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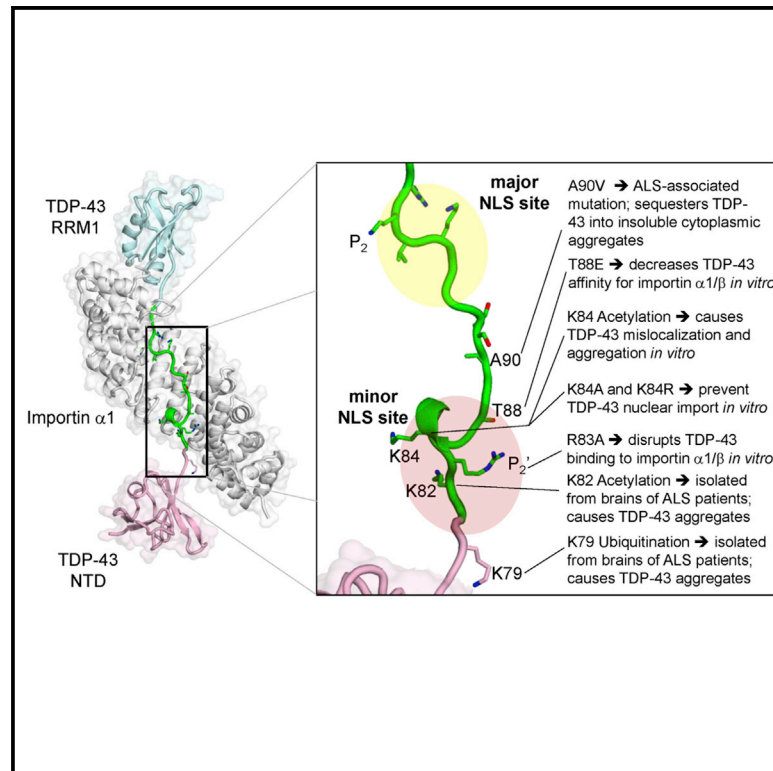
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Recognition of the TDP-43 nuclear localization signal by importin $\alpha 1/\beta$

Graphical abstract



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In brief

Doll et al. describe how the human importin $\alpha 1/\beta$ heterodimer recognizes the nuclear localization signal (NLS) of TDP-43. The paper explains the pathogenic role of post-translational modifications and mutations near the TDP-43 minor NLS site that are linked to ALS and sheds light on the chaperone activity of importin $\alpha 1/\beta$.

Highlights

- The TDP-43 NLS engages importin $\alpha 1$ through a bipartite binding mechanism
- Importin $\alpha 1$ minor NLS-binding site is essential for TDP-43 association
- Importin $\alpha 1/\beta$ disrupts the TDP-43 N-terminal domain (NTD) dimerization interface
- Phosphorylation near the TDP-43 minor NLS site reduces affinity for importin $\alpha 1/\beta$



Article

Recognition of the TDP-43 nuclear localization signal by importin $\alpha 1/\beta$

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SUMMARY

Cytoplasmic mislocalization of the TAR-DNA binding protein of 43 kDa (TDP-43) leads to large, insoluble aggregates that are a hallmark of amyotrophic lateral sclerosis and frontotemporal dementia. Here, we study how importin $\alpha 1/\beta$ recognizes TDP-43 bipartite nuclear localization signal (NLS). We find that the NLS makes extensive contacts with importin $\alpha 1$, especially at the minor NLS-binding site. NLS binding results in steric clashes with the C terminus of importin $\alpha 1$ that disrupts the TDP-43 N-terminal domain (NTD) dimerization interface. A putative phosphorylation site in the proximity of TDP-43 R83 at the minor NLS site destabilizes binding to importins by reducing the NLS backbone dynamics. Based on these data, we explain the pathogenic role of several post-translational modifications and mutations in the proximity of TDP-43 minor NLS site that are linked to disease and shed light on the chaperone activity of importin $\alpha 1/\beta$.

INTRODUCTION

In amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD), the principal aggregating protein is TDP-43. This RNA-binding protein is predominately nuclear (Zhang et al., 2009), although a pool of TDP-43 is constantly exported from the nucleus as part of ribonucleoprotein particles (RNPs) (Coyne et al., 2015; Majumder et al., 2016; Sephton et al., 2011). TDP-43 begins to self-oligomerize in the cytoplasm, likely when localized to stress granules (Colombrita et al., 2009), although more recent work suggests that TDP-43 may aggregate through phase separation independent of stress granules (Gasset-Rosa et al., 2019). Impairment in nuclear import of TDP-43 coupled with continued export of the protein contributes to increasing the cytoplasmic concentration of the protein, while the pool of nuclear TDP-43 is correspondingly depleted (Gasset-Rosa et al., 2019). This disruption in cellular homeostasis facilitates the growth of large TDP-43 aggregates (Yang et al., 2010).

TDP-43 function in a cell is intimately linked to RNA metabolism. The protein binds and stabilizes nascent mRNA in the nucleus (Tollervey et al., 2011) and matured mRNA in the cytoplasm (Colombrita et al., 2012). The mRNA binding activity is conferred by two RNA recognition motifs (RRMs) that occur in tandem (RRM1 and RRM2), separated by a linker sequence (Maris et al., 2005). TDP-43 contains a nuclear localization signal (NLS) immediately downstream of the N-terminal domain (NTD) (Johnson et al., 2008; Winton et al., 2008a). Classical NLSs can

bind to the nuclear import adaptor importin $\alpha 1$ at two distinct sites, also known as the major and minor NLS binding grooves (Conti et al., 1998; Marfori et al., 2011). Association with an NLS cargo and concomitant recruitment of the receptor importin β (Cingolani et al., 1999) frees the importin β binding domain (IBB) of importin $\alpha 1$ (Kobe, 1999), leading to the assembly of a trimeric nuclear import complex (e.g., importin $\alpha 1/\beta$ /TDP-43). The IBB functions as its own importin $\alpha 1$ NLS recognized with nanomolar affinity by importin β (Lott and Cingolani, 2011). Importin $\alpha 1$ has been shown to associate with cytoplasmic TDP-43, including phase-separated TDP-43 (Gasset-Rosa et al., 2019; Liu et al., 2021). Other importin α isoforms (Pumroy and Cingolani, 2015), such as $\alpha 3$ and $\alpha 4$, were shown to interact with TDP-43 *in vitro* (Nishimura et al., 2010) and based on proximity labeling studies (Chou et al., 2018). Furthermore, importin $\alpha 3$, alone or with importin β , was found to reverse TDP-43 aggregation mediated by arginine-rich dipeptide repeats (DPRs) *in vitro* (Hutten et al., 2020).

In addition to serving a role as import factors, importin $\alpha 1$ and β (also referred to as karyopherins) were shown to possess disaggregase activity toward TDP-43 fibrils, capable *in vitro* of disassociating pre-formed fibrils and returning TDP-43 to the soluble phase (Guo et al., 2018). Potent antiaggregase activity was reported for importin $\beta 2$ (also known as Kap $\beta 2$), which imports PY-NLS cargos without an adaptor (Lee et al., 2006). This karyopherin possesses NLS-dependent disaggregase activity toward aggregates of FUS (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018), a second



