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Computational and Biochemical Identification of a Nuclear Pore Complex Binding Site on the Nuclear Transport Carrier NTF2

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Nuclear transport carriers interact with proteins of the nuclear pore complex (NPC) to transport their cargo across the nuclear envelope. One such carrier is nuclear transport factor 2 (NTF2), whose import cargo is the small GTPase Ran. A domain highly homologous to the small NTF2 protein (14 kDa) is also found in a number of additional proteins, which together make up the NTF2 domain containing superfamily of proteins. Using structural, computational and biochemical analysis we have identified a functional site that is present throughout this superfamily, and our results indicate that this site functions as an NPC binding site in NTF2. Previously we showed that a D23A mutant of NTF2 exhibits increased affinity for the NPC. The mechanism of this mutation, however, was unknown as this region of NTF2 had not been implicated in binding to NPC proteins. Here we show that the D23A mutation in NTF2 does not result in gross structural changes affecting other known NPC binding sites. Instead, the D23 residue is located in an evolutionarily important region in the NTF2 domain containing superfamily, that in NTF2, is involved in binding to the NPC.
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Nuclear pore complexes (NPCs) regulate the traffic of proteins and nucleic acids into and out of the nucleus. The NPC is a large proteinaceous structure spanning the two lipid bilayers of the nuclear envelope and is one of the largest macromolecular structures in cells. In both yeast and mammals, approximately 30 proteins assemble to form the core NPC and most, if not all, of these nucleoporins are present in 8–48 copies per NPC.[1,2] The nuclear transport of most cargo is mediated by transport carriers that bind the cargo, facilitate interactions with the NPC, move the transport complex through the NPC, and finally release their cargo on the other side of the NPC. Transport carriers bind to nucleoporins containing phenylalanine/glycine (FG) motifs, and 11 out of 30 vertebrate nucleoporins contain more than five of these FG repeats.[2] Often this FG motif is found within a larger structural motif such as FxFG or GLFG, which are two of the most common motifs found (reviewed by Suntharalingam & Wente).[3]

NTF2 was one of the first nuclear transport carriers discovered and is responsible for importing RanGDP into the nucleus.[4–7] NTF2 is conserved in all eukaryotes; the yeast and rat homologues share 40% sequence identity and rat NTF2 can functionally replace the essential yeast NTF2 protein.[8] The NTF2 sequence (14 kDa) also comprises a

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Abbreviations used: NPC, nuclear pore complex; NTF, nuclear transport factor; NXF, nuclear export factor; ET, evolutionary trace; rms, root-mean-square.

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conserved domain that is found in several larger proteins with widely differing functions. NTF2 domain containing proteins include delta-5-3-ketosteroid isomerases, scylatone dehydratases, and the beta subunit of ring hydroxylating dioxygenases.[9] This family also contains the Ras GAP SH3-domain-binding protein (G3B2), which contains an RNA binding domain in addition to the NTF2 domain.[10] The NTF2 domain is also found in nuclear carriers that, like NTF2, interact with the NPC. These carriers comprise the NXF (nuclear export factor) family of export carriers that includes the critical mRNA export carrier TAP which forms a heterodimer with p15/NXT1.[11,12] The NTF2 domain of NXF members has been shown to be involved in both hetero-dimerization and interactions with the NPC.[11,13]

Previously, we reported that a point mutation (D23A) in NTF2 increases this carrier's binding to the NPC, resulting in this mutant carrier importing its cargo (RanGDP) faster and more efficiently than wild-type NTF2.[14] However, the molecular mechanism of this mutation was unknown, as the crystal structure of NTF2 bound to a peptide containing FG repeats revealed that a region of NTF2 far removed from D23 interacted with nucleoporin FG repeats.[15] Here we report that the region around D23 constitutes an additional NPC binding site.

