Review

Floppy but not sloppy: Interaction mechanism of FG-nucleoporins and nuclear transport receptors

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ABSTRACT

The nuclear pore complex (NPC) forms a permeability barrier between the nucleus and the cytoplasm. Molecules that are able to cross this permeability barrier encounter different disordered phenylalanine glycine rich nucleoporins (FG-Nups) that act as a molecular filter and regulate the selective NPC crossing of biomolecules. In this review, we provide a current overview regarding the interaction mechanism between FG-Nups and the carrier molecules that recognize and enable the transport of cargoes through the NPC aiming to understand the general molecular mechanisms that facilitate the nucleocytoplasmic transport.

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1. Introduction to the nucleocytoplasmic transport and the transport paradox

The exchange of biomolecules between the nucleus and the cytoplasm takes place through the nuclear pore complex (NPC). It is typically considered that molecules below 40 kDa are able to freely diffuse through the NPC. Molecules above this molecular weight require the interaction with nuclear transport receptors (NTRs) to successfully cross through the NPC (see review in Ref. [1]). However, this size limit appears to be rather a gradient, than a strict cut-off limit [2,3]. NTRs, independent of being loaded with a cargo, are able to cross the permeability barrier of the NPC which

is primarily formed by a group of proteins called FG-nucleoporins (FG-Nups). NPC crossing is an energy independent process but the directionality of the molecules being actively transported by NTRs depends on a RanGTP/GDP gradient formed between the nucleus and the cytoplasm [4]. RanGTP is more abundant in the nucleus, and can bind to import complexes, causing the cargo release in the nucleoplasm. Vice versa, when bound to export NTRs, RanGTP increases the affinity for export cargoes. The exported cargo can then be released from the export complex by RanGTP hydrolysis in the cytoplasm. The high concentration of RanGTP in the nucleus and of RanGDP in the cytoplasm is caused by the nuclear localization of RanGEF and RCC1, that exchange GDP for GTP, and the cytoplasmic localization of RanGAP, which hydrolyzes RanGTP into RanGDP. Cytoplasmic RanGDP is then recycled back to the nucleus by the NTR NTF2 (see review of RanGTP/GDP cycle in Ref. [5,6]).

The crossing of the NPC by NTRs with or without cargoes is a fast and selective process. Single molecule fluorescence microscopy

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experiments have enabled the measurement of the translocation time through the NPC of different cargoes by following the trajectories of labeled molecules (reviewed in Ref [7]). In this way, it was directly measured that molecules of different sizes can cross the NPC on millisecond timescales [8,9]. During this time, the NTRs interact with phenylalanine glycine motifs (FG-motifs) from the FG-Nups, on their way throughout the NPC barrier, undergoing multiple binding and unbinding events.

Using the latest published structure of the NPC [10], we can estimate the dimension of the NPC that is occupied by the FG-Nups of the central channel (Fig. 1A). We approximately measured a radius of 22.5 nm and a height of 24 nm for the inner ring of the NPC resulting in a calculated volume of around 38200 nm^3. Then we calculate the number of FG, GF and F residues, which are motifs or residues that could engage in the binding to NTRs, present in the disordered region of the FG-Nups based on the known stoichiometry at the NPC [11]. The estimated concentration of FGs at the central NPC barrier is ~160 mM. If we then consider in addition the GF residues (~80 mM) we would have a local concentration of binding sites of ~240 mM which increases up to ~260 mM if we consider the F residues only. Moreover, different *in vitro* equilibrium studies of FG-Nups and NTRs have reported high binding affinities from the nM the μM range [12–16]. As high affinity values are often associated with tight specific long-living complexes, it seems as a paradox that NTRs are not mainly stuck to the surface of the FG-Nup barrier, but can actually migrate through it very fast.

To further understand the nucleocytoplasmic transport, it is key to study the interaction mechanism between FG-Nups and NTRs. In this review, we provide an overview of the characteristic features of FG-Nups and NTRs with a focus on the latest studies on the interaction between FG-Nups and NTRs. This brings us closer to elucidating the nucleocytoplasmic transport process and to answer how fast and specific transport could be achieved. We will argue that this fundamental concept is largely robust and independent of what "model" one assumes for the structure of the actual, so far elusive permeability barrier.

2. Characteristic features of FG-nucleoporins and nuclear transport receptors

2.1. FG-nucleoporins, the molecular doorkeepers of the NPC

FG-Nups line the central channel of the NPC (Fig. 1B) and are composed of at least a folded domain, involved in anchoring the protein to the NPC, and an intrinsically disordered domain (IDD) protruding into the channel [17]. IDDs or whole intrinsically disordered proteins (IDPs, here for simplicity all called IDP) are proteins that lack a stable secondary and tertiary structure and are disordered under native conditions [18,19]. Typically, these structural features are associated with a high net charge and a low hydrophobicity, which allows them to remain unfolded in solution under physiological conditions [19]. FG-Nups however, contain on average high mean hydrophobicity, which is comparable to the levels of hydrophobicity present in globular proteins. FG-Nups also contain a very low net charge, which is lower than in typical disordered domains [20,21]. Furthermore, FG-Nups contain multiple F residues across their sequence, many of which are in direct neighborhood to a G (Fig. 1C) [17,22]. Based on the different amino acids flanking the F residues different types of FG-motifs have been classified: FxFG, GLFG, PxFG, SxFG and FG are the most frequently seen FG-motifs but less abundant FG-motifs have also been identified in some FG-Nups [21,23,24]. In many cases, however, it still remains elusive to what extent the nature of those repeats directly correlates to a specific function.