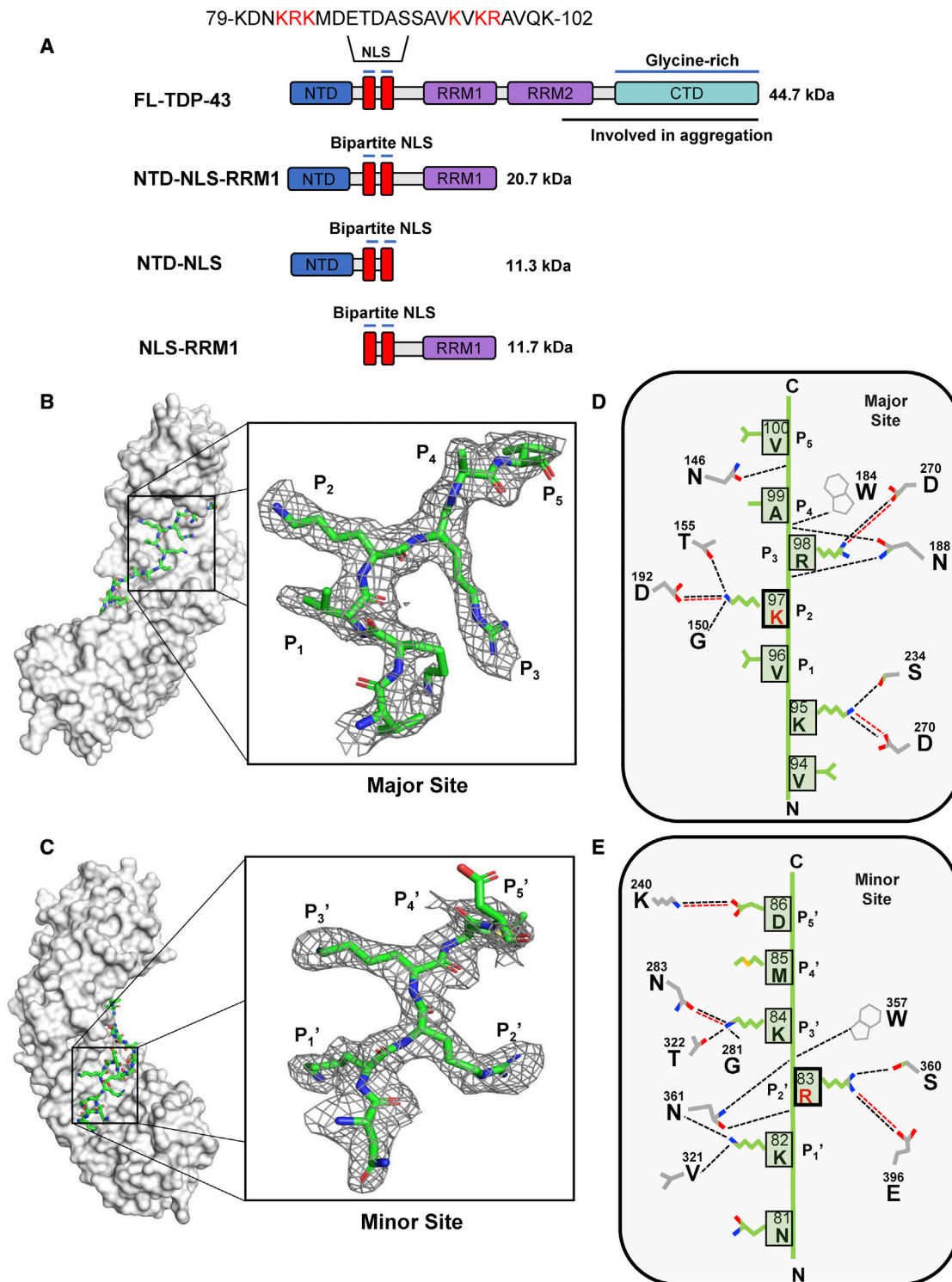


Figure 1. The TDP-43 NLS engages importin α 1 through a bipartite mechanism

(A) Schematics of all TDP-43 constructs that were used in this work.

(B and C) Crystal structure of the TDP-43 NLS (green) bound to importin α 1 (gray), major site view. The P₁-P₅ residues are shown in the inset, along with electron density from a Polder map contoured at 2.0 σ . (C) Rotated view of panel (B) showing the density for the TDP-43 NLS at the minor NLS site.

(legend continued on next page)

aggregation-prone RNA-binding protein that contributes to a significant proportion of ALS cases. Interestingly, while Kap $\beta 2$ alone is necessary and sufficient to disaggregate FUS *in vitro*, importin β disaggregase activity requires the adaptor importin $\alpha 1$ (Guo et al., 2018) or $\alpha 3$ (Hutten et al., 2020), as neither the receptor nor the adaptor alone is sufficient to dissolve TDP-43 aggregates (Guo et al., 2018).

Cytoplasmic TDP-43 aggregation is a complex process that is thought to depend on several concomitant events, including an imbalance in nucleocytoplasmic transport, aberrant post-translational modifications, and mutations, and that involves both the NTD and the prion-like C-terminal domain (CTD) (Prasad et al., 2019). A recent cryoelectron microscopy (cryo-EM) helical reconstruction of aggregated TDP-43 extracted from the frontal cortex of two individuals suffering from ALS with FTLD revealed an amyloid-like filament structure comprising a single protofilament spanning CTD residues 282–360 (Arseni et al., 2022). This low-complexity domain adopts a double-spiral-shaped fold consisting of 10 short β strands, distinct from TDP-43 filaments formed *in vitro* (Cao et al., 2019; Li et al., 2021). The stacking of TDP-43 double spirals gives rise to parallel inter-molecular β sheets that extend along the helical axis, generating amyloid-like structures, thought to be linked to TDP-43 pathogenesis (Berning and Walker, 2019). Unlike the CTD, which is intrinsically disordered in the unaggregated protein but forms pathogenic amyloid-like structures in the aggregated TDP-43, the NTD is well folded (Afroz et al., 2017; Mompeán et al., 2016; Wright et al., 2020) and likely functions as a dimer (Chang et al., 2012; Shiina et al., 2010; Wang et al., 2013), with the dimerization interface occurring within the seventh β strand of the NTD (Jiang et al., 2017). Biophysical analysis using the single-molecule fluorescence technique revealed the NTD is thermodynamically stable and undergoes reversible oligomerization that enhances the propensity of the CTD to form amyloid-like structures (Tsoi et al., 2017). Similarly, Wang et al. (2018) found the NTD enhances inter-molecular contacts between molecules promoting phase separation of the CTD and partitioning into membraneless organelles. These studies contradict previous reports that NTD-mediated oligomerization of TDP-43 represents a physiological process that may antagonize pathological aggregation (Afroz et al., 2017; Jiang et al., 2017).

Aggregated TDP-43 is subjected to heavy ubiquitination and phosphorylation (Hasegawa et al., 2008; Kametani et al., 2016). The majority of the literature focuses upon phosphorylation within the prion-like CTD (Eck et al., 2021). However, there is evidence that phosphorylation may also occur at the N terminus of TDP-43 (Kametani et al., 2016; Wang et al., 2018). Interestingly, T88, S91, and S92 within the TDP-43 NLS have been shown to be phosphorylated by casein kinase 1 δ *in vitro* (Kametani et al., 2009). Further, S92 was phosphorylated by a truncated version of this kinase in a cellular model of TDP-43 aggregation (Nonaka et al., 2016). NLS phosphorylation is known to regulate NLS exposure and presentation to importins (Nardozi et al., 2010),

and there is evidence that phosphorylation is linked to the aggregation of RNA-binding proteins (Kurischko and Broach, 2017).

In this paper, we elucidate how importin $\alpha 1$ recognizes the TDP-43 NLS and determine the effect of NLS phosphorylation on the conformational dynamics of the NLS and binding affinity for the importin $\alpha 1/\beta$ heterodimer.

RESULTS

The TDP-43 NLS engages importin $\alpha 1$ through a bipartite binding mechanism

We generated several TDP-43 constructs containing the NLS (Figure 1A) and co-expressed them in bacteria with the mammalian importin $\alpha 1$ lacking the IBB domain (Δ IBB-importin $\alpha 1$) followed by one-step purification on glutathione beads. Except for the full-length (FL) TDP-43, which is aggregation prone (Wright et al., 2020), all TDP-43 constructs containing the NLS copurified with Δ IBB-importin $\alpha 1$. Crystals of the NTD-NLS:- Δ IBB-importin $\alpha 1$ complex diffracted X-rays to 2.2 Å resolution and were used to collect a complete dataset (Table S1). The structure, solved by molecular replacement, revealed strong electron density for TDP-43 residues bound to importin $\alpha 1$ major (Figure 1B) and minor (Figure 1C) NLS-binding sites that correspond to TDP-43 residues 94–100 and 81–87, respectively. The linker between these two regions (residues 89–95) had weak density in a conventional difference density map but was visible in an unbiased maximum entropy map (Gull and Daniell, 1978) (Figure S1), sufficient to trace the entire NLS mainchain and identify the position of putative phosphorylation sites at T88, S91, and S92. Unexpectedly, the TDP-43 NTD (residues 1–77) was not visible in the electron density, although SDS-PAGE analysis of melted crystals indicated it was present in the crystal (Figure S2). The final model of importin $\alpha 1$ bound to the TDP-43 NTD-NLS was refined to an $R_{\text{work}}/R_{\text{free}}$ of 18.96%/21.51% at 2.2 Å resolution (Table S1).

The minor NLS pocket of importin $\alpha 1$ makes the majority of bonds with TDP-43 NLS

In the crystal structure, the TDP-43 NLS is oriented anti-parallel to importin $\alpha 1$, with the N terminus at the minor NLS binding site and the C terminus at the major NLS-binding site. K97 occupies the critical P_2 position at the major NLS-pocket, with R83 at the P_2' position, as seen in other bipartite NLS (Marfori et al., 2011) (Table S2). The total buried surface area of the interface between importin $\alpha 1$ and the TDP-43 NLS calculated from the crystal structure is 1,317.1 Å², and the complex is predicted to be thermodynamically stable with a positive free energy of dissociation, $\Delta G_{\text{diss}} \sim 1.9$ kcal/mol (Krissinel and Henrick, 2007). Although the TDP-43 NLS bound to importin $\alpha 1$ superficially resembles the classical bipartite nucleoplasmin NLS (Conti and Kuriyan, 2000; Fontes et al., 2000), the total number and distribution of bonds differ between the two bipartite NLSs is quite different. The TDP-43 NLS makes six salt bridges, 24 H-bonds, and 214 van der

(D and E) Minor site view. The P_1' – P_5' residues are shown in the inset, along with electron density from a Polder map contoured at 2.0 σ . Diagram of the TDP-43 NLS:importin $\alpha 1$ major (D) and minor (E) site interface detailing key residues of the Arm repeats within H-bonding distance of a given NLS residue. Dashed black lines indicate H-bonds, while dashed red lines indicate salt bridges.