Results

Stewart and co-workers identified on NTF2 a hydrophobic cavity, centered around W7, that binds FG-containing nucleoporin sequences.[15–17] We found previously that a mutation of residue D23 in NTF2 to an alanine results in increased binding of NTF2 to the NPC.[14] Surprisingly, the NTF2 crystal structure indicates that the D23 residue is spatially far removed from this W7 hydrophobic cavity shown to bind NPC proteins.[17]

To determine if the D23A mutation possibly results in global structural changes in NTF2 that affect the W7 region, we crystallized the D23A mutant to compare its structure to the wild-type NTF2. Notably, however, the D23A mutation had little effect on the overall structure of NTF2 with a rms deviation of 0.3399 Å for the C^{α} backbone (Figure 1A and C). The major alteration was at the mutated residue itself, showing the expected change from aspartic acid to alanine (Figure 1B). The substitution of an alanine for aspartic acid 23 disrupts the hydrogen bond between D23 and H66 seen in the wild-type structure. It has been shown that disruption of this bond (H66A) has introduced structural changes at some distance from H66.[18] In particular, regions 20–30, 55–68, and 90–95, in the H66A mutation were shown to have a higher rms deviation as compared to wild-type than other parts of the protein. In the D23A mutant, regions 52–68 and 90–95 also had particularly large rms deviations (Figure 1C and D). Additionally, regions 108–110 and the C-terminal residues had large rms deviations as compared to the rest of the protein. Interestingly, the D23A mutation had very little effect on the C^{α} positions of residues 20–30 compared to wild-type NTF2, unlike that seen in the H66A mutation. However, the effect on rms deviation of residues 52–68 was much more dramatic in the D23A mutation than seen in the H66A structure (Figure 1C and D). The removal of the negatively charged D23 resulted in a ∼1 Å shift of residues 62–66 allowing the hydrophobic side-chains of I64 and H66 to maximize their interactions with the hydrophobic pocket lined by numerous aromatic residues (Figure 1B–D). The removal of the negative charge has also increased the hydrophobicity of this area of the protein. Thus, these results demonstrated that the D23A mutation does not increase NTF2's binding for the NPC *via* alterations in the hydrophobic cavity around W7, a region known to bind nucleoporins.

We and others have noted that the D23 residue (and its equivalent) is extremely conserved in the NTF2 protein across species and also in the NTF2 domain superfamily.[14,17] We performed computational analysis to determine if any of the residues surrounding D23 are also conserved, possibly indicating functional significance. We utilized the evolutionary trace (ET), which uses sequence homology and structural data to determine potential functional regions of a protein.[19–21] An ET is performed by grouping members of a protein family into classes based on a sequence identity tree. Residues that are invariant within every class, but are variable between the classes, are termed "class specific" or trace residues. Trace residues are then mapped on the crystal structure of the protein of interest. Clusters of class-specific residues on a protein indicate an evolutionarily privileged site, which is likely to be important functionally.[22,23]

An ET of the NTF2 family identified D23 as a very highly evolutionary conserved residue. It was one of the first amino acids to appear on the ET (rank = 10). Notably, H66 was identified much later in the ET (rank = 23), indicating that D23 is more functionally conserved and may have an additional role other than its structural role in forming an H-bond with H66. An isoleucine residue (I64) located adjacent to D23 also appeared early in the ET (rank = 14). Notably, residue I64 of NTF2 has been previously identified as contributing to RanGDP binding.[24] In general, the ET revealed an evolutionary conserved cluster of residues located in the D23 region, indicating that this region is likely to be functionally important (Figure 2A). In addition, the ET revealed two other evolutionary conserved regions, one that corresponds to the dimerization domain of NTF2 and another that corresponds to the Ran binding domain of NTF2 (Figure 2B). Interestingly, the W7 region of NTF2 implicated in binding nucleoporins was much less conserved than these other three regions. W7 did not appear until much later in the trace, indicating that D23 and I64 are more conserved than W7 throughout the entire NTF2 family.