The primary sequence of the IDP region of FG-Nups does not have a high sequence conservation, which is often the case for disordered proteins that show a high amino acid substitution rate [25]. A bioinformatic study of FG-Nup sequences showed that the intrinsic disorder of FG-Nups is a conserved feature. However, FG-Nups also showed rapid evolution rates compared to other proteins. Another feature that seems to be conserved across FG-Nups is the hydrophilic inter-motif sequences of 10–20 amino acids present between the FG-motifs [23]. Those "linkers" also show a high amino acid substitution rate but maintain hydrophilic physicochemical properties [23].

The physicochemical properties of FG-Nups are directly related to a topic of central relevance to the field, i.e. how the actual permeability barrier is formed. The structure of the permeability barrier, which depends in part on the general question if FG-Nups interact with each other (are cohesive) or to what degree they rather repel each other (which could yield to brush type structures), still hasn't converged to a single model that the whole field can easily agree on [17,26–33]. At the core of this problem is that the study of the barrier *in situ* is extremely challenging because it is a dynamic structure with a size below the resolution limit for conventional light microscopy. Several high resolution cryo-electron tomography 3D maps of the NPC have meanwhile been presented [10,34]. While most folded proteins can already be fitted into the maps with secondary structure resolution, the actual transport conduit shows up as a large empty channel, because the dynamic structures are averaged out and remain invisible to electron tomography technology. In light of a lack of technology to directly visualize the permeability barrier and the inability to purify entire functional NPCs, scientists have designed various elegant approaches to study how the permeability barrier might be formed *in vivo*. FG-Nups have shown to be able to adapt several single and macromolecular states, ranging from collapsed to extended states as single proteins in solution, as well as brushes and films when mounted on surfaces [26,28,35–37]. In addition, FG-Nups can undergo transitions to supramolecular assemblies giving rise to tough hydrogels, as well as amyloid fibers [21,38,39]. Based on these different studies a large set of models have been developed. In this review, we will not get into the discussion of these models, and rather point the reader to interesting reviews on this topic [20,40,41]. Instead, we will focus on the one thing that most models agree on: The required interaction between NTRs and FG-Nups to cross the selective barrier of the NPC and the roughly known concentration of FGs in the permeability barrier. We will first describe the structural features of different NTRs that are required for the successful NPC crossing followed by an overview of what we learned from recent structural and biochemical studies about the interaction between FG-Nups and NTRs.