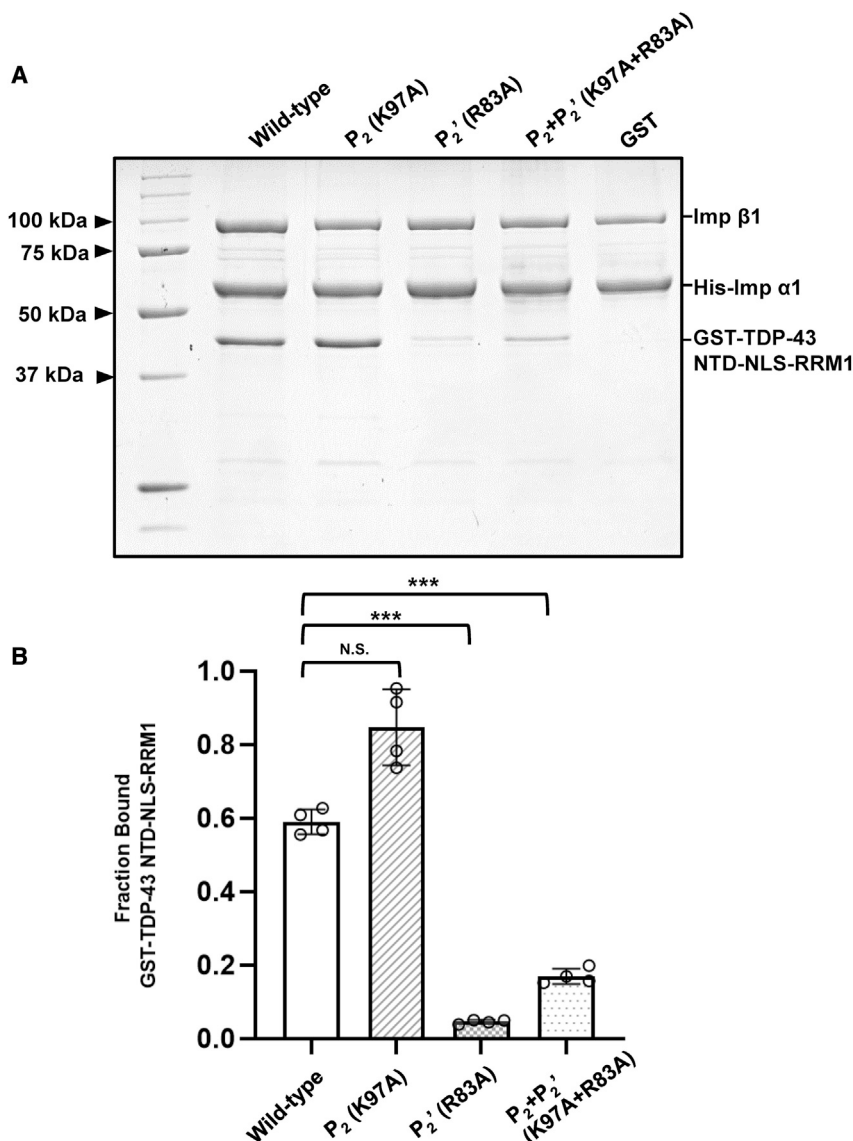


Figure 2. R83 at the P₂' site is responsible for NLS selectivity

(A) Representative SDS-PAGE gel of the pull-down experiment in which WT TDP-43 NTD-NLS-RRM1 and variants carrying ala-mutations at P₂ (K97A), P₂' (R83A), and P₂+P₂' (K97A + R83A) were co-expressed with the importin α1/β heterodimer.

(B) Quantification of TDP-43 NTD-NLS-RRM1 bands from replicate SDS-PAGE gels (n = 4). Band intensity was measured through ImageJ (Abramoff et al., 2004). Histogram columns indicate the ratio of TDP-43 band intensity to normalized importin α1 intensity, with standard deviation (error bars). Individual ratios from replicate gels are shown (circles). Significant (p < 0.01) deviation from the WT TDP-43:importin α1 ratio was determined through a two-tailed Student's t test assuming unequal variance; ***p < 0.001; n.s., not significant.

Thus, TDP-43 NLS combines binding determinants seen in recognition of classical bipartite NLSs (Pumroy et al., 2015; Sankhala et al., 2017), as well as a deeper interaction at the minor NLS-binding site and particularly the P₂' position.

In cellulo pull-down identifies the P₂' as a critical determinant for importin α1/β binding

Given that TDP-43 is more bonded to importin α1 at the minor NLS site than the major, we sought to probe the effect of point mutations at P₂ and P₂' positions. We introduced single and double alanine mutations at K97 (P₂), and R83 (P₂') of TDP-43 NTD-NLS-RRM1 (Figure 1A), which is the largest TDP-43 fragment generated in this study proved to remain soluble when expressed in isolation. On the other hand, a significant portion of recombinant importin α and β expressed in bacteria are misfolded and/or

inactive, affecting the reproducibility of biochemical experiments made using individually purified importins. The best way to purify correctly folded and active importin β is by binding it (*in vitro* or *cellulo*) to the IBB domain of importin α. Thus we devised a co-expression system where importin α1 and β were co-expressed from a polycistronic plasmid in bacteria in the presence of a second expression plasmid expressing wild-type (WT) or mutated TDP-43 constructs bearing ala-substitutions in the NLS. This system is ideal for probing the balance between importin α/β-binding and TDP-43 aggregation. As a vital control, we established that mutations in the NLS did not alter TDP-43 expression (Figure S3), allowing for a direct comparison of binding interactions with importin α1/β. The trimeric importin α1/β/TDP-43 complex was then captured on Ni beads through a histidine tag in importin α1 (Figure 2A), and the relative intensities of all three bands were quantified densitometrically (Figure 2B). Interestingly, we found that the R83A mutation at P₂' position, but not K97A at

Waals interactions with importin α1, as determined by PDBsum (Laskowski, 2009), versus only 14 H-bonds and 132 van der Waals interactions of the nucleoplasmin NLS. Furthermore, the TDP-43 NLS appears to have stronger bonding at the minor NLS-binding site than at the major site. Residues P₁'–P₅' (Figure 1E) engage in three salt bridges, 11 H-bonds, and 67 van der Waals interactions, while residues P₁–P₅ (Figure 1D) form two salt bridges, eight H-bonds, and 87 van der Waals interactions. The opposite scenario was observed for the nucleoplasmin NLS (Conti and Kuriyan, 2000; Fontes et al., 2000), which makes stronger bonding at the major NLS-binding pocket (e.g., nine H-bonds and 84 van der Waals interactions) than at the minor site (e.g., five H-bonds and 21 van der Waals interactions). Like nucleoplasmin, most known bipartite NLSs make more contacts with importin α at the major NLS-binding pocket and are disrupted by a single substitution of the Lys residue at P₂ position (Kralt et al., 2015; Lokareddy et al., 2015; Pumroy et al., 2015; Sankhala et al., 2017) (Table S2).