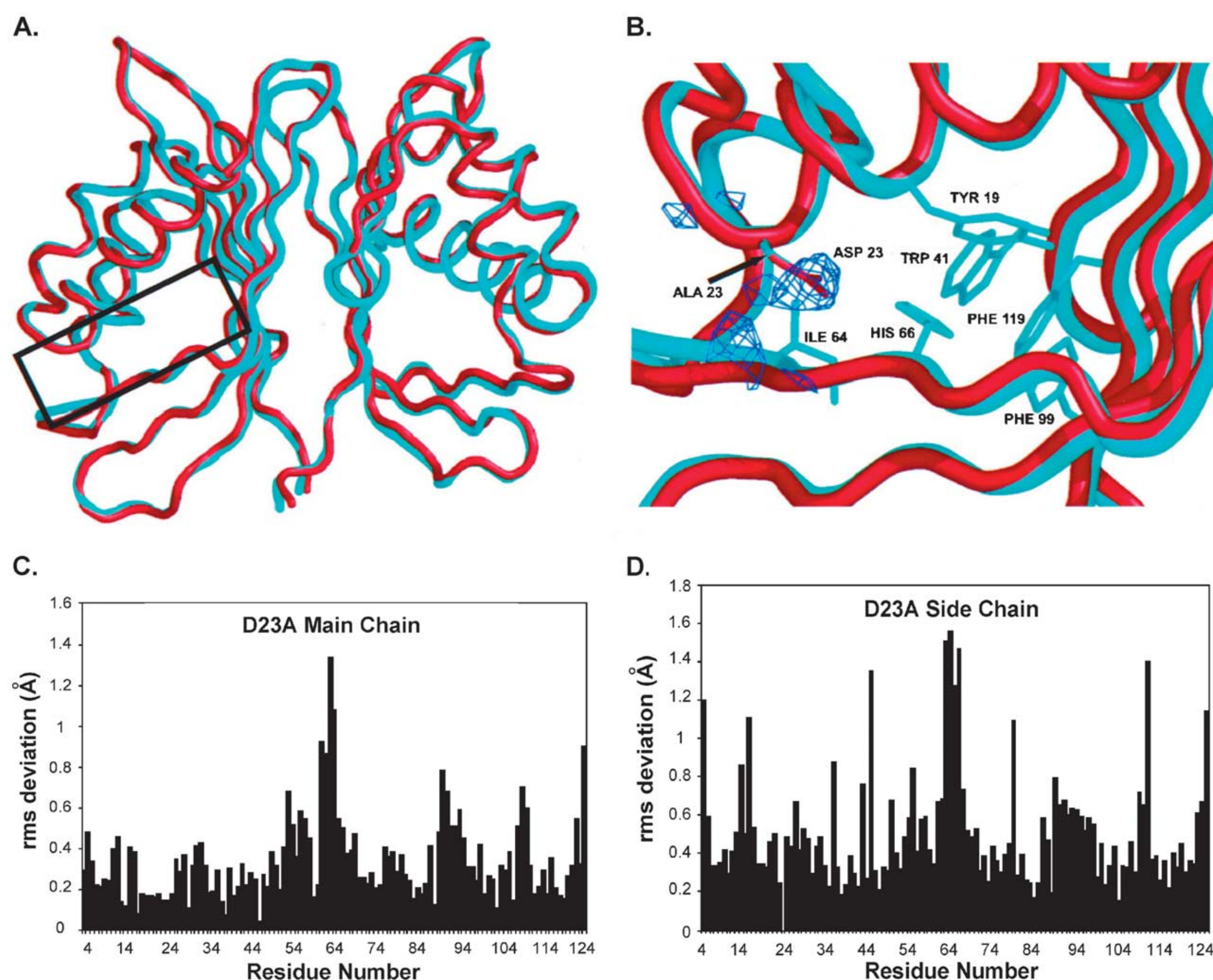
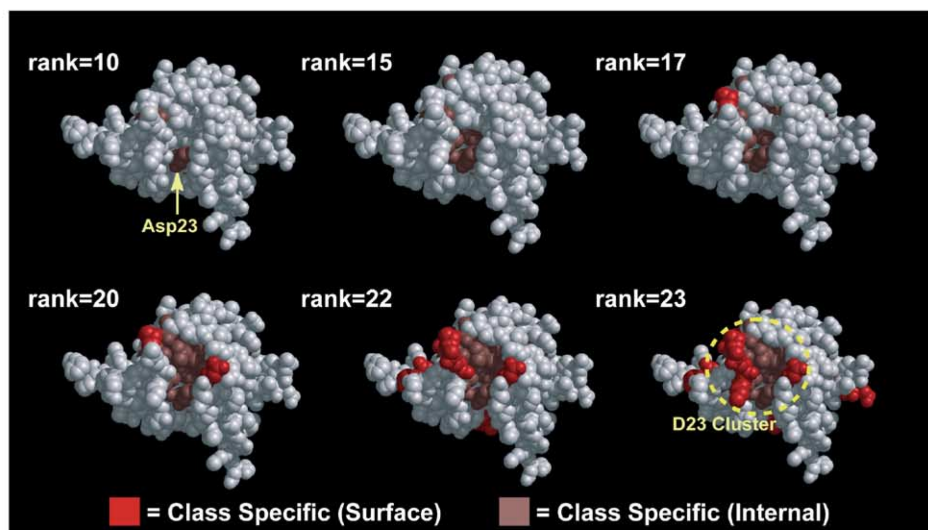


