Thermodynamic characterization of the multivalent interactions underlying rapid and selective translocation through the nuclear pore complex

**Ryo Hayama¹,†, Samuel Sparks²,†, Lee M. Hecht¹, Kaushik Dutta³, Jerome M. Karp², Christina M. Cabana¹, Michael P. Rout¹,*, David Cowburn²,*

¹Laboratory of Cellular and Structural Biology, The Rockefeller University New York, New York, USA, ²Depts. of Biochemistry and of Physiology & Biophysics, Albert Einstein College of Medicine, Bronx, New York, USA, ³ New York Structural Biology Center, New York, New York, USA.

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To whom correspondence should be addressed: David Cowburn, ²Depts. of Biochemistry and of Physiology & Biophysics, Albert Einstein College of Medicine, Bronx, New York, USA, Tel.: (718) 430-8621; Email: cowburn@cowburnlab.org

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**ABSTRACT**

Intrinsically disordered proteins (IDPs) play important roles in many biological systems. Given the vast conformational space that IDPs can explore, the thermodynamics of the interactions with their partners is closely linked to their biological functions. Intrinsically disordered regions of Phe–Gly nucleoporins (FG Nups) that contain multiple phenylalanine–glycine repeats are of particular interest, as their interactions with transport factors (TFs) underlie the paradoxically rapid yet also highly selective transport of macromolecules mediated by the nuclear pore complex (NPC). Here, we used NMR and isothermal titration calorimetry (ITC) to thermodynamically characterize these multivalent interactions. These analyses revealed that a combination of low per-FG motif affinity and the enthalpy–entropy balance prevents high-avidity interaction between FG Nups and TFs underlie the paradoxically rapid yet also highly selective transport of macromolecules mediated by the nuclear pore complex (NPC). Here, we used NMR and isothermal titration calorimetry (ITC) to thermodynamically characterize these multivalent interactions. These analyses revealed that a combination of low per-FG motif affinity and the enthalpy–entropy balance prevents high-avidity interaction between FG Nups and TFs, while the large number of FG motifs promotes frequent FG–TF contacts, resulting in enhanced selectivity. Our thermodynamic model underlines the importance of functional disorder of FG Nups. It helps explain the rapid and selective translocation of TFs through the NPC and further expands our understanding of the mechanisms of “fuzzy” interactions involving IDPs.

Intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs), constitute ~30-40% of the human proteome and are involved in many protein signaling and regulation processes (1). IDPs/IDRs can interact with their targets with high specificity, and yet often with low affinity and high reversibility. There is a broad interest in quantifying the thermodynamic driving forces governing IDP interactions. Many IDPs undergo a disorder-to-order transition upon binding to their targets (2), while others form ‘fuzzy complexes’ (3) where significant residual disorder is maintained in the interacting state. Due to their essential role in many biological processes, a better understanding of the energetics of IDP interactions is needed (4).

Many IDP interactions are mediated by short linear motifs (SLiMs) that engage with receptor molecules. Because SLiMs do not have extensive interaction interfaces to induce high enthalpy, SLiM-containing IDPs often utilize multiple motifs to participate in multivalent interactions (5). This mitigates the conformational entropy loss upon binding, and
enhances individually weak monovalent interactions, resulting in higher overall affinity (avidity) and specificity (6,7). One example of an IDR that utilizes multiple short linear motifs are disordered domains of Phe-Gly nucleoporins (FG Nups) which line the central channel of the nuclear pore complex (NPC) (Figure 1A). FG Nups typically contain 5-50 FG motifs separated by spacer residues (8). These FG repeat regions collectively form a selectively permeable barrier for macromolecular transport through the NPC. Specific cargos can translocate rapidly and efficiently through the NPC by binding to cognate transport factors (TFs). TFs make contacts with multiple FG repeat motifs, allowing them to diffuse rapidly and selectively through the central channel (9,10). The passage of non-specific macromolecules, which lack similar interactions, is impeded (11). Selectivity for TFs arises, in part, because the surface of TFs contains multiple hydrophobic pockets for FG motif interaction. However, the binding of multiple FG motifs to a TF containing multiple interaction sites could lead to high avidity complexation and thus long residence times, and such interactions would be incompatible with the rapid transport rates observed in vivo (~5-10 ms) (12,13). The thermodynamics underlying FG-TF interactions that enable TFs to translocate rapidly yet selectively through the NPC (often referred to as the “transport paradox” (14)) has been poorly characterized, and so it remains unclear what mechanism prevents TFs from “sticking” to multiple FG motifs through such avidity effects.