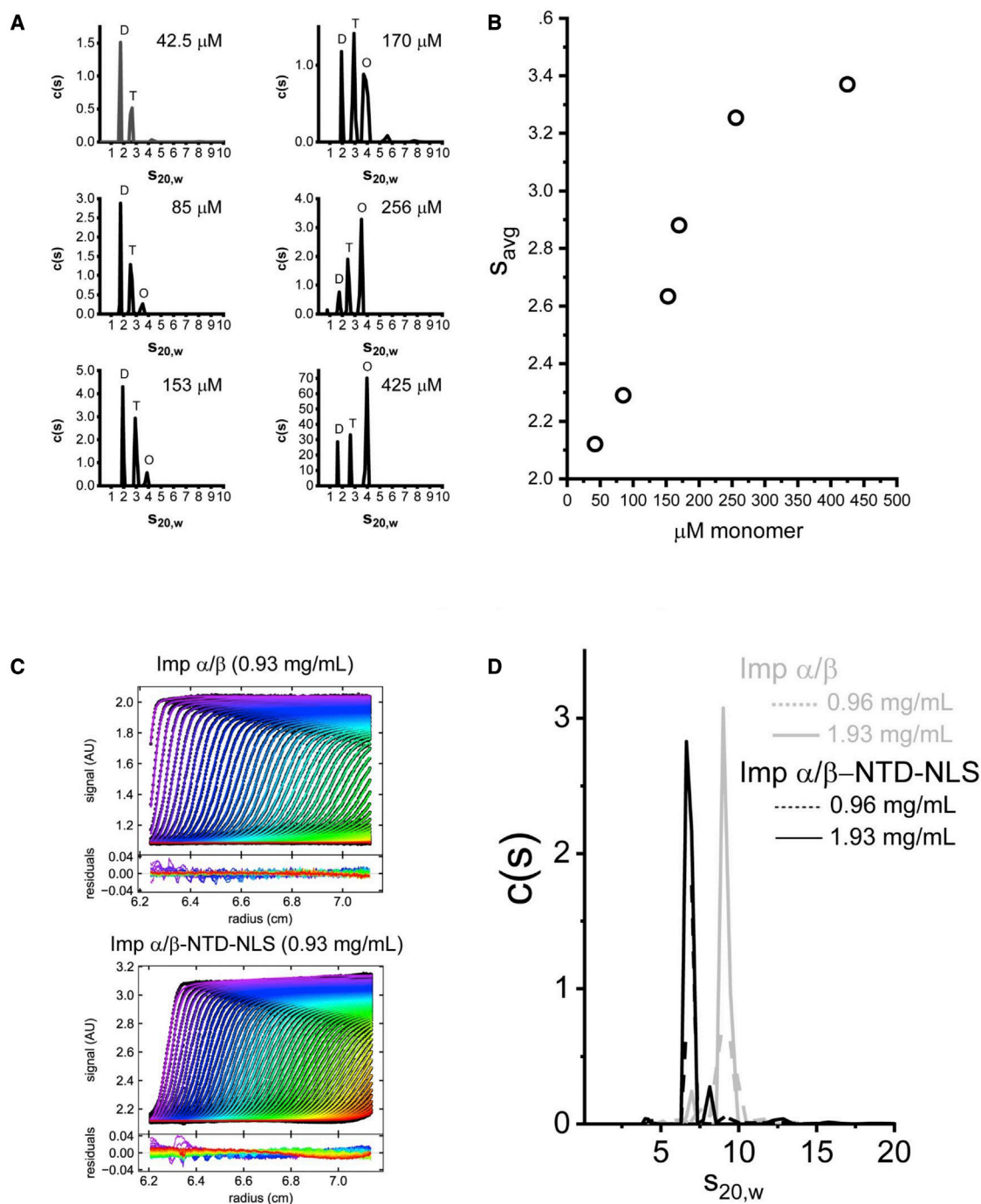


Figure 3. SV-AUC analysis of TDP-43 NTD-NLS and its complex with importin α/β

(A) Sedimentation coefficient distributions ($c(s)$) derived from SV-AUC analysis of a range of concentrations for TDP-43 NTD-NLS alone (black lines, 42.5 μ M, 85 μ M, 153 μ M, 170 μ M, 256 μ M, and 425 μ M monomer) at 20°C. Peaks are assigned to apparent dimer (D), tetramer (T), and octamer (O) species. The corresponding experimental SV-AUC data fit with the Lamm equation, and residuals are shown in Figure S4.

(B) Plot of signal-weighted average (s_{avg}) as a function of loading concentration, in μ M monomer. Values at each concentration were derived from integration of the $c(s)$ distributions shown in (A), illustrating the concentration-dependent behavior of the NTD-NLS alone.

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P₂, disrupted binding to the importin α 1/ β heterodimer. Thus, the minor site of TDP-43 NLS plays a crucial role in binding to importin α 1/ β .

Importin α 1/ β binding disrupts NTD-NLS oligomerization

The 80 residues N-terminal of the TDP-43 minor NLS box form the NTD (Figure 1A). To identify a possible link between NLS recognition and TDP-43 aggregation, we investigated the oligomeric state of the TDP-43 NTD-NLS in a range of concentrations (42.5–425 μ M) using analytical ultracentrifugation sedimentation velocity analysis (SV-AUC). Consistent with previous findings (Chang et al., 2012; Jiang et al., 2017; Mompean et al., 2017; Shiina et al., 2010; Wang et al., 2013), we observed that the NTD is dimeric in solution, displaying a concentration-dependent tendency to form tetramers and larger oligomers (Figures 3A and S4). Plotting the average sedimentation coefficient as a function of NTD-NLS concentration revealed a sigmoidal dependence consistent with a recent crystal structure that revealed a supramolecular helical packing built by five copies in the asymmetric unit (Wright et al., 2020). We then investigated the oligomeric state of TDP-43 NTD-NLS in complex with the importin α 1/ β , or just Δ IBB-importin α 1. We used isoform α 1, which was previously reported to exert anti-aggregase activity toward TDP-43 aggregates (Guo et al., 2018). We assembled homogeneous complexes of importin α / β and α / β /NTD-NLS by co-expression in bacteria and subjected them to sedimentation velocity analysis (Figure 3C). We found the apo-importin α / β complex exists as a dimer of \sim 330 kDa, in a physiological range of concentration (e.g., importin α and β concentration in a cell was estimated to be \sim 3 and 1 μ M, respectively; Riddick and Macara, 2005). The heterodimer dimerization was likely mediated by importin α 1 tendency to dimerize (Miyatake et al., 2015). In contrast, the pre-formed α / β /NTD-NLS complexes were always monomeric, consistent with a 1:1:1 binding of the three proteins (Figure 3D). The same result was obtained by performing SV-AUC analysis of the Δ IBB-importin α 1:NTD-NLS complex, which also migrated as a monodisperse species of $s = 2.086$ S corresponding to a 1:1 complex of 67.2 kDa, remarkably close to the expected mass of 66.6 kDa (Figure S5). Thus, the NLS-dependent association of importin α 1 with the NTD is sufficient to disrupt TDP-43 NTD oligomerization, rendering the import cargo monomeric.

TDP-43 NLS is loosely connected to two small globular domains

The architecture of the TDP-43 NLS in the context of the upstream NTD and downstream RRM1 (Figure 1A) was investigated by subjecting homogeneous complexes of importin α 1 bound to either domain to size-exclusion chromatography coupled with small-angle X-ray scattering (SEC-SAXS) (Acerbo et al., 2015). In a concentration range between 8 and

10 mg mL⁻¹, pre-formed complexes of both NTD-NLS: Δ IBB-importin α 1 and NLS:RRM1: Δ IBB-importin α 1 gave scattering profiles suitable for biophysical analysis. From the Guinier fits of the scattering intensities, we were able to calculate a radius of gyration (R_g) for each complex. The NTD-NLS and NLS-RRM1 complexes had an R_g of 36.37 ± 0.15 Å and 38.28 ± 0.26 Å, respectively (Figures 4A and 4C; Table S3). We determined that the volume of correlation (V_c) mass for NTD-NLS and NLS-RRM1 complexes were 64.8 kDa and 67.5 kDa, respectively, which are close to the expected mass of each complex (66.6 kDa for NTD-NLS: Δ IBB-importin α 1 and 67.0 kDa for NLS-RRM1: Δ IBB-importin α 1). The distance distribution function $P(r)$ calculated from data indicates a maximum diameter (D_{max}) of 140.3 Å for NTD-NLS: Δ IBB-importin α 1 complex and 136.7 Å for the NLS-RRM1: Δ IBB-importin α 1 complex (Figures 4A and 4C). The Kratky plots of each complex suggest a degree of globularity (Figures S6A and S6B), likely provided by importin α 1 and either the folded NTD or RRM1. To visualize the structural organization of the NTD-NLS and NLS-RRM1 complexes in solution, we calculated an electron density from the scattering data using Density from Solution Scattering (DENSS) (Grant, 2018). The SAXS electron densities were shaped asymmetrically (Figures 4B and 4D), allowing us to place the solenoid structure of importin α 1 and the TDP-43 domains flanking the NLS. The deposited structures of the TDP-43 NTD (Mompean et al., 2016) or RRM1 (Kuo et al., 2014) domains were connected to the TDP-43 NLS described previously (Figure 1B), and the modeled TDP-43 fragments spanning NTD-NLS and NLS-RRM1 fit within the electron density generated by DENSS.

To determine the reliability of our models, we generated a theoretical scattering profile for each and compared them with the observed scattering of the respective complex. We found that the NTD-NLS: Δ IBB-importin α 1 model fit the experimental data with a χ^2 value of 1.39, which was indicative of a strong agreement between the model and experimental data. The NLS-RRM1: Δ IBB-importin α 1 model fit the experimental data with a χ^2 value of 2.06, again suggesting a good agreement between our model and experimental data (Figures 4B and 4D). We further constructed a composite model of TDP-43 NTD-NLS-RRM1 (residues 1–179) bound to Δ IBB-importin α 1 (shown in Figure 7) that revealed both the NTD and RRM1 are loosely connected from the importin α 1 Arm core as “knots on a rope.” This is in agreement with previous SEC-SAXS data using FL TDP-43, in which the entire protein appears highly flexible if not unfolded (Wright et al., 2020). Thus, SEC-SAXS and molecular modeling revealed that the domain flanking the NTD-43 NLS does not make stable and direct contacts with importin α 1. Nonetheless, importin α 1 is sufficient to disrupt NTD dimerization and assembly into higher-order structures upon binding.

(C) Experimental SV-AUC data (circles) fit with the Lamm equation (lines) as implemented in the program SEDFIT (Schuck, 2000) for importin α / β alone (upper, RMSD = 0.004) and importin α / β -NTD-NLS (lower, RMSD = 0.006). Below each panel are the residuals for the fit data. For clarity, only every third data point is shown. Data were obtained with 280 nm detection at 20°C. Figures were created using the program GUSSI (Brautigam, 2015).

(D) Sedimentation coefficient distributions $c(s)$ derived from SV-AUC data for importin α / β alone (gray dotted line, 0.95 mg/mL; gray solid line, 1.93 mg/mL) and importin α / β -NTD-NLS (black dotted line, 0.93 mg/mL; black solid line, 1.93 mg/mL) at 20°C. Peaks values are observed at 9.24 $s_{20,w}$ (\sim 351 kDa) for importin α / β alone and 6.93 $s_{20,w}$ (\sim 174 kDa) for importin α / β -NTD-NLS, respectively.