Figure 1. The D23A mutation does not alter the structure of NTF2. Wild-type and D23A NTF2 were expressed in *Escherichia coli* and purified as described.[33] Wild-type NTF2 crystals were grown as described.[34] Small crystals of the wild-type NTF2 were used to cross-seed the crystals of D23A. The best D23A crystals were grown in 10% (w/v) PEG 8000, 25 mM magnesium acetate, and 100 mM sodium acetate (pH 4.7). The crystals were harvested after one week, transferred to cryo-protectant containing mother liquor plus 20% (v/v) MPD, and flash frozen at 100 K. A data set to 2.5 Å resolution was collected using a MAC Science DIP2030 image plate system with double mirror optics mounted on a Rigaku rotating anode (Cu Kα), and processed and merged with DENZO and SCALEPACK, respectively.[35] The space group was $P2_12_12_1$ with unit cell dimensions $a=55.8$ Å, $b=56.9$ Å, $c=85.9$ Å, $\alpha=\beta=\gamma=90°$. An initial structural model was obtained by rigid body minimization of the wild-type NTF2 structure (PDB ID, 1OUN) with the D23A data set. Model building was done using O.[36] The model was refined in CNS.[37] Water molecules were fitted using the CNS automatic water_pick function and visually inspected using O. The final R_{cryst} value was 21.9% with an R_{free} of 26.1%. Coordinates have been deposited under RCSB PDB accession code 1U5O. A, C^{α} superposition of wild-type (wt) (red) and D23A (blue) NTF2 homodimers. The black box represents a zoom window whose contents are shown in B. B, Zoom view of the pocket where the D23A mutation occurs. The electron density represents an F_o-F_c map contoured at 2σ showing difference density around the D23A residue. The illustration was made using PyMol (http://www.pymol.org). C, Plots of the rms deviation for the C^{α} backbone for the D23A mutant compared with wild-type NTF2 for one of its two chains. The overall rms deviation was 0.3399 Å for the entire molecule. D, A plot of the rms deviation for the side-chains of the D23A mutant compared with wild-type NTF2 for one of its chains. The overall rms deviation was 0.5050 Å for the entire molecule. It was not possible to compare side-chain deviations at the mutated residue so its value is zero.

To determine if other conserved residues in the D23 cluster affect NPC binding, we made a series of point mutations of amino acid residues identified in the ET. To test NPC binding in the context of intact nuclear envelopes and NPCs, immunofluorescence microscopy studies were done with Flag-tagged wild-type NTF2 and the point mutants. We have shown that His and Flag epitopes have minimal effect on the function of NTF2 by the fact that tagged NTF2 can support nuclear import *in vitro* and *in vivo*.[14] HeLa cells were permeabilized with digitonin and washed, then wild-type or mutant FLAG-NTF2 was incubated with the cells. The cells were then washed, fixed and visualization of the bound NTF2 was done with an anti-FLAG antibody.

As previously shown, the D23A mutant binds the NPC to a greater extent than wild-type (Figure 3). To determine the contribution of I64 to NPC

A.