RESULTS

We characterized the interaction between FG Nups and NTF2 with a series of constructs containing a variable number of FSFG motifs by sequentially replacing FSFG motifs with SSSG motifs (Figure 1B). This F->S mutation is known to negate interaction with TFs (15,16). We characterized their interaction with NTF2 by both NMR and ITC. We first titrated NTF2 against each FSFG construct. Control titrations with the SSSG6 variant yielded no observable heat (Figure 1C), no chemical shift perturbations (CSPs), and no peak intensity changes (Figure 1E), confirming that the SSSG motif and the spacer regions display no affinity to NTF2. In contrast, heteronuclear single quantum coherence (HSQC) peaks corresponding to FSFG motif residues underwent selective chemical shift changes upon increasing concentration of NTF2, although the spacer residues remained largely unperturbed, consistent with previous observations (Figure 1F) (17,18).

Affinity measurements by NMR and ITC were consistent for each construct (Figure 2A, 2B, S1, S2, and Table S1), demonstrating they reported on the same chemical reactions. Data from both methods fit to a simple binding model and did not require use of more complex equilibrium models. The affinity of the single FG-motif construct (FSFG1) to NTF2 was very weak ($K_D = 4.35$ mM observed by NMR). This is consistent with the affinity measured for the interaction between importin-β and a human Nup153 F->A mutant with only a single phenylalanine residue ($K_D = 7.3$ mM) (18). Though the accumulated strength of multivalent interactions could substantially increase avidity (6), the affinity of NTF2 for the divalent construct FSFG2 was only ~2 fold greater than for the monovalent FSFG1. As the FSFG valency increased further, the apparent affinity continued a modest increase, plateauing after four motifs; even twelve FSFG repeats (FSFG12) exhibited a similar affinity to that of FSFG6 (Figure 2B, Table S1). Thus, multivalency had a much weaker effect on the overall affinity than expected.

Importantly, titrations with FSFG1 yield the per-motif affinity to an NTF2 molecule (which contains multiple FG contact sites). For multivalent FSFG constructs, this per-motif affinity would remain constant in the absence of cooperativity, and the overall molecular $K_D$s would reflect the ensemble of all equivalent FSFG motifs interacting and rebinding to NTF2 before escaping to bulk by diffusion (19). Our experiments lacked temporal resolution to fully describe such microstates in the ensemble. Thus, we emphasize that the measured $K_D$s account for contributions made by all underlying microscopic interaction equilibria with distinct
Thermodynamics of FG-Transport Factor Interaction

individual \( K_D \)s, including ones involving simultaneous engagement of multiple FG motifs to a single NTF2. Affinity measurements indicate that FSFG displays modest avidity to NTF2, implying that even the divalenty interacting state is at best scarcely, or transiently populated.

The interaction of a TF with any of the FG motifs in a construct localizes the rest of the FG chain into the vicinity of the TF, increasing the concentration of FG motifs available for subsequent interactions or rebinding events. If the additional enthalpy from the intramolecular interaction significantly exceeds the loss of conformational entropy of the chain, strong avidity would result (6,20). To assess how changes in the local concentration of FSFG motifs would affect the overall affinity, we tested a set of FSFG\(_2\) variants, altering the distance between the two FSFG motifs (Figure 1B, right and Table S3). The affinity for NTF2 decreased toward that of FSFG\(_1\) with increased inter-motif distance (Figure 2C). Separations larger than two repeats induced no significant binding enhancement. Thus, the modest affinity gain observed in Figure 2B can be described as the result of an increased intramolecular contacts induced by the local concentration of FSFG motifs (Figure 2C). This excludes an interaction mechanism where each FSFG motif binds independently to a different molecule of NTF2, where no change in the measured affinity would be expected. Our results also suggest that FSFG motifs proximal to the initially bound FG motif more effectively contribute to the enhancement of overall affinity than those that are distant. Importantly, the effect of large local concentration increases the probability that an FG molecule will be in the ‘interacting’ state through rebinding (Figure 2D) (19).