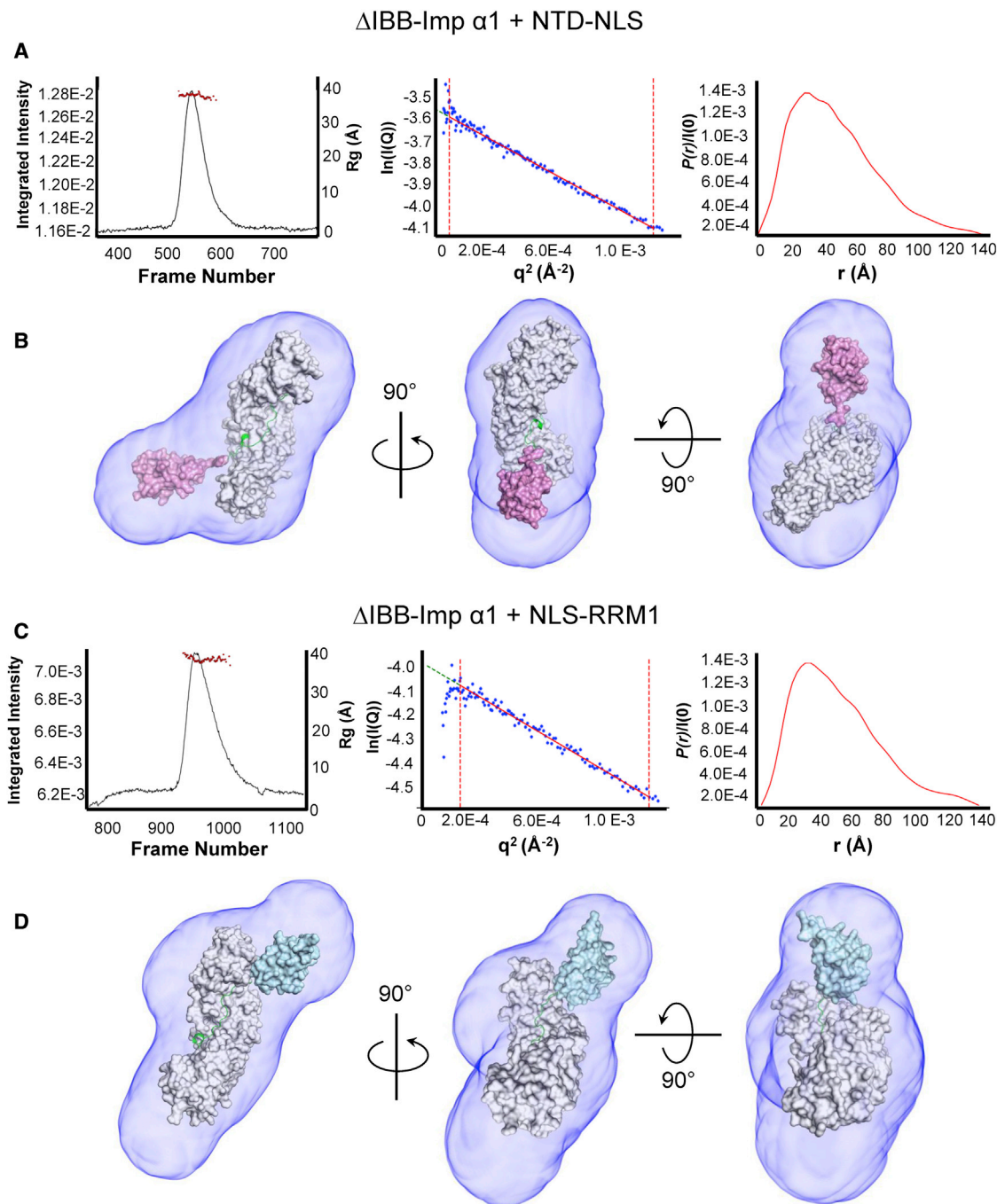


Figure 4. Solution structures of TDP-43:ΔIBB-importin α 1 complexes

(A) Left, experimental SEC-SAXS profile of the TDP-43 NTD-NLS:ΔIBB-importin α 1 complex (black trace) overlaid with Rg distribution across the peak (red circles). Center, Guinier region of the intensity $I(q)$ to the scattering vector (q^2). The q_{\max} (Rg) cutoff was 1.3. Right, $P(r)$ function with D_{\max} of 140.3 Å. (B) Model of the TDP-43 NTD-NLS:ΔIBB-importin α 1 complex fit within the electron density generated by DENSS. ΔIBB-importin α 1 is shown in gray, the TDP-43 NLS in green, and the NTD in magenta. The comparison of the scattering profile predicted for the model to the empirical scattering of the complex produced a χ^2 value of 1.39. (C) Left, experimental SEC-SAXS profile of the TDP-43 NLS-RRM1:ΔIBB-importin α 1 complex (black trace) overlaid with Rg distribution across the peak (red circles). Center, Guinier region with q_{\max} (Rg) cutoff of 1.3. Some repulsion was observed within the sample at low q , which was omitted from the Guinier region. Right, $P(r)$ function with D_{\max} of 136.7 Å. (D) Model of the TDP-43 NLS-RRM1:ΔIBB-importin α 1 complex fit within the electron density generated by DENSS. ΔIBB-importin α 1 is shown in gray, the NLS is in green and the RRM1 is colored in cyan. The comparison between predicted and observed scattering produced a χ^2 value of 2.06.

Phosphorylation of the TDP-43 NLS reduces affinity for importin $\alpha 1$

The linker sequence in TDP-43 NLS that connects the major site residues to the minor site is subject to phosphorylation at T88, S91, and S92 (Kametani et al., 2009; Nonaka et al., 2016) (Figure 5A). While the function of NLS phosphorylation is not entirely understood, the addition of this post-translational modification within the linker may inhibit association with importin $\alpha 1$ (Nardozzi et al., 2010). To test this hypothesis, we designed a microtiter plate binding assay to probe the affinity of the TDP-43 NTD-NLS-RRM1 WT and phosphomimetic constructs for the importin $\alpha 1/\beta$ heterodimer. To generate the phosphomimetics, we individually mutated T88, S91, and S92 in the NLS linker to glutamic acid. We also generated a hyperphosphorylated phosphomimetic in which all three residues were mutated to glutamic acid. We found that all TDP-43 constructs bound the importin $\alpha 1/\beta$ heterodimer with nanomolar affinity, with WT TDP-43 NTD-NLS-RRM1 having $K_D = 65.8 \pm 14.3$ nM. The pT88 mutant produced the most significant reduction in affinity for the importin $\alpha 1/\beta$ heterodimer, over 3-fold relative to the WT ($K_D = 218.1 \pm 27.6$ nM) (Figure 5B). Mutations at S91 and S92 had a minor role on NLS affinity ($K_D = 120.7 \pm 33.5$ nM and 87.8 ± 8.5 nM, respectively) (Figures 5C and 5D). Interestingly, when all three mutations were introduced in the same construct, the reduction in affinity was the greatest, over 5-fold relative to the WT ($K_D = 350.2 \pm 1.0$ nM) (Figure 5E). Thus, the phosphorylation site that resides next to the critical minor NLS box dramatically affects binding affinity for the importin $\alpha 1/\beta$ heterodimer; together, all phosphorylations appear to play an additive role in weakening the association to the importin $\alpha 1/\beta$ heterodimer.

Phosphorylation in TDP-43 NLS modulates conformational dynamics

To rationalize the role of phosphorylation in the TDP-43 NLS linker, we performed long-timescale-all-atom molecular dynamics (MD) simulations of the unmodified TDP-43 NLS and NLS with phosphorylation at T88, S91, and S92. In each regard, we considered the NLS both independent of the importin $\alpha 1$ adaptor and in complex with importin $\alpha 1$. For the latter experiments, the relative binding energy for each system was calculated from the TDP-43:importin $\alpha 1$ trajectories using the Molecular Mechanics Poisson-Boltzmann Surface Area (MM/PBSA) (Homeyer and Gohlke, 2012; Miller et al., 2012) approach to explore the interaction affinity of each phosphorylated TDP-43 NLS with importin $\alpha 1$. To analyze the dynamical behavior of TDP-43 NLS we measured the root-mean-square fluctuation (RMSF) from simulations of free NLS, not bound to importin $\alpha 1$ (Figures 5F–5I). The latter data indicated that phosphorylation of the TDP-43 NLS within the linker sequence could increase the RMSF of particular NLS residues relative to reference positions determined for an unmodified NLS. Interestingly, RMSF peaks were observed at the tract of basic residues (the KRK sequence) bound at the importin $\alpha 1$ minor site for NLS in which T88 was phosphorylated (Figure 5F) and T88/S91/S92 were all modeled as phosphorylated (Figure 5I). Structurally, phosphorylation of T88 was found to disrupt a short helix at the minor NLS binding site. This helix is characteristic of NLS that engages the minor site (Nakada et al., 2015), and perturbing this structure through phosphorylation could explain the

increase in RMSF and the consequent reduction in binding affinity (Figures 5B and 5E). A second RMSF peak was observed over the basic tract KVKR at the importin $\alpha 1$ major site when T88 was phosphorylated, which was not observed for any other phosphorylation event (Figures 5F–5I). Phosphorylation of S91 and S92 did not produce significant deviations in position across the entire NLS, indicating that these NLS behave similarly to the unmodified NLS (Figures 5G and 5H). For each of the complexes simulated in Figure 5, we performed cross-correlation analysis of the simulated structures against the experimental SAXS density (Figure S7). We saw consistent correlations, suggesting that alternate conformations were not observed in our simulations.