2.2. Structural features required for NPC crossing

Binding to NTRs is the major route for cargoes to get across the NPC. To do so, NTRs can recognize and bind nuclear localization and nuclear export signals (NLS and NES respectively) from different cargoes. Many of these carrier proteins belong to the Importin-β/β-karyopherin superfamily (for review see Refs. [42,43]). Members of this superfamily contain an overall high structural similarity but low, between 15 and 20%, sequence identity [44]. β-karyopherins like the import receptor Importin-β and Transportin or the export receptor CRM1 are composed of a tandem series of HEAT repeats (reviewed in Ref. [45]). HEAT is an acronym that comes from the different proteins where this motif was originally found: Huntingtin, elongation factor 3, the A subunit of the protein phosphatase 2A and the kinase TOR1. Each HEAT motif is composed of approximately ~30-40 amino acids that form two amphiphilic α-helices (A-helix and B-helix) (Fig. 1D) with one side containing hydrophobic residues and the other side being enriched in hydrophilic amino
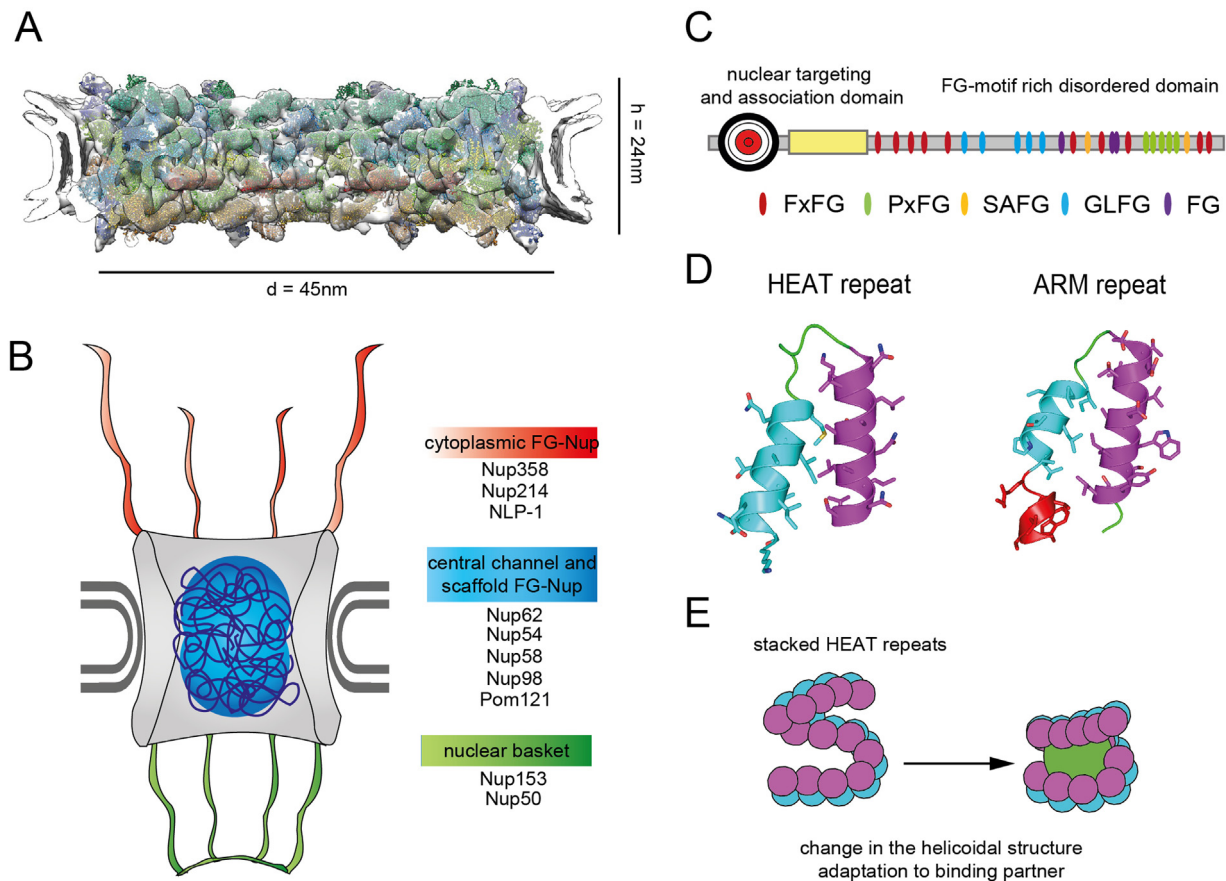
Fig. 1. Cartoon of the NPC and the main features of FG-Nups and NTRs. A) Transversal section of the NPC structure with an estimated diameter of 45 nm and height of 24 nm of the central channel of the NPC (PDB:5IJN overlaid with a cryo-EM subtomogram average EMD-8087 [10]). B) Cartoon of the NPC indicating the location of FG-Nups from the cytoplasmic (red) central channel (blue) and nuclear basket (green). C) Example of FG-Nup containing a folded nuclear targeting and association domain and a disordered FG-motif rich region. The different FG-motifs are color coded and distributed through the FG-Nup sequence. D) Left, secondary structure of the HEAT repeat 5 of Importin-β (PDB:1QGK [46]). Each HEAT repeat is formed by two helices, an A-helix (cyan) and a B-helix (purple) which are oriented in an antiparallel manner. Right, structure of the ARM repeat 5 of Importin-α (PDB:1EE5 [55]). Structure showing the helix H1 (red), H2 (cyan) and H3 (purple), the last two having an antiparallel orientation as it is the case for the HEAT repeats. E) Cartoon representing an NTR composed of stacked HEAT forming a helicoidal structure. The pitch of the helicoid changes when it is bound to a protein by mildly modifying the angle of the repeats.

acids. Both helices are linked by a turn and oriented in an antiparallel manner so that the hydrophobic surfaces of each helix are facing each other [46,47]. The tandem of HEAT repeats form a solenoid structure which contains the A and B helices arranged in the concave and convex surfaces respectively. This structural HEAT repeat composition provides the proteins with different levels of structural elasticity [48] which enables the NTRs to adopt different conformations resulting in curvature changes so that the solenoid adopts a helicoidal structure [42,49] (Fig. 1E). These structural changes have been reported to vary depending on the binding partner the karyopherin is bound to. For example, the level of compaction of NTRs like Importin-β increases when bound to the auto-inhibitory binding domain of Importin-α (IBB) when the import complex Importin-β/Importin-α/cargo is formed [46]. Structural studies have revealed that the binding of RanGTP to Importin-β also causes a conformational change in the helicoidal structure that inhibit the binding to Importin-α or cargoes by an allosteric mechanism [46,50]. The exportin CRM1 also undergoes a conformational change upon binding to RanGTP, in this case the conformational change upon RanGTP binding is required for the formation of the export complex. All these structural studies highlight the ability of NTRs to adapt and change the pitch of the helicoid upon binding to the different binding partners. It has been speculated that this structural rearrangements of the helicoid could store energy and would allow highly specific high energy interactions

and at the same time due to the high level of flexibility it enables big structural changes in the helicoid with small movements of the repeats [42,51]. In addition to these conformational changes, NTRs have also been shown to undergo structural fluctuations in which the level of exposed hydrophobic protein surface fluctuates reversibly depending on the composition of the medium, having a larger exposed hydrophobic surface in a hydrophobic environment [52]. This structural "breathing" may be useful to adapt to the different environments that the NTRs encounter i.e. a more aqueous in the cytoplasm and more hydrophobic in the transport conduit of the NPC.