To explore the effect of increased local concentration on the NTF2 interaction surface, we mapped the NTF2 residues interacting with our FSFG constructs by NMR. As observed previously (21), CSPs were observed at, but not limited to, the crystallographically observed site around Phe5 of NTF2 (22) and extended along the hydrophobic groove bridging the two monomers (Figure 3A and Figure S4). TROSY-HSQC titrations with FSFG\(_1\), FSFG\(_3\), and FSFG\(_6\) showed the same residues of \([\text{\textsuperscript{2}H,\text{\textsuperscript{15}N}}]\) NTF2 were involved in the interaction (Figure 3B) and the affected residues shifted linearly regardless of the valency of the FSFG constructs, indicating a single binding mode regardless of FG valency. To determine the number of spectrally distinguishable binding modes contributing to the CSPs, singular value decomposition (SVD) analysis was used. (23). SVD analysis from each titration produced only two non-noise components (free and interacting), indicating an apparent two-state reaction (Figure S5). We calculated NTF2 site-affinity for all significantly perturbed residues, collectively representing the interaction surface (Figures 3A, S4-S7), using the raw and SVD noise filtered data, the latter of which yielded better precision in the derived \( K_D \) (Figure S7).

Surprisingly, for \([\text{\textsuperscript{2}H,\text{\textsuperscript{15}N}}]\) NTF2 titrations with varying concentration of FSFG\(_1\), fitting of the 61 residues displaying significant CSPs to a global \( K_D \) value yielded 19.9 ± 0.4 mM (Figures S7A). This dissociation constant is likely a lower-bound in our assay conditions, as we were limited by the solubility of the FSFG\(_1\) construct. Higher solubility was achieved using a single repeat FSFG peptide; nevertheless, a similar result to that of FSFG\(_1\) was obtained with this peptide (Figures S4-7). These titrations probed saturation of spatially distinct, independent interaction sites.

Our results indicate that residues on NTF2, in different areas of the molecule (Figure S4E), have individual \( K_D \)s of similar values and can be fit to a single global value with high precision (Figure S7). Our previous simulations demonstrated that FSFG motifs make multiple transient and exploratory contacts with the interaction surface of NTF2 (24). Taken together, the single binding mode detectable by SVD suggests that the exchange rates of FG motifs contacting the NTF2 surface are faster than the experimental detection (~ms) such that the observed CSPs reflect a single time-averaged binding mode. In other words, while multiple interactions and rebinding events occur rapidly, we only detect average bulk behavior in the timescale of our experiments. The difference in observed \( K_D \) between this titration of NTF2 sites and the titration of \([\text{\textsuperscript{15}N}}]\) FSFG\(_1\) (above) (Figure 2B and Table S1) further suggests that the NTF2 interaction surface is composed of multiple
Thermodynamics of FG-Transport Factor Interaction

contact sites. This difference is illustrated by a simple kinetic modelling of FSFG1-NTF2 interaction (supporting information, and Figure S8).

The NTF2 per-site $K_D$ decreased with FSFG valency (Figures S6 and S7), with FSFG$_3$ and FSFG$_6$ displaying ~7.5 and ~60-fold increases, respectively, compared to FSFG1. As there was neither an emergence of additional residues involved in the interaction at higher valency (Figure 3B) nor additional binding modes appearing by SVD analysis (Figure S5), multivalency appears to simply induce an additive effect. The increased frequency of contacts formed enhances specificity but without significantly changing the mode of interaction. Taken together, FSFG motifs interact with a large surface patch with near uniformly distributed low interaction potentials, lacking a well-defined energy minimum.