In addition to RMSF, we also calculated the change in free energy of binding for the interaction between unmodified and phosphorylated NLS with importin $\alpha 1$ (Figure S8). Phosphorylation caused a reduction in the free energy of binding, the most significant of which occurred for the hyperphosphorylated NLS. This observation suggested that a thermodynamic approach could not sufficiently explain the disruption of the predicted helix at the importin $\alpha 1$ minor binding site through conformational deviation.

Kinetic maps by Markov state modeling

To better understand how phosphorylation affects the conformational dynamics of the TDP-43 NLS, we generated kinetic maps generated by Markov state modeling. Mean first-passage time (MFPT) was derived from Markov state models (MSMs) (Bhattacharya and Lin, 2019; Huang et al., 2017), representing how phosphorylation impacts the kinetics of the TDP-43 NLS. These maps revealed that the TDP-43 NLS adopts different metastable states and distinct MFPTs between states due to phosphorylation. The arrows in Figure 6 represent the first transition time between different states for the WT NLS (Figure 6A) and each phosphorylated state (Figures 6B–6E). The lowest MFPT belongs to the WT system (<333.0 ns), suggesting the unphosphorylated NLS, which binds importin $\alpha 1$ with the highest affinity, also has the highest probability of transitioning to the conformation that is favored for binding to importin $\alpha 1$. On the other hand, the longest MFPT belongs to hyperphosphorylated TDP-43 NLS (<1862.6 ns) and indicates that the spontaneous transition between different states, relative to other phosphorylated NLS peptides, is less probable, representing a rigid structure with a low affinity for binding to importin $\alpha 1$ (Figure 6E). Empirically, this results in a reduced affinity for importin $\alpha 1/\beta$, which in our binding experiment had over 5 \times higher K_D for TDP-43 carrying three phosphomimetic mutations at T88/S91/S92 relative to the WT (Figure 5E). Consistent with the experiment in Figure 5, phosphorylation at T88, proximal to the critical minor NLS-binding site, stabilized a short α helix at the minor NLS site (Figure 6B), affecting the binding affinity for the importin $\alpha 1/\beta$ heterodimer. Overall, these data suggest that phosphorylation in TDP-43 NLS residues not directly involved in binding to the Arm core but positioned in the proximity of the minor NLS-binding pocket destabilizes binding to importin $\alpha 1/\beta$ by reducing the NLS backbone dynamics.

DISCUSSION

In this paper, we have determined the structure and recognition of the TDP-43 NLS by the importin $\alpha 1/\beta$

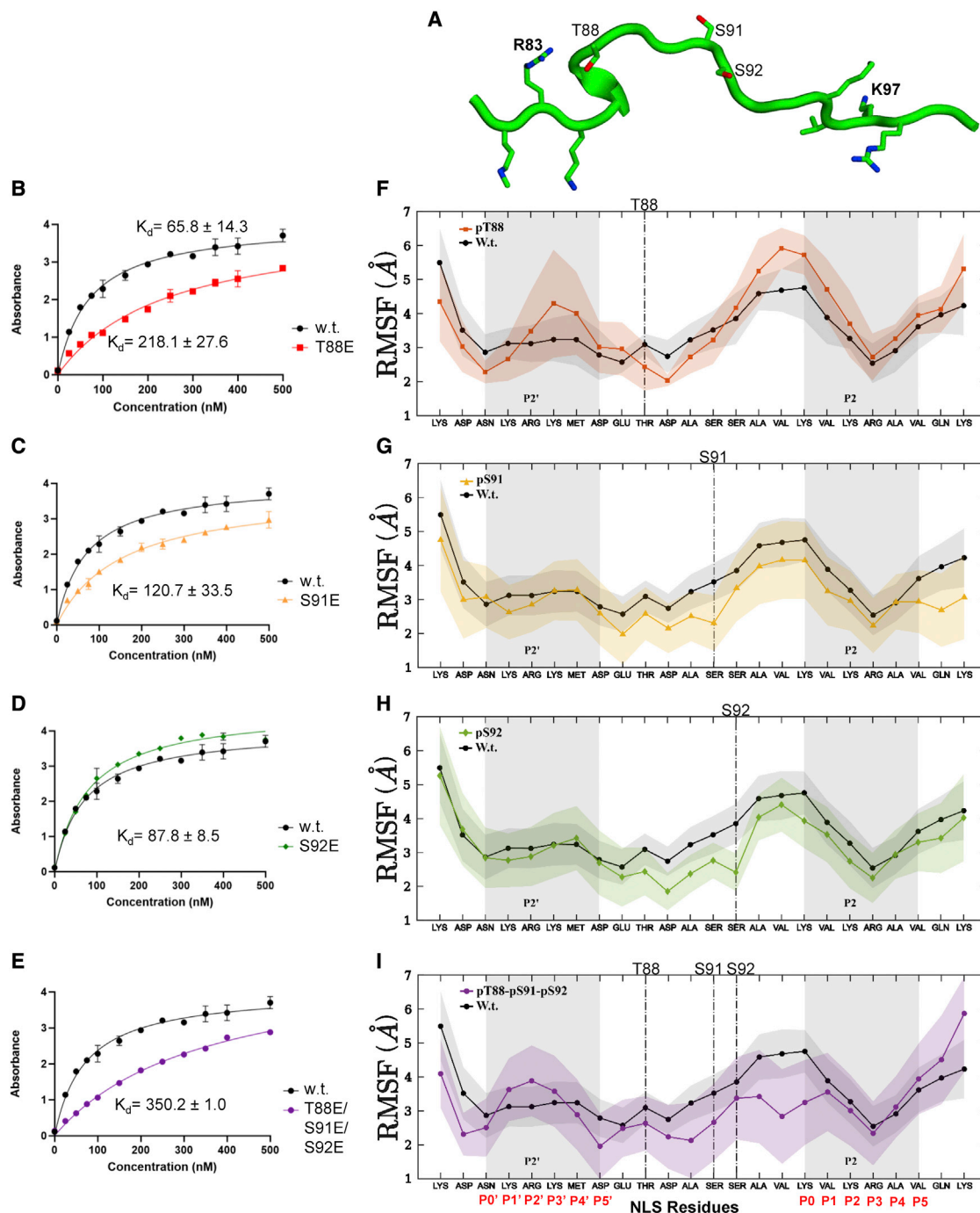


Figure 5. NLS phosphorylation reduces affinity for import $\alpha1/\beta$

(A) Ribbon diagram of the TDP-43 bipartite NLS highlights putative phosphorylation sites at T88, S91, and S92.

(B–E) Binding isotherms of importin $\alpha1/\beta$ binding to TDP-43 NTD-NLS-RRM1 containing the (B) T88E phosphomimetic (red curve), (C) S91E phosphomimetic (orange curve), (D) S92E phosphomimetic (green curve), and (E) T88E + S91E + S92E hyperphosphorylation mimetic (purple curve). The positive control, importin $\alpha1/\beta$ binding to unphosphorylated TDP-43 NTD-NLS-RRM1, is shown as a black curve in all four panels. All K_D values are the average of at least two independent experiments, each with at least two independent replicates. Errors are calculated as the standard deviation from the mean.

(F–I) Carbon α root-mean-square fluctuation (RMSF) was measured across 6 μ s for each isolated TDP-43 NLS system in units of \AA . In each panel, NLS residues at P_1 – P_5 (major NLS box) and P_1' – P_5' (minor NLS box) are highlighted in gray. Dashed lines refer to the putative phosphorylation sites T88, S91, and S92. The RMSF curve is shown for the (F) phosphorylated T88 (red trace), (G) phosphorylated S91 (orange trace), (H) phosphorylated S92 (green trace), and (I) hyperphosphorylated T88 + S91 + S92 (purple trace), relative to the RMSF of wt TDP-43 (black trace). The color-coding of RMSF curves in (F–I) matches the binding curves in (B–E).