B.

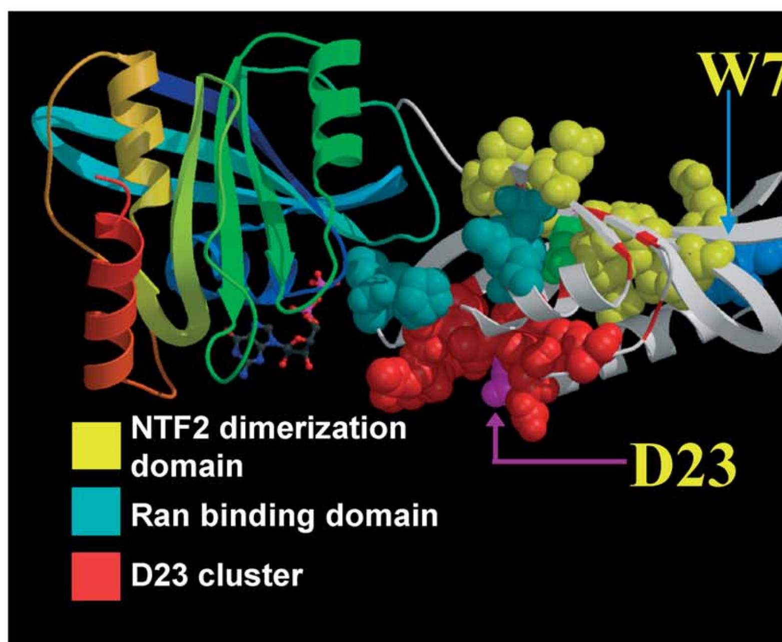


Figure 2. The evolutionary trace identifies a functionally conserved region (D23 cluster). The standard FASTA tool was used to gather sequence fragments that matched NTF2. The list was truncated when repetitive proteins were observed. Sequence alignment and dendrogram construction was carried out with the GCG multiple sequence alignment tool PILEUP. The evolutionary trace was run with the program TRACE. The input consisted of the multiple sequence alignment (MSA) PILEUP, the crystallography structure of NTF2 and RanGDP was the reference protein in the MSA. The output of the ET is a list of conserved and class-specific residues broken down as internal or external positions on the protein surface. The latter distinction is defined by the percentage solvent exposure of the side-chain as calculated by the ACCESS program. The TRACE output produces files readable by RasMol. A, The ET defines D23 as a highly conserved residue and defines the D23 cluster on the NTF2 structure. B, Mapping of trace residues onto the NTF2 monomer bound to RanGDP (structure from Stewart *et al.*[24]). The ET defined three functionally conserved regions on NTF2. The NTF2 dimerization region is depicted in yellow. The Ran binding domain is in light blue. The D23 cluster is in red. The W7 residue is colored blue and D23 is purple.

binding, we made two mutations of this residue. We substituted either an alanine for the isoleucine, or a glutamine instead, so that the side-chain would remain approximately the same size. I64Q NTF2 showed dramatically less binding to the NPC than wild-type NTF2, and the I64A mutation abolished all visible binding of NTF2 to the NPC (Figure 3). In experiments not shown, we determined by gel

filtration chromatography that mutations of I64 did not impair homodimerization of NTF2 and both mutants eluted with the expected dimeric size of 28 kDa, indicating that the I64 mutations do not cause a gross loss of structural integrity.

We also tested the ability of these mutants to bind recombinant NPC protein and Ran. As previously determined, wild-type NTF2 and the D23A mutation both bound RanGDP and Nup153C (a fragment of an FG repeat containing nucleoporin), with D23A showing similar Ran binding but enhanced binding to Nup153C (Figure 4).[14] Confirming the results of others, we found the W7A mutation decreased the binding of NTF2 significantly to Nup153C.[15,16] The double mutation of D23A/W7A did not compensate for the loss of nucleoporin binding caused by the mutation of W7, indicating that the enhanced binding of the D23A mutation is not seen when the W7A mutation is present. We also mutated D23 to asparagine to eliminate the acidic charge while leaving the side-chain with relatively the same size. We found that this mutation behaved like D23A, in that it increased binding of NTF2 to the NPC and NPC proteins without affecting the ability of NTF2 to bind RanGDP (Figure 4, and data not shown). These results indicate that it is the loss of a negative charge at residue D23 that results in increased NPC binding of NTF2 rather than a change in the size of the D23 side-chain (see Discussion).