A different example of an Importin molecule that crosses the NPC and is formed by stacked consecutive α-helical repeats is Importin-α. Importin-α recognizes and binds to the NLS from the cargoes functioning as an adaptor protein between the cargo and Importin-β. Importin-α is formed of consecutive armadillo repeats (ARM) [53]. ARM repeats consist of three α-helices (H1, H2 and H3) (Fig. 1D) where helices H2 and H3 are placed in an antiparallel manner, as it is the case for the A-helix and B-helix of the HEAT repeats, and the helix H1 is placed slightly perpendicular to H2 and H3. As for stacked HEAT repeats, the stacking of different ARM repeats form a solenoid structure in which the concave surface is involved in ligand binding [54,55]. ARM and HEAT repeats contain seven highly conserved hydrophobic residues that form the hydrophobic core of the repeat (for a comparative review between HEAT and
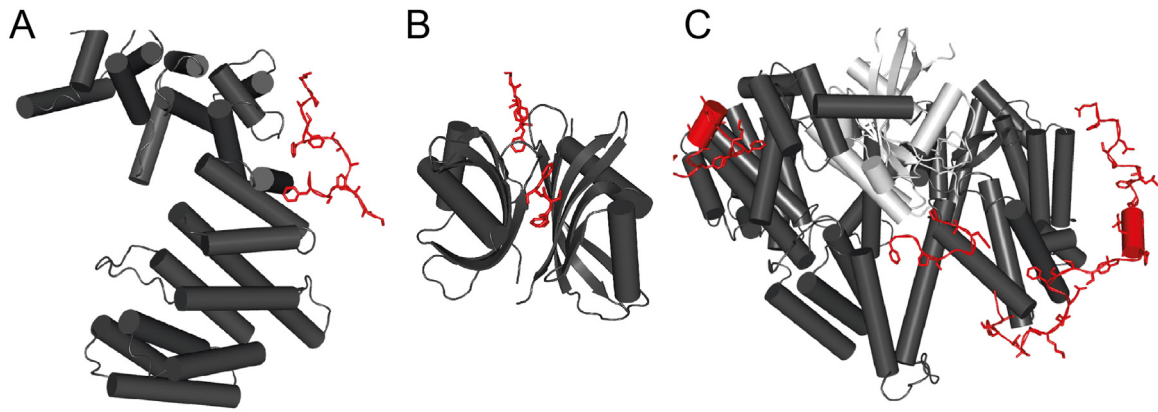
Fig. 2. Structures of NTRs bound to FG-motifs. A) Crystal structure of Importin-β (grey cartoon) bound to an FxFG motif (red) in between the HEAT repeats number 5–6 and 6–7 (PDB:1F59 [60]). B) Structure of NTF2 dimer (grey) bound to an FxFG peptide (red), the FxFG peptide binds to the hydrophobic groove formed in the binding interface between the NTF2 monomers (PDB:1GYB [57]). C) Crystal structure of CRM1/RanGTP (grey/light grey) bound to a 117 amino acid long C-terminal sequence of Nup214 (red). Nup214 wraps around the outer surface binding at different hydrophobic pockets connecting the C-and the N-terminal site of CRM1 (PDB:5DIS [66]).
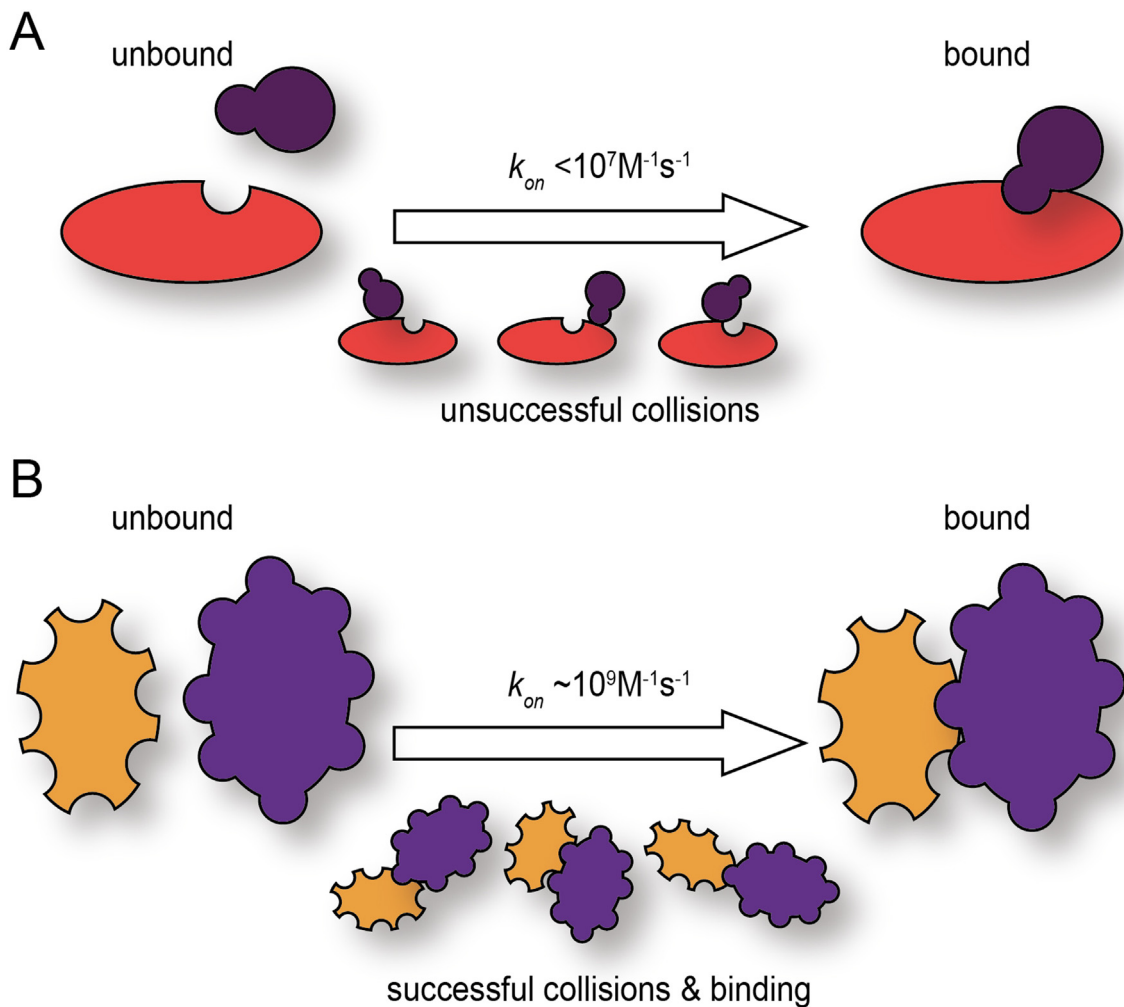