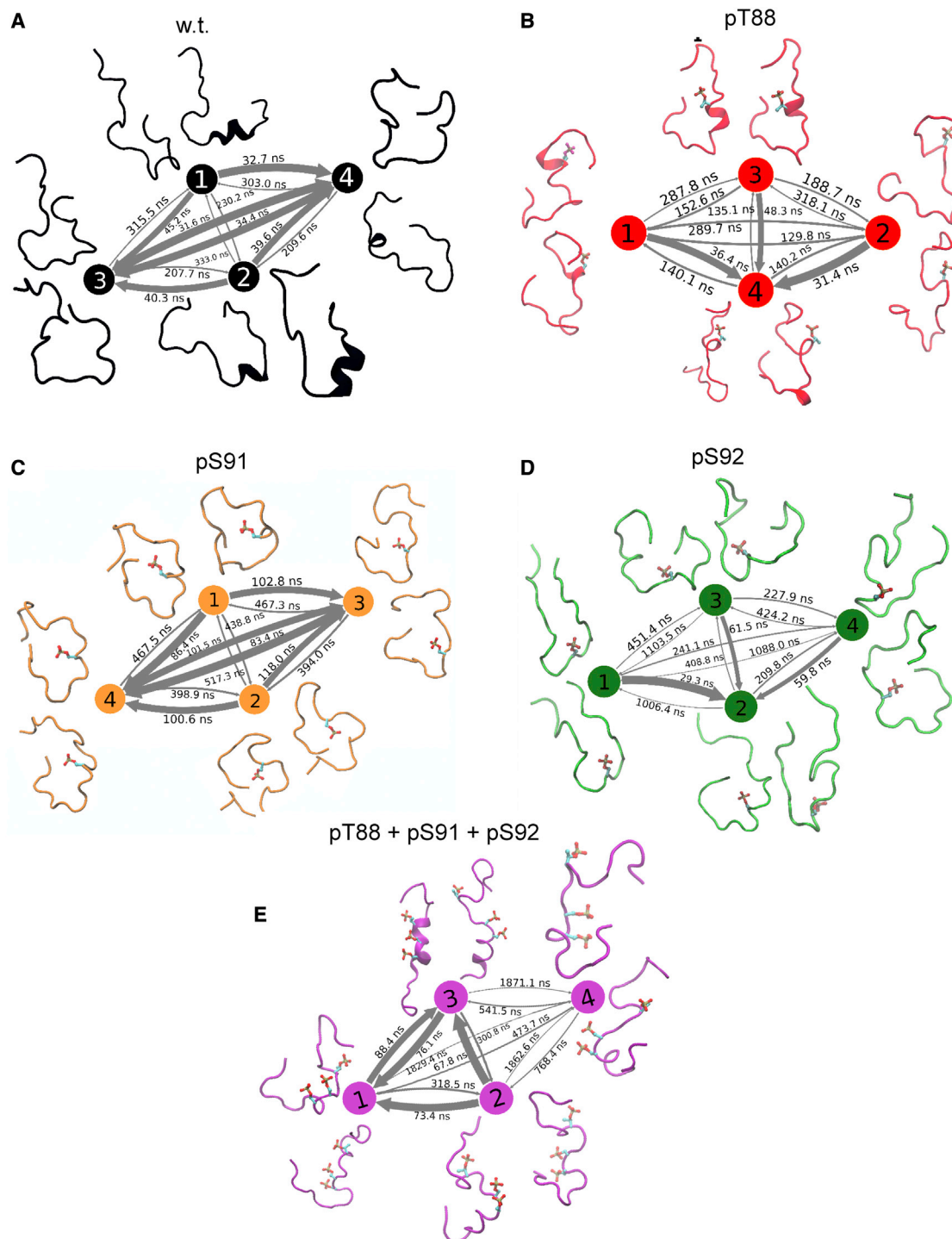


Figure 6. Kinetic maps from Markov state model analysis of each TDP-43 NLS system

(A–E) The maps show peptides in secondary structure representation, with phosphorylation sites shown in licorice representation. In each map, arrows connecting states represent the mean first-passage time (MFPT) listed next to each arrow. Thicker arrows represent faster transition rates along a given direction and therefore illustrate higher transition probabilities as measured from Markov state models (MSMs). (A) Unphosphorylated NLS binds Δ IBB-importin $\alpha 1$ with the lowest MFPT (<333.0 ns) and has the highest probability of transitioning to the conformation favored for binding importin $\alpha 1$. (B) When phosphorylated at T88, the NLS had generally reduced transition times between states. (C) Phosphorylation at S91 yielded a transition map most similar to the wild-type, albeit with different NLS conformations. (D) The transition map of TDP-43 NLS with phosphorylated S92 featured a paucity of fast kinetics with different states preferred than those in panel (B). (E) The longest MFPT belongs to the hyperphosphorylated TDP-43 NLS (<1,862.6 ns), suggesting that the spontaneous transition between different states is less probable, as expected for a rigid structure with a low affinity for binding to importin $\alpha 1$.

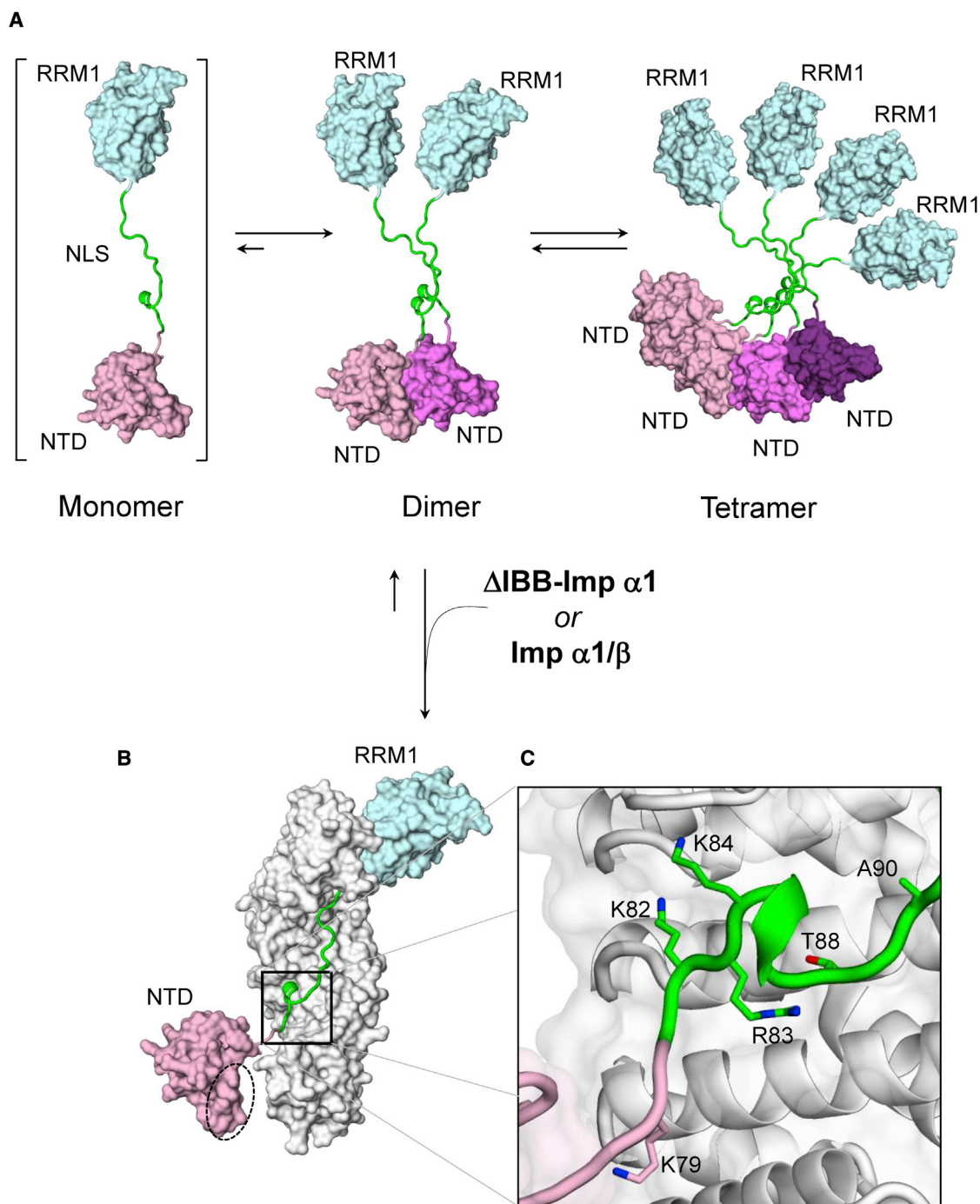


Figure 7. Proposed role of importins in TDP-43 NTD aggregation

(A) TDP-43 exists in a concentration-dependent equilibrium between dimers, tetramers, and oligomers (not shown). Only the NTD-NLS-RRM1 fragment modeled from SEC-SAXS is shown.

(B) Δ IBB-importin α 1, or the importin α 1/ β complex, breaks the NTD dimer by binding the NLS with nanomolar affinity. The NTD dimerization interface is shown by a dashed circle.

(C) A magnified view of the minor NLS-binding region highlighting all residues implicated in TDP-43 loss of function.

heterodimer. Our work investigates the interplay between TDP-43 recognition by importins and the events that lead to its aggregation.