Interestingly, if I64 was mutated to an alanine (I64A), binding to Nup153C was almost completely abolished while NTF2 binding to Ran was greatly reduced but still apparent. In contrast, we found that I64Q loses the ability to bind Ran completely, and has much reduced, but still apparent, binding to Nup153C.

Discussion

Previously, we reported that a D23A mutation in NTF2 results in increased binding of mutant NTF2 to FG repeat containing nucleoporins and the NPC.[14] The residue D23, however, is some distance away from the hydrophobic region centered around W7, originally reported to be the sole NPC binding site of NTF2.[15,16,25] Surprisingly, when we crystallized the D23A NTF2 protein and compared its structure to that of the wild-type structure, there was little change in the backbone of the mutant (Figure 1). Thus, the crystal structure of D23A NTF2 revealed that the mutation did not cause gross structural changes capable of increasing the binding affinity of NTF2 for nucleoporins *via* alteration of the W7 region of the molecule. However, localized rms deviation of residues surrounding the hydrophobic cavity near D23 revealed that the removal of the negatively charged D23 resulted in a ∼1 Å shift of residues 62–66 allowing the hydrophobic side-chains of I64 and H66 to maximize their interactions with the hydrophobic pocket (Figure 1B–D). The removal of the negative charge has also increased the hydrophobicity of this area of the protein. Thus, the D23A mutation results in a subtle structural change maximizing hydrophobic interactions in the pocket and thus resulting in tighter binding to NPC proteins.

The H66A mutation engineered by Clarkson *et al*. resulted in a decrease in Ran binding.[18] Based on the crystallography data of NTF2-RanGDP interaction it was proposed that this loss of Ran binding to the H66A mutation is due to a change in residues D92 and D94.[24] Surprisingly, the D23A mutation showed a similar shift in residues 90–95 (Figure 1C and D), but we did not observe a decrease in Ran binding. This may be due to the increased hyrdophobicity of D23A NTF2 in the cavity surrounding F72 of Ran that may compensate for the loss of binding in residues 92 and 94.

NTF2 comprises a highly conserved evolutionary protein motif found in numerous dissimilar proteins. The structures of NTF2 and scylatone dehydrogenase are remarkably similar even though their sequence homology is quite low, indicating that they diverged early in evolution.[17,26] Interestingly, D23 is conserved in scylatone dehydratase and is associated with its enzymatic activity, indicating that this conserved residue likely serves different functions in different proteins.[17] The ET utilizes sequence, rather than structural,

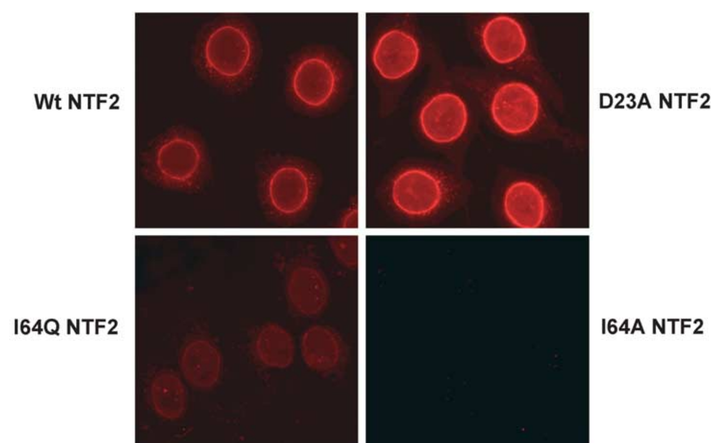


Figure 3. Mutations in the D23A cluster reduce binding to the NPC. Immunofluorescence microscopy was performed as described.[14] Digitonin-permeabilized HeLa cells were incubated with 0.25 μM (dimer) wild-type or mutant NTF2. After washing and fixation, the bound NTF2 was detected by indirect immunofluorescence microscopy with an anti-FLAG (Sigma) antibody followed by a TRITC-labeled anti-mouse second antibody (Jackson Labs).