Fig. 3. Cartoon representing the different association rate constants (k_{on}) for different binding modes. A) Representation of the typical bimolecular binding that requires proper orientation of the binding partners to achieve a successful collision and binding. B) Representation of a diffusion limited protein-protein binding where the binding partners contain a highly reactive surface in which any collision encounter leads to a successful binding event.

ARM repeats see Ref. [56]). Nevertheless, not all the molecules that have the ability to cross the NPC are formed of solenoids forming helicoidal structures and are part of the β-karyopherin superfamily. The NTR nuclear transport factor 2 (NTF2) for example is a key component of the nucleocytoplasmic transport required for trans-

port of RanGDP to the nucleoplasm [57,58]. NTF2 is formed by two homo-dimers containing a bent β-sheet and three α-helices, one longer and two shorter ones [59]. The hydrophobic cavity formed between the homo-dimers forms in combination with its neighboring surface the interaction interface between NTF2 and RanGDP.

Despite the structural similarities or differences within NTRs, all transport receptors have the common ability to cross the NPC with a cargo by interacting with the FG-motifs of the FG-Nups located at the NPC. In the following sections, we will focus on describing the interaction mechanism between FG-Nups and NTRs and its implication in the context of the nucleocytoplasmic transport.

3. Interaction between FG-Nups and NTRs

3.1. FG-Nup NTR interaction, multiple sites with multiple strengths

Over the years numerous crystal structures of different NTRs with short peptides containing FG-motifs have revealed many structural details of the interaction between FG-Nups and NTRs. For example, it has been shown that Importin-β has at least two main hydrophobic FG binding pockets between the A-helix of HEAT repeat 5 and 6 and of 6 and 7 that bind to FxFG peptides [60] (Fig. 2A). The binding groove formed by the HEAT repeat 5 and 6 has also been crystallized bound to a peptide containing GLFG motifs [61]. Due to the structural features of many NTRs, of stacked repeats, there are multiple binding groves available to bind with FG-Nups. Molecular Dynamics (MD) simulations have shown that FG peptides engage with binding sites in agreement with previous X-ray crystallographic results and have in addition identified at least 6 more possible FG-motif binding sites in Importin-β [62], highlighting the multivalent nature of the interaction between FG-Nups and NTRs. Atomic force microscopy measurements of Nup153 and Importin-β showed that different binding pockets of the NTRs have different affinities for FG-Nups [63]. Moreover, there is also evidence that the conformational plasticity of NTRs can tune the interaction strength to FGs. The crystal structure of Importin-β bound to RanGTP and an FG peptide indicated that the structural changes caused in Importin-β upon RanGTP binding induced the movement of the A-helix from the HEAT repeat 5. This may facilitate the release of the FxFG peptide from the binding groove [60]. This effect on the FG binding to Importin-β/RanGTP complex was also observed by single-molecule force measurements [63]. These observations form the basis for a model in which RanGTP can facilitate Importin-β release from the NPC [33].

Not only NTRs formed out of stacked repeats are able to bind with FG-Nups. In the case of NTF2, it has been shown that the successful import of RanGDP into the nucleus was dependent on the ability of NTF2 to interact with FxFG containing nucleoporins [57]. The crystal structure of NTF2 with a short FxFG peptide showed that both Fs were buried in the hydrophobic groove formed between the two NTF2 monomers [64] (Fig. 2B). Further NMR studies confirmed the data obtained by X-ray crystallography and indicated an additional secondary FxFG binding site [65].