To understand what factors prevent strong avidity, we examined the energetics of interactions by ITC. The increase in negative $\Delta G$ with higher valency was modest, with only a ~27% gain with FSFG$_6$ relative to FSFG$_1$ (Figure 4A and Table S1). We observed enthalpy-driven interactions for each FSFG construct. Since the interactions are both low-affinity and low-enthalpy, we had to balance the sensitivity and the degree of complexation in order to maintain the data quality. As a result, these ITC experiments were performed at low $c$-values ($c = n[M_0]/K_D$, where $[M_0]$ is the concentration of the titrand and $n$ is the molecular stoichiometry). However, low $c$-values are not necessarily limiting, as long as sufficient saturation is achieved and the value of $n$ or of $\Delta H$ is separately determined by other methods (25,26). Moreover, NMR measurements can cross-validate the $K_D$ determined by ITC. Due to lack of sufficient saturation, the lower affinity constructs (FSFG$_1$-FSFG$_3$) were excluded from thermodynamic analysis (Table S1). For FSFG$_4$-FSFG$_6$, we set $n = 1$ based on dynamic light scattering (DLS) titration measurements which indicated that FSFG$_6$ and NTF2 predominantly form a complex of 1:1 molecular stoichiometry at high sample concentrations (Figure S9). This confirms that the local concentration effect favors intra- rather than inter-molecular FG Nup-TF interaction (Figure 2C). For FSFG$_{12}$, DLS indicates that two molecules of NTF2 can interact simultaneously with a single FSFG$_{12}$ polypeptide (Figure S9), thus, we set $n = 2$. This indicates that after some number of FSFG motifs (greater than six) a second TF molecule would be able to interact, likely due to the loss of steric and/or excluded volume constraints. Importantly, the $K_D$s of FSFG$_6$ and FSFG$_{12}$ were similar, suggesting that the linkage between the two adjacent TF-interactions is minimal beyond a certain distance. Thus, the affinity of TF interactions with FG Nups can remain weak even in the NPC milieu where large number of FG motifs are clustered on single FG Nups.

The overall heat released increased with valency, as expected (Figure S2). A prominent negative $\Delta H$ for higher valency constructs further indicates that more frequent intramolecular contacts form (Figure 4B and Table S1) as the local concentrations of FSFG motifs increase (supporting information, Figure S10, and Table S3). Notably, though hydrophobic associations are usually driven by a favorable change in solvent entropy; FSFG-NTF2 interactions instead display a “non-classical” hydrophobic interaction driven by enthalpy (27). This type of interaction has been attributed to water molecules at hydrophobic surfaces participating in weaker hydrogen bonding relative to bulk solvent and therefore their displacement (by potentially enthalpically favorable contacts between the interaction partners) is overall enthalpically favorable (27). Our result suggests an unexpected and important role of protein hydration and solvent dynamics in FG-TF interaction, raising an interesting question on the origin of the enthalpy in FG-TF interactions.

Many multivalent interactions involve ligands with relatively rigid spacers, at lengths similar to the distances between receptor interaction sites, resulting in large cumulative enthalpy with minimal loss in conformational entropy, and so strong avidities (28). In our system, the magnitude of $\Delta H$ that increased with increasing FSFG valency was almost perfectly offset by an increasing $-T\Delta S$ (Figure 4C), exhibiting apparent enthalpy-entropy compensation (29). This trend again reflects the
additive nature of the system (30) i.e. the frequency of qualitatively similar contacts increases with valency. We conclude that this modest avidity is maintained because the effect of increased local concentration of FSFG motifs around NTF2 is countered by the entropic costs of restricting the conformational freedom of the chain, preventing stable multi-bound states (Figure 4D).

**DISCUSSION**

The inherent issue in IDP interactions is how they cope with the loss of conformational entropy upon binding. High entropy state of IDPs may appear as a considerable energetic obstacle to their functions. Here, we propose instead that FG Nups exploit this entropy loss for functionality. The weak single FG motif affinity and the enthalpy-entropy balance operative at higher valency states prevent strong avidity - enabling rapid and reversible interactions. Simultaneously, enhanced specificity is achieved through high local concentration of FG motifs, permitting higher frequency of contacts (longer global residence time at a high rate of exchange). We propose this combined effect as a reasonable explanation to the “transport paradox” (14).