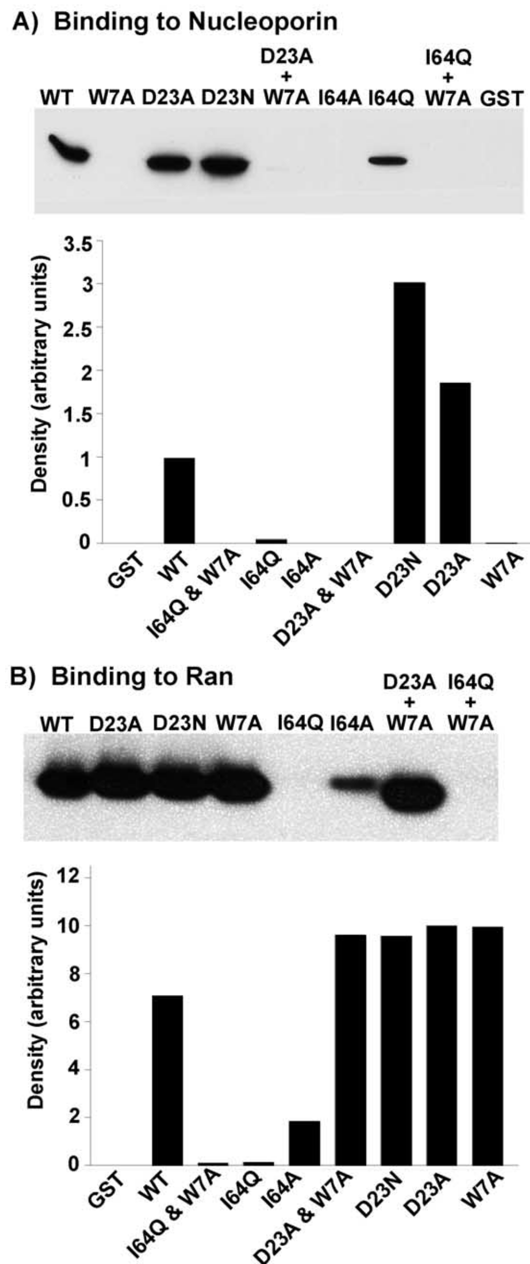


Figure 4. Mutations in the D23 cluster affect binding to nucleoporins and RanGDP. Wild-type or mutant His-Flag NTF2 was purified[14] and incubated with purified GST-Ran[38] or GST-Nup153C.[39] (A) GST-pull-downs were with 2.5 μM GST-153C mixed with 2.5 μM (dimer) human wild-type or mutant NTF2 and 30 μl of glutathione beads in 200 μl of transport buffer (20 mM Hepes (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT) with 0.1% (v/v) Tween for 45 minutes at room temperature. After the binding reaction, the beads were collected by centrifugation (13,000 rpm for 30 seconds at room temperature). The supernatant was discarded and then beads were resuspended in SDS-PAGE sample loading buffer (0.5 M Tris, 6% (w/v) SDS, 0.1 M DTT, 5% (v/v) glycerol, and 0.1% (w/v) bromophenol blue). Samples were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-FLAG antibody (Sigma) to detect FLAG-NTF2. Lower panel, quantification of densitometry of NTF2 binding to Nup153. (B) GST-pull-downs with GST-Ran were conducted in the same manner as (A). Lower panel, quantification of NTF2 binding to Ran.

homology so scylatone dehydrogenase and other enzymatic NTF2 family members were not included in the ET. However, our ET results indicate that this region is still functionally conserved in transport carrier members of the family, such as TAP and p15, and in the G3B2 protein which has no known carrier function.