More recently, Port et al. showed a crystal structure of the exportin CRM1 bound to RanGTP and a 117 amino acid long disordered Nup214 [66]. Here, the C-terminal region of Nup214 appeared bound to the outer convex surface of CRM1 and it binds to the NTR with three FG regions in different hydrophobic pockets of CRM1 [66] (Fig. 2C). Interestingly, these FG regions interact with the N- and the C- terminal sites of CRM1 when bound to RanGTP and the authors suggested that this may be a mechanism of stabilizing CRM1/RanGTP export complex in a closed conformation and that Nup214 may be acting as a molecular clamp [66]. It is remarkable that the structure of Nup214 bound to CRM1/RanGTP showed high quality electron density in the FG-Nup binding sites. Notably, the structures did not reveal substantial secondary structure in the Nup, and it remains up to investigation, if the crystallization process trapped a specific state, or if indeed these regions lock specifically onto CRM1. Independent biochemical studies from different groups have indicated a possible different behavior of CRM1 export complexes compared to other NTR complexes when it comes to FG-Nup binding. In one of the studies the permeability properties of FG-Nup hydrogels, which have been formed under certain *in vitro* experimental conditions, for different NTRs and NTR complexes was tested [39]. Hydrogels formed of Nup214 showed that the complex of GFP-CRM1-NES bound to RanGTP penetrated the hydrogel minimally and the signal was mainly detected on the gel surface. In contrast, a deeper penetration of GFP-CRM1 alone or CAS/GFP-Importin-α bound to RanGTP was observed [39]. This would be in agreement with previous reports that suggested that Nup214 is a terminal binder for the CRM1 export complexes [67]. Moreover, similarly to the crystal structure of Nup214 and CRM1/RanGTP, the yeast homolog of Ran-binding protein RanBP3, Yrb2p that mediates the formation of export complexes also showed a similar binding with Xpo1p, the yeast homolog of CRM1, where two FG regions where also found to be bound to the N- and the C-terminal sites in the crystal structure [66,68]. Related to the export complex, it has also been shown that CRM1/NES/RanGTP bound to Nup358/RanBP2 can form a complex in which 2 FG-motif regions which are around 200 amino acids apart appear to engage with CRM1, potentially reducing the flexibility of the FG-Nup [69].

3.2. Insights into the binding of dynamic FG-Nup NTR complexes

IDPs are highly dynamic proteins in solution that are populating an ensemble of multiple conformations, their flexibility is often used by nature to form highly dynamic complexes (see review in Ref. [70]). Multiple conventional methodologies are very useful studying strong static complexes but the characterization of weak transient interactions with fast dissociation rate constants (k_{off}) are in some cases not easily detected.

Multi-parameter single-molecule FRET (Förster resonance energy transfer) (smFRET) is a highly sensitive fluorescent method that can investigate changes in distances and conformational dynamics of single molecules labeled site-specifically with a donor and an acceptor dye forming a FRET pair [71,72]. Any changes in the conformation that would lead to a change in the distance between the donor and the acceptor dyes can be monitored as a change in the efficiency of energy transferred from the donor to the acceptor dye. In addition, these measurements are also able to show if the complex under study is static (does not undergo any conformational change during the measuring time) or dynamic (multiple conformations take place during the measuring time leading to the detection of the average conformation). The smFRET study of the conformational state of FG-Nups shows that they are highly dynamic proteins [73]. Notable, the average distance distribution of the disordered ensemble and the average dynamics were retained even when the FG-Nups were bound to the folded NTR, a phenomen also in agreement with furtherMD simulations and nuclear magnetic resonance (NMR) [73,74]. It appears, that F residues of the FG-Nup necessary for the binding were preexposed on the surface and ready to bind the hydrophobic pockets of the NTR so that no major changes in the conformational ensemble of the FG-Nup are necessary to form a highly dynamic FG-Nup•NTR complex [73,74].

