(95)90181-7)
- Ribbeck, K., G. Lipowsky, H.M. Kent, M. Stewart, and D. Görlich. 1998. NTF2 mediates nuclear import of Ran. *EMBO J.* 17:6587–6598. <http://dx.doi.org/10.1093/emboj/17.22.6587>

- Riddick, G., and I.G. Macara. 2005. A systems analysis of importin- α - β mediated nuclear protein import. *J. Cell Biol.* 168:1027–1038. <http://dx.doi.org/10.1083/jcb.200409024>
- Rout, M.P., J.D. Aitchison, A. Suprapto, K. Hjertaas, Y. Zhao, and B.T. Chait. 2000. The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J. Cell Biol.* 148:635–651. <http://dx.doi.org/10.1083/jcb.148.4.635>
- Sakiyama, Y., A. Mazur, L.E. Kapinos, and R.Y.H. Lim. 2016. Spatiotemporal dynamics of the nuclear pore complex transport barrier resolved by high-speed atomic force microscopy. *Nat. Nanotechnol.* 11:719–723. <http://dx.doi.org/10.1038/nnano.2016.62>
- Schoch, R.L., L.E. Kapinos, and R.Y.H. Lim. 2012. Nuclear transport receptor binding avidity triggers a self-healing collapse transition in FG-nucleoporin molecular brushes. *Proc. Natl. Acad. Sci. USA.* 109:16911–16916. <http://dx.doi.org/10.1073/pnas.1208440109>
- Stewart, M. 2007. Molecular mechanism of the nuclear protein import cycle. *Nat. Rev. Mol. Cell Biol.* 8:195–208. <http://dx.doi.org/10.1038/nrm2114>
- Strawn, L.A., T. Shen, N. Shulga, D.S. Goldfarb, and S.R. Wentz. 2004. Minimal nuclear pore complexes define FG repeat domains essential for transport. *Nat. Cell Biol.* 6:197–206. <http://dx.doi.org/10.1038/ncb1097>
- Sun, C., G. Fu, D. Ciziene, M. Stewart, and S.M. Musser. 2013. Choreography of importin- α /CAS complex assembly and disassembly at nuclear pores. *Proc. Natl. Acad. Sci. USA.* 110:E1584–E1593. <http://dx.doi.org/10.1073/pnas.1220610110>
- Tauchert, M.J., C. Hémonnot, P. Neumann, S. Köster, R. Ficner, and A. Dickmanns. 2016. Impact of the crystallization condition on importin- β conformation. *Acta Crystallogr. D Struct. Biol.* 72:705–717. <http://dx.doi.org/10.1107/S2059798316004940>
- Timney, B.L., B. Raveh, R. Mironska, J.M. Trivedi, S.J. Kim, D. Russel, S.R. Wentz, A. Sali, and M.P. Rout. 2016. Simple rules for passive diffusion through the nuclear pore complex. *J. Cell Biol.* 215:57–76. <http://dx.doi.org/10.1083/jcb.201601004>
- Tokunaga, M., N. Imamoto, and K. Sakata-Sogawa. 2008. Highly inclined thin illumination enables clear single-molecule imaging in cells. *Nat. Methods.* 5:159–161. <http://dx.doi.org/10.1038/nmeth1171>
- Tu, L.C., G. Fu, A. Zilman, and S.M. Musser. 2013. Large cargo transport by nuclear pores: implications for the spatial organization of FG-nucleoporins. *EMBO J.* 32:3220–3230. <http://dx.doi.org/10.1038/emboj.2013.239>
- von Appen, A., J. Kosinski, L. Sparks, A. Ori, A.L. DiGuilio, B. Vollmer, M.T. Mackmull, N. Banterle, L. Parca, P. Kastriitis, et al. 2015. In situ structural analysis of the human nuclear pore complex. *Nature.* 526:140–143. <http://dx.doi.org/10.1038/nature15381>
- Vovk, A., C. Gu, M.G. Opferman, L.E. Kapinos, R.Y.H. Lim, R.D. Coalson, D. Jasnow, and A. Zilman. 2016. Simple biophysics underpins collective conformations of the intrinsically disordered proteins of the nuclear pore complex. *eLife.* 5:5. <http://dx.doi.org/10.7554/eLife.10785>
- Wagner, R.S., L.E. Kapinos, N.J. Marshall, M. Stewart, and R.Y.H. Lim. 2015. Promiscuous binding of Karyopherin β 1 modulates FG nucleoporin barrier function and expedites NTF2 transport kinetics. *Biophys. J.* 108:918–927. <http://dx.doi.org/10.1016/j.bpj.2014.12.041>
- Weis, K., C. Dingwall, and A.I. Lamond. 1996. Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities. *EMBO J.* 15:7120–7128.
- Yamada, J., J.L. Phillips, S. Patel, G. Goldfien, A. Calestagne-Morelli, H. Huang, R. Reza, J. Acheson, V.V. Krishnan, S. Newsam, et al. 2010. A bimodal distribution of two distinct categories of intrinsically disordered structures with separate functions in FG nucleoporins. *Mol. Cell. Proteomics.* 9:2205–2224. <http://dx.doi.org/10.1074/mcp.M000035-MCP201>
- Yang, W., and S.M. Musser. 2006. Nuclear import time and transport efficiency depend on importin β concentration. *J. Cell Biol.* 174:951–961. <http://dx.doi.org/10.1083/jcb.200605053>
- Yang, W., J. Gelles, and S.M. Musser. 2004. Imaging of single-molecule translocation through nuclear pore complexes. *Proc. Natl. Acad. Sci. USA.* 101:12887–12892. <http://dx.doi.org/10.1073/pnas.0403675101>
- Yoshimura, S.H., M. Kumeta, and K. Takeyasu. 2014. Structural mechanism of nuclear transport mediated by importin β and flexible amphiphilic proteins. *Structure.* 22:1699–1710. <http://dx.doi.org/10.1016/j.str.2014.10.009>
- Zachariae, U., and H. Grubmüller. 2008. Importin- β : structural and dynamic determinants of a molecular spring. *Structure.* 16:906–915. <http://dx.doi.org/10.1016/j.str.2008.03.007>