It was not surprising that D23 (rank = 10) was identified by the ET, since many structures that have an NTF2 fold contain a conserved H-bond between the D23 and H66 residues of NTF2, which is probably important for structural integrity.[17] However, H66 was not identified until much later in the trace (rank = 23 *versus* rank = 10 for D23), indicating that D23 may be functionally conserved for reasons other than forming the H-bond with H66. Additionally, computational analysis by the ET method revealed a clustered region of conserved amino acid residues located near D23 (Figure 2), indicating that this larger region is also likely to be functionally important. In particular, I64 (rank = 14) was identified by the ET as being functionally important. Mutational studies based on the ET results identified this region in NTF2 as being involved in both RanGDP binding and binding to the NPC. The D23A mutation may result in tighter nucleoporin binding by causing subtle changes that allow the region to increase in hydrophobic interactions with FG repeats.

There are multiple lines of evidence that the W7 (F5 in yeast) region of NTF2 is involved in binding to the nuclear pore complex. Mutational studies have conclusively shown that W7 binds to FG repeats and is involved in NTF2 transport.[16,25,27] In addition, structural work in yeast has shown F5, Q43 and Q45 bind FG repeats and Q43 and Q45 are completely conserved as Q45 and Q47 in humans.[15] Interestingly, the ET does not recognize this region as being functionally conserved. The ET ranks of Q45 and Q47 are 22 and 26, respectively, and W7 appears much later with an ET rank of 44. These results indicate that this region may not be functionally important throughout the entire NTF2 family. In NTF2 specifically, it has been proven that this region is involved in binding to nucleoporins.[16] Our results indicate that the W7 region must be functional for the D23 binding region to interact with nucleoporins, since the D23A mutation did not rescue the NPC binding of the W7A mutation. Conversely, however, an intact W7 residue cannot compensate for an I64A mutation.

Previous crystallography and mutational studies identified the hydrophobic region of NTF2 clustered around W7 as being important for binding to the NPC.[15,16] However, numerous lines of evidence have indicated that there are additional regions of NTF2 that can interact with the NPC. Ribbeck *et al*. found that the rate of transport through the NPC by the W7A NTF2 was highly diminished compared to wild-type NTF2; however, the mutant still moved through the NPC significantly faster than GFP, a protein of similar size.[27] They concluded that there must be other NPC binding sites still functional in

the W7A NTF2 mutant. Importantly, an NMR study of the binding of a FG-containing peptide to NTF2 revealed three regions of interaction on the NTF2 molecule.[28] These investigators concluded that the majority of FG repeat binding took place at the W7 region of NTF2, but that there are also two additional regions of binding. One of those regions overlaps significantly with the D23 cluster we identified here and surrounds the hydrophobic pocket into which the aromatic ring of Ran F72 inserts into NTF2. In particular D23 was identified as being involved in binding to FG sequences and also R120, W41, and G43, which, according to our ET results, are in the D23 region of NTF2.

Crystallography studies previously identified I64 as being involved in NTF2 binding to RanGDP,[24] and here we demonstrate that I64 is involved in binding to both Ran and FG repeats of nucleoporins. Morrison *et al.* concluded from their NMR study that the phenylalanine of FG repeats may be inserting into the same hydrophobic pocket that F72 of Ran inserts into when RanGDP binds NTF2.[28] I64 is located in this hydrophobic cavity, and thus our results are consistent with their observation. It should be noted that members of the NXF family of RNA export carriers do not bind Ran, but this region may serve an analogous role in these proteins and act as a secondary binding site for the NPC.[11,12] An alternative explanation is that D23 and I64 bind FG repeats *via* their main-chains, which are located on the exterior of the molecule. The interior of the hydrophobic cavity may only be involved in binding Ran.

Our identification of a second site on NTF2 is consistent with the finding that other transport carriers possess multiple NPC binding sites. Importin β has two distinct NPC binding sites[29] and the mRNA export carrier TAP binds to the NPC both through its UBA regions and its NTF2-like domain.[11,12,30–32] Our results imply that the NTF2 domain of TAP also contains more than one NPC-interacting domain. The identification of multiple weak binding sites on transport carriers is consistent with the model of carriers tumbling through the NPC, contacting different FG repeats with different portions of their structures.[29]

Acknowledgements

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