MD simulations of FxFG peptides binding to the hydrophobic groove between NTF2 monomers showed that not only the FG motifs bound NTF2 but also the spacer residues between the FG-peptide where able to interact transiently, possibly contributing to the binding of the neighboring FG-motif [75]. A notable finding from these MD simulations was also that the FG-motifs, while being bound to NTF2, showed some local motion along the binding site, which was described as sliding of the FG-motif at the NTF2 contact site. FG-motifs did not interact in a static defined position but had a high degree of motion while bound to the binding
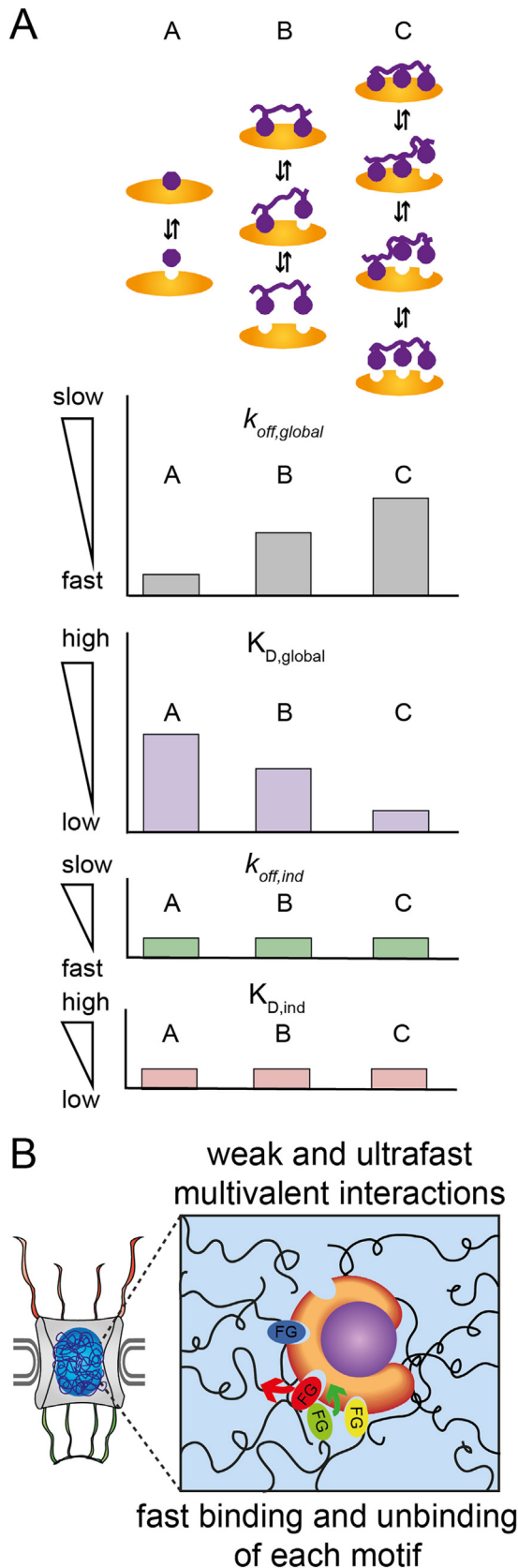
Fig. 4. Cartoon illustrating the differences between $k_{off,global}/k_{off,indiv.}$ and $K_{D,global}/K_{D,single}$ for molecules with different valency and cartoon of NTR crossing the NPC. A) Cartoon of 3 molecules (orange): a monovalent (molecule A), divalent (molecule B) and trivalent (molecule C) that bind to their binding partners (purple) that are also mono-,di- and trivalent. These three molecules contain the same binding sites and bind to the same motifs the only difference is the valency.

groove of NTF2 [75]. They also observed that FG-motifs sliding on the hydrophobic groove could slide out and then either slide back into the bound state or they would unbind from NTF2. With these results Raveh et al. proposed a slide-and-exchange mechanism of binding between FG-Nups and NTRs. In this slide-and-exchange mechanism the transition from a strongly interacting state into a weakly interacting state enables the hydrophobic pocket to be accessible for the return of an FG-motif from the weakly interacting state back into the strongly interacting state, or in other words, it enables easy displacement of an FG-motif by a different one [75].

3.3. Reconciling kinetics and equilibrium constants with nucleocytoplasmic transport time scales

The above described results highlight the multivalent nature of the binding between FG-Nups and NTRs, where multiple F residues can engage with different binding pockets of the same NTR and that binding of FG-motifs across the NTR surface is a common feature for FG-Nup NTR binding. Due to the formation of multivalent complexes most equilibrium measurements report on the avidity ($K_{D,global}$) of the formed complex, which is the result of the combination of the binding strengths of the binding events that take place simultaneously by the different FG-motifs of an FG-Nup and the different hydrophobic pockets of the NTR. This shall not be confused with the affinity ($K_{D,ind}$) which is here defined as the binding strength of a single binding event (single F to a single binding pocket).

Many binding reactions between proteins cover a wide range of association rate constants (k_{on}) ($\sim10^4$–$10^7\,M^{-1}\,s^{-1}$) [76]. Typically, fast protein-protein interaction values are frequently in the range of 10^6–$10^7\,M^{-1}\,s^{-1}$ [77]. This is because in order to achieve a successful binding, the right orientation between the binding partners is required and this may need several collisions between the proteins (Fig. 3A). Considering that $K_D = k_{off}/k_{on}$ one can calculate an upper limit for the $k_{off,max}$. From this $k_{off,max}$ value the dissociation half-life, which is equal to $0.693/k_{off}$, for a bound protein complex can be calculated. In order to calculate the dissociation half-life of a FG-Nup/NTR complex, if we take 1 μM as the avidity of an average FG-Nup/NTR complex and a k_{on} of $10^7\,M^{-1}\,s^{-1}$ one would obtain a $k_{off,max}$ of $10\,s^{-1}$ which corresponds to a dissociation half-life of $\sim$70 ms. Considering that during the crossing of NTRs through the NPC multiple binding and unbinding events must take place, the calculated half-life is orders of magnitude higher than what it is required in order to be in agreement with the measured NPC crossing times of molecules (approximately 5 ms) [8,9].

Stopped-flow spectroscopy association kinetic experiments enabled to measure k_{on} values for different FG-Nups and NTRs near the diffusion limit ($\sim10^9\,M^{-1}\,s^{-1}$), a result in agreement with Brownian Dynamics (BD) simulation experiments [73]. Diffusion limited protein binding however, can only occur when both proteins are able to bind upon any collisional encounter (Fig. 3B).