Our results also provide new details for NPC transport models that describe the NPC as a virtual energetic gate (31,32) By providing microscopic details, our work along with other recent studies indicate that collective low-affinity FG-TF interactions are functionally relevant to the transport mechanism (17,18). This contrasts models assigning emphasis on high avidity ones (33).

Importantly, high concentration of FG motifs in the NPC and their fluctuations around a TF likely facilitate TF hopping between different FG Nups. When multiple FG motifs from two or more different FG Nups simultaneously engage to a TF at different sites (i.e. intermolecular interaction), the interactions at those sites are very weak ($K_D \sim$ millimolar) and individual FG motifs would have short residence times, τ, on a single interaction site ($\tau = 1/k_{off}$, where $k_{off}$ is the off-rate constant). τ is estimated to be $<1$ µs assuming $K_D = 1$ mM and based on limits of the on-rate constant, $k_{on}$, from stopped-flow analysis ($\sim 10^9$ M$^{-1}$S$^{-1}$) (18). Thus, the lifespan of such a multivalent complexation would be short (i.e. a few µs). When multiple FG motifs from the same chain engage to an NTF2 molecule at different sites (intramolecular interaction), the enthalpy-entropy balance disfavors divalent and higher valency (high avidity) interactions because of the entropic penalty associated with the intramolecular interaction. In both scenarios, FG motifs would easily undergo rapid exchange due to competition from other FG Nups. Thus, it appears that the combination of multivalency and low per NTF2-site affinity provides a balance between the selectivity achieved through increased avidity and the fast exchange rates required for transport. While other effects such as sequence heterogeneity among FG Nups, end-tethering to the NPC scaffold, and inter-FG Nup cohesiveness (34-36) may additionally modulate the FG-TF interactions, entropic resistance to static complexes would still play a fundamental role in the selective translocation.

Our study also expands our mechanistic understanding of “fuzzy” interactions involving IDPs (3). The design principle characterized here could be extended to chemical applications, such as artificial molecular sorting machines (37) and steric inhibitors utilizing multivalency (6).

**EXPERIMENTAL PROCEDURES**

**Plasmids**

FSFG$_6$ and SSSG$_6$ DNA constructs codon-optimized for bacterial expression were synthesized (IDT). Gene fragments from the synthesized plasmids were ligated into pET21b or pET24a vector. The FSFG$_{12}$ plasmid was created by inserting a FSFG$_6$ fragment into a FSFG$_6$ plasmid. Other FSFG variants were created by site-directed mutagenesis from the two parental constructs. For NTF2, the genomic sequence from *S. cerevisiae* was used. All the proteins were C-terminally tagged with hexa-histidine Their primary sequences are listed in the supporting information.

**Protein purification**

FSFG constructs were purified as described previously (17) (see supporting information) and NTF2 was expressed and purified in an identical manner except that urea was removed from all
the buffers. [U-$^{15}$N] and [U-$^{13}$C, $^{15}$N] samples were prepared using M9 media containing $^{15}$N NH$_4$Cl and [U-$^{13}$C] glucose (Cambridge Isotopes) as needed. For [H, $^{15}$N] NTF2 the sample was prepared in M9 media containing 99% D$_2$O and $^{15}$N NH$_4$Cl with natural abundance glucose as the sole carbon source (38). The purification of NMR labeled samples was identical to the protocol for their natural abundance counterparts. However, for [H, $^{15}$N] NTF2, additional steps were needed to exchange the unobservable N$^2$H to N$^1$Hs (see supporting information). Protein concentrations were measured by BCA assay kit (ThermoScientific) following the product instructions. Amino acid analysis was conducted for select FSFG constructs and NTF2, and the measured concentrations were consistent with those measured by BCA assay.

**Nuclear Magnetic Resonance**

All NMR experiments were conducted on Bruker spectrometers at 800 MHz and 298 K with samples prepared in Buffer A (20 mM HEPES-KOH, pH 6.8, 150 mM KCl, 2 mM MgCl$_2$), unless otherwise noted. NMR data was analyzed using NMRPipe (39) and CCPNMR Analysis (40). Titration experiments were performed in Buffer A using a fixed concentration of $^{15}$N labeled sample and by preparation of separate samples for each titration point. Sample concentrations for [H, $^{15}$N] NTF2 were either 250 μM or 500 μM while concentrations for [$^{15}$N] FSFG$_3$ ranged from 20 μM to 120 μM. A majority of our experimental conditions were within the optimal range of titrant concentrations suggested by Granot (41) with the exception for titrations involving FSFG$_1$ as well as [H, $^{15}$N] NTF2 titration with FSFG$_3$ due to the very low affinity nature and limited solubility of both components. As a result, derived $K_{D}s$ for those titrations should be considered as lower-bound estimates. Chemical shifts for the FSFG residues and all the assigned NTF2 residues were extracted from each titration point and the CSP were calculated as $\sqrt{(δ^{15}N \times 0.11)^2 + (δ^{1}H)^2}$, where $δ^{15}$N and $δ^{1}$H are the $^{15}$N and $^{1}$H chemical shift changes with respect to the free state (42). The CSP was plotted as a function of titrant concentration and fit to a standard equation (42)

$$\Delta \delta = \frac{\Delta \delta_{max} ((P_0 + X + K_D) - \sqrt{(P_0 + X + K_D)^2 - (4 \times P_0 \times X)})}{2 \times P_0}$$  

(equation 1)

where $\Delta \delta_{max}$ is the maximum chemical shift change, $P_0$ is the fixed protein concentration and $X$ is the titrant concentration. The fitting was performed by Prism (Graphad Software) with the above equation to derive $K_D$. This general equation was also used to calculate the final saturation using the protein concentrations at the last titration point, i.e. 

$$\text{Final saturation} = \frac{(P_0 + X_{max} + K_D) - \sqrt{(P_0 + X_{max} + K_D)^2 - (4 \times P_0 \times X_{max})}}{(2 \times P_0)}$$

Final saturations for the ITC experiments were calculated in the same manner. Assignments of NTF2 used standard triple resonance approaches (43,44) including HNCA, HNCACO, HNACAB, CBCACONH, HCNA, and HNCOCA.

The single repeat FSFG peptide was synthesized and reverse-phase HPLC purified by the Proteomic Resource Center at Rockefeller University. A stock solution was prepared by dissolving the lyophilized sample into Buffer A.

**Isothermal titration calorimetry**

All ITC experiments were conducted in Buffer A at 25 °C on a MicroCal Auto-iTC200 (Malvern). In all experiments, the concentration of FSFG construct was fixed and NTF2 was titrated in (see Table S2). See supporting information for additional details.

The raw heat evolution data were integrated and analyzed by the ITC module (Malvern) within the Origin program (OriginLab). Heats of dilution of FSFG constructs and NTF2 were separately measured for each experiment (see Figure S3) before curve fitting. The corrected curves were fitted using Origin software, using a non-linear least squares algorithm based on a model with a single class of independent, equivalent sites to determine the molecular stoichiometry $n$, the $K_D$, and the enthalphy ($\Delta H$). For FSFG$_4$ - FSFG$_6$, $n$ was set at 1 and for FSFG$_{12}$, $n$ was set at 2, based on the molecular stoichiometry determined by DLS.
(See Fig. S9) while the $K_D$ and the $\Delta H$ were allowed to freely float in the fitting procedure. For the constructs with very low affinity and low enthalpy (i.e. FSFG$_1$ – FSFG$_3$), fixing $n$ at 1 yielded poor fits to the experimental data and curve fitting also failed to converge on single $n$ and $\Delta H$ values when $n$ was freely fit. This is due to the low degree of complexation (Table S2) limited by the low affinity and the low enthalpy of interactions, as well as the limitations of NTF2 solubility. Thus, for those constructs, $n$ and $\Delta H$ could not be determined separately, though during the fitting procedure the products of $n$ and $\Delta H$ ($n\Delta H$, total enthalpy change) can be calculated and were reported since they are known to stay approximately normal in low $c$-value systems (25,26). However, $K_D$s are insensitive to errors in $n$ at low $c$-values (26) and were reliably obtained for those constructs by ITC as demonstrated by the cross-validation with the NMR results (Figure 2B and Table S1).