We also assume that the k_{on} for each motif molecules is equal and we exclude cooperative binding in our model. Because of this, the binding affinity ($K_{D,single}$) and dissociation rate constant ($k_{off,single}$) for each motif individually is the same. However, the $k_{off,global}$ for molecule A will be faster than for the divalent or the trivalent molecule because molecule B and C require that more motifs unbind from the entire molecule in order to achieve the dissociation of the complex. In this way, the $k_{off,global}$ increases (slower unbinding) with increasing valency. Due to the increase in the $k_{off,global}$ the molecules with higher valency will have a higher avidity (lower $K_{D,global}$) even if the affinity of each motif ($K_{D,single}$) is equal in all the molecules. B) Cartoon illustrating the crossing of an NTR through the NPC barrier interacting with multiple FG-motifs from different FG-Nups. Due to the low affinity of each binding pocket of the NTR and the fast binding and unbinding kinetics multiple binding and unbinding events can take place during a single NPC crossing. The arrows next to the FG-motifs indicate a FG leaving the NTR binding pocket (red) and a FG that will bind to the same pocket (green).

How is this possible? The orientation and association kinetics of binding proteins can be favored by long-range electrostatic interactions [78,79]. Association kinetic experiments at different ionic strength conditions enable the estimation of a basal k_{on}, at infinite ionic strength where all the electrostatic interactions are shielded. The estimated k_{on} basal for Nup153 and Importin-β was largely unaffected by the shielding of electrostatic forces. BD simulation experiments,where the electrostatic contribution during the simulation was excluded, agreed with the stopped-flow kinetic results. Therefore, the remaining explanation is the existence of a highly reactive surface of the FG-Nup, leading to a k_{on} limited by the translational diffusion of the proteins where binding takes place everytime when the molecules come into contact. The FG-Nup/ NTR dis-so-ci-a-tion half-life us-ing the mea-sured k_{on} in this case would be of 0.7 ms. Moreover, BD simulations also tested the effect on the k_{on} in the absence of apolar desolvation, which accounts for the hydrophobic effect or the segregation of water molecules from the non-polar surfaces. When the hydrophobic effect was not considered in the simulations, the k_{on} was affected by more than two orders of magnitude indicating a strong relationship between water solvation and fast binding between F residues of FG-Nups and hydrophobic pockets of the NTRs [73].

Due to the multivalent nature of the interaction between FG-Nups and NTRs, any measured k_{off} will represent the dissociation rate constant of the entire molecule ($k_{off,global}$ of Fig. 4A), meaning that each individual binding site must be unbound for the complex to fall apart. Due to this, the measured k_{off} for a single site ($k_{off,ind}$ of Fig. 4A) will be orders of magnitude faster than for the entire molecule [80]. Instead of using the measured avidity value of 1 µM we can also make use of the affinity value for a single site, which was suggested to be in the µM to mM range [15,73,74,81]. For an affinity of 1 mM and a k_{on} of $10^9\,\mathrm{M^{-1}\,s^{-1}}$ the dissociation half-life would be of ∼0. 7 µs, a value that would easily enable an NTR to cross the NPC undergoing multiple binding and unbinding events during a single transport event.

These findings indicate that the interaction of multiple low affinity binding events between individual FG-motifs and NTR binding pockets,with ultrafast kinetics, are able to form highly dynamic complexes that would allow the fast crossing of NTRs through the NPC (Fig. 4B).

4. Conclusions and future perspectives

The mechanistic understanding of the nucleocytoplasmic transport is limited by the complexity of the NPC and the lack of experimental methods to directly monitor the conformation and dynamics of FG-Nups *in situ* during nucleocytoplasmic transport.

In this review, we have summarized the main characteristics of the major players of the nucleocytoplasmic transport: FG-Nups and NTRs. We have briefly highlighted the main features that are required from both parts to perform their function. We have also summarized the latest results obtained in the field that increase the understanding of how fast yet specific transport across the NPC can be achieved. Multivalent interaction of multiple low affinity ultrafast binding and unbinding events taking place between the FG-Nups and the NTRs that allow the rapid exchange of FG-motifs from the different NTR binding pockets [73,75]. Despite the advances in the understanding of the nucleocytoplasmic transport many questions remain open, besides the fundamental question on how the permeability barrier is built. For example, how do sequence heterogeneity correlate with specific function, specific NTR interactions, or potentially spatially distinct mechanisms in the NPC is still not fully understood, and complicated by the effect, that the permeability barrier is highly robust, and functions even when multiple proteins are removed [82,83]. Thus several regulatory mechanism still remain to be discovered.

Another remaining open question derived from the previously described BD simulation experiments brings the attention to the study of the effect of the solvation by water, the most abundant molecule in the cytoplasm, which may play a crucial role in the interaction mechanism between FG-Nups and NTRs and other protein-protein interactions. It will also be interesting to elucidate what are the implications of the structural modulation of NTRs in the different stages of the nucleocytoplasmic transport. How could the modulation of the number and the type of binding grooves that are available to bind FG motifs, due to the conformational changes of the NTR, can affect the kinetics affecting the dwell times at the NPC. This could regulate the NTR release from the permeability barrier or could even modulate the interaction with different FG motifs in different FG-Nups that could even potentially shape different transport routes through the nuclear pore complex.

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