Gibbs free energy ($\Delta G$) and its entropic component ($T\Delta S$) were calculated using the following equations:

$$\Delta G = RTln(K_D) \quad \text{(equation 2)}$$

$$\Delta G = \Delta H - T\Delta S \quad \text{(equation 3)}$$

where $R$ is the ideal gas constant and $T$ is the absolute temperature (298 K). Means and standard errors of the mean (SEMs) of the thermodynamic parameters were calculated for each FSFG construct and are reported in Table S1.

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**Author contributions:** †R.H. and S.S contributed equally to this work. R.H., S.S., D.C. and M.R. conceived the project. R.H., S.S., L.H. and K.D. performed experiments and R.H., S.S., L.H. and C.C. prepared the samples. R.H., S.S., J.M.K., D.C., analyzed the data and R.H., S.S. and M.R. made figures and wrote the manuscript with D.C.
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FOOTNOTES

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Figure 1: Systematic analysis of multivalent interaction between FSFG constructs and NTF2. (A) Schematic diagram of the NPC-mediated transport. (B) Design of FSFG constructs with varying degrees of valency (left) and with varying distance between two FSFG motifs (right). (C to F) Characterization of the interaction between NTF2 and SSSG₆, FSFG₁, FSFG₃, and FSFG₆ by ITC (C and D) and NMR HSQC titration experiments (E and F). The ITC curves represent the baseline-corrected, normalized, and reference subtracted dataset (See Figure S3 for details and Table S2 for experimental conditions). The [¹⁵N] SSSG₆ (100 µM) HSQC spectra (E) was performed at 900 MHz, in the presence (Blue) and absence (Red) of 150 µM NTF2. The [¹⁵N] FSFG₁ (120 µM), [¹⁵N] FSFG₃ (40 µM), and [¹⁵N] FSFG₆ (20 µM). The concentrations for NTF2 vary with the maximum concentrated tested indicated by the color bar. Titrations were acquired at 800 MHz.
Figure 2: Quantitative analysis of NTF2 interaction with various FSFG constructs. (A) Titration curves derived from NMR titration experiments in Figure 1F, displaying changes in chemical shifts (Δδ) versus concentration of NTF2. The solid line represents the fit to Equation 1 used to determine the apparent dissociation constant (K_D) (see Figure S1 and Table S3 for final saturation achieved). (B) K_D values for each of the FSFG1-FSFG12 constructs (Fig. 1B, left) by NMR and ITC. Standard errors of the curve fitting and standard errors of the mean are plotted for NMR and ITC, respectively (see Table S1). (C) K_Ds determined by NMR titration experiments of FSFG constructs with varying distance between two FSFG motifs (Fig. 1B, right). Concentrations of [\(^{15}\)N] FSFG constructs were fixed at 60 µM for all titrations. The infinity sign indicates the K_D for FSFG1 construct. (D) Schematic diagram of the local concentration effect promoting the ‘interacting state’.
Figure 3: NMR analysis of NTF2. (A) Chemical shift perturbations at ~1:1 molar ratio of $[^{2}H,^{15}N]$ NTF2:FSFG$_6$ mapped onto the structure of NTF2 (PDB: 1GYB). Several selected residues are shown in ball and stick representations with their associated titration curves and $K_D$ (locally fit) from the reconstructed dataset after noise filtering by SVD. See Figures S4 to S7 for additional information. (B) Chemical shift perturbations observed at indicated concentrations for each assigned residue in NTF2 from experiments with FSFG$_6$ (top), FSFG$_3$ (middle) and FSFG$_1$ (bottom) as the titrant.
Figure 4: Thermodynamics of FSFG-NTF2 interaction. (A) Gibbs free energy ($\Delta G$) for the interactions between FSFG constructs and NTF2. (B) Gibbs free energy ($\Delta G$), enthalpy ($\Delta H$) and entropy ($-T\Delta S$) for the interactions between FSFG$_{4}$-FSFG$_{12}$ constructs and NTF2 measured by ITC. (C) Enthalpy-entropy compensation curve for interactions for the aforementioned constructs, with a linear fit (See Figure S11). (D) Schematic diagram of the enthalpy-entropy balance that prevents high avidity interactions.
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