We show that the TDP-43 NLS employs a bipartite binding mechanism to engage importin α 1/ β with $K_D = 65.8 \pm 14.3$ nM. The TDP-43 NLS sequences that bind at the major and minor

NLS binding sites of importin α 1 are in agreement with those of previously published bipartite NLSs (Marfori et al., 2011). However, unlike classical bipartite NLSs, mutation of K97 at the P₂ position did not affect binding to importin α 1/ β , which was disrupted solely by mutation of R83 at the P₂' position. A similar phenomenon was previously reported for the NLSs of inner nuclear membrane proteins Heh1 and Heh2 (Lokareddy et al., 2015) and suggested the minor site of TDP-43 NLS plays a crucial role in binding energetics to importin α 1/ β . Our structural and biochemical findings agree with previous work investigating TDP-43 nuclear import in cell-based assays (Ayala et al., 2008; Pinarbasi et al., 2018; Winton et al., 2008a). These studies used TDP-43 constructs with poly-alanine mutations at the basic residues of the minor site KRK and major site KVKR sequences (Figures 1A and Table S2). In cell lines, over-expression of the Δ NLS construct in which the minor site sequence was disrupted resulted in depletion of nuclear TDP-43 and formation of cytoplasmic TDP-43 inclusions. Disruption of the major site also caused nuclear depletion, but this did not appear as substantial as when the minor site was affected (Ayala et al., 2008; Winton et al., 2008a). Similarly, in a more recent study, single K84A and K84R mutations at position P₃' prevented the nuclear import of a reporter protein consisting of the red fluorescent protein fused to TDP-43-NLS (RFP-TDP-43-NLS), whereas K95A and K95R mutants at P₀, in the major binding site, were imported to the nucleus as efficiently as the WT RFP-TDP-43-NLS (Khosravi et al., 2020). Thus, recognition of the TDP-43 NLS depends chiefly on binding to the minor NLS-pocket of importin α 1 and, consequentially, perturbation of the TDP-43 minor NLS tract is expected to play a critical role in slowing TDP-43 nuclear import.

In the attempt to rationalize how the NLS binding crosstalks to the TDP-43 NTD, we crystallized Δ IBB-importin α 1 with the NTD-NLS construct. Although the crystals included the NTD, we found no discernable density N-terminal of the minor NLS box, suggesting the NTD is loosely connected to the NLS and does not make contact with importin α 1. SEC-SAXS confirmed that both TDP-43 domains flanking the NLS (e.g., NTD and RRM1) do not directly contact importin α 1. In agreement with a recent study (Wright et al., 2020), TDP-43 NTD and RRM1 can be thought of as beads on a string, independently sampling different conformations, while importin α 1 stabilizes the extended bipartite NLS. Interestingly, the TDP-43 NTD has been shown to mediate dimerization of the protein and form higher-order oligomers (Afroz et al., 2017; Chang et al., 2012; Jiang et al., 2017; Mompeán et al., 2016; Shiina et al., 2010; Wang et al., 2013; Wright et al., 2020). However, we did not observe dimerization of the TDP-43 NTD-NLS construct in complex with importin α 1/ β , or just Δ IBB-importin α 1, which lacks the autoinhibitory IBB domain (Lott and Cingolani, 2011). This is remarkable given that the NTD does not interact with importin α 1 directly. Our SAXS model of the Δ IBB-importin α 1:NTD-NLS complex suggests a dimeric NTD is incompatible with importin α 1 association, as a second NTD protomer would clash with the C-terminal Armadillo repeats 8–10 of importin α 1 (Figure 7B). The residues responsible for NTD dimerization are just upstream of the NLS (Jiang et al., 2017), and, although they do not appear to bond importin α 1 directly, the protein presents a steric barrier, likely sufficient to disrupt dimerization. This observation may explain how

the importin α 1/ β heterodimer prevents TDP-43 aggregation *in vitro* (Guo et al., 2018). This activity is NLS dependent and requires both the FL importin α 1 and the receptor importin β , while individual importin α 1 or β were not sufficient. In hindsight, importin β removes the IBB autoinhibition from importin α 1, exposing the minor NLS pocket for TDP-43 binding and disrupting NTD dimerization. It is also possible that, concomitantly with importin α 1-mediated disruption of TDP-43 NTD aggregation, importin β , a 97 kDa protein, also engages the rest of TDP-43, possibly shielding the CTD and further preventing its aggregation. In other words, the observed chaperone activity of importin α 1/ β toward TDP-43 aggregates may arise from different biochemical activities harbored in both importins.

Our data provide a reading frame to explain the deleterious effect of post-translational modifications and mutations in the proximity of the minor NLS site previously reported in the literature. Acetylation of K82, at position P₁' (Figure 7C), and ubiquitination of the nearby K79 (at position P₋₁') were found in a sarkosyl-insoluble fraction of TDP-43 aggregates isolated from brains of ALS patients (Kametani et al., 2016). Acetylation of K84 at the P₃' position (Garcia Morato et al., 2022) resulted in TDP-43 cytoplasmic mislocalization, and aggregation propensity in HEK293E cells, strengthening the connection between modification of the TDP-43 minor NLS box and pathogenicity. Similarly, as previously pointed out, K84A and K84R mutations largely prevented the nuclear import of an RFP-TDP-43-NLS reporter, indicating K84 is crucial for the function of the TDP-43 NLS (Khosravi et al., 2020). C-terminal of the minor NLS box, the ALS-associated A90V mutation was found to sequester the endogenous TDP-43 into insoluble cytoplasmic aggregates (Winton et al., 2008b). This mutation is three-dimensionally close to the minor site R83, given the turn in the NLS backbone at the minor site (Figure 7C). All these modifications and mutations likely destabilize the recognition of the minor NLS box, preventing importin α 1/ β association that disrupts healthy nucleocytoplasmic trafficking and promotes cytoplasmic aggregation.

Beyond the phenomenological observation that a mutation or post-translational modification is linked to aggregation, in this paper, we sought to investigate how changes to just one of the many residues in TDP-43 NLS making contacts with importin α 1 can have such a pleiotropic effect. To this end, we investigated three putative phosphorylation sites in the TDP-43 NLS linker at T88, S91, and S92, previously identified as targets of casein kinase 1- δ (Kametani et al., 2009; Nonaka et al., 2016). Although none of these three residues makes direct, strong contacts with importin α 1 Arm core, phosphorylation of the threonine within the linker sequence (T88) produced the most significant reduction in affinity for importin α 1. This was in agreement with our MD simulations of the interaction between importin α 1 and phosphorylated TDP-43 NLS, in which phosphorylation at T88 produced a consistently high RMSF relative to phosphorylation of other residues within the NLS. Interestingly, T88 is close to the minor NLS binding site (Figure 7C). MD simulations indicate that phosphorylation within the NLS linker reduces the spontaneous transition of the NLS backbone to more kinetically favorable states, making binding to importin α 1 less probable. Specifically, phosphorylation at T88 stabilizes a short α helix in the immediate proximity of the minor site that has a pleiotropic

negative effect on binding affinity for importin $\alpha 1/\beta$. This effect was exacerbated by having three phosphorylations in the NLS linker that further stabilized secondary structure states having unfavorable binding characteristics for importin $\alpha 1/\beta$, enhancing cytoplasmic retention that leads to aggregation. This finding is particularly relevant in the disease state, in which aggregated TDP-43 becomes hyperphosphorylated, with most phosphorylation sites in the CTD (Kametani et al., 2016). Our results contrast with a recent report that phosphorylation of TDP-43 CTD suppresses condensation and aggregation (Grujic da Silva et al., 2022) instead of enhancing it (Hasegawa et al., 2008). Notably, Grujic da Silva et al. used *in vitro* phosphorylated TDP-43 lacking the three phosphorylation sites in the NLS reported by Kametani et al. (2009) that, as shown in this paper, modulate the affinity of TDP-43 for the importin $\alpha 1/\beta$ complex.

We propose that, under normal conditions, importin $\alpha 1/\beta$ antagonizes TDP-43 aggregation via two concomitant mechanisms: promoting its import into the nucleus and preventing NTD dimerization by steric hindrance (Figures 7A and 7B). Perturbations in the NLS that affects the minor NLS site reduce binding affinity for importin $\alpha 1/\beta$ and serve two pathogenic functions: on one side, slowing down TDP-43 nuclear import, as a weaker NLS is also less likely to be imported into the cell nucleus (Fanara et al., 2000); on the other, reducing importin $\alpha 1/\beta$'s ability to stabilize monomeric TDP-43 NTD, leading to large aggregates. The literature is split on the actual role of the NTD dimerization vis à vis TDP-43 aggregation. While certain reports have suggested that TDP-43 NTD dimerization is necessary for its physiological functions like RNA splicing and may antagonize pathological aggregation (Afroz et al., 2017; Jiang et al., 2017), others have argued that the NTD dimerization is involved in TDP-43 aggregation (Shiina et al., 2010; Wang et al., 2013). Recent biophysical work has shown that NTD undergoes reversible oligomerization that enhances the propensity of the CTD to form amyloid-like structures (Tsoi et al., 2017; Wang et al., 2018). This paper supports the latter hypothesis and attempts to interpret the NTD dimerization in the context of importin $\alpha 1/\beta$ recognition. NTD self-association brings TDP-43 molecules closer together, enhancing the local concentration of CTDs. This intrinsically disordered portion begins the formation of amyloid-like structures (Arseni et al., 2022), and larger and larger oligomers eventually become phase-separated aggregates. Post-translational modifications in the NLS also reduce the affinity for importins, reducing importin $\alpha 1/\beta$ chaperone activity toward TDP-43 aggregates (Guo et al., 2018). Overall, the cell is overwhelmed by the disruption of TDP-43 homeostasis, promoting the formation of inexorably larger and larger inclusions that drive the disease state.

Limitations of the study

In this study, we characterize the recognition of the TDP-43 NLS by human importins $\alpha 1/\beta$ or just a fragment of importin $\alpha 1$ lacking the IBB domain. One limitation of our work is that we limited our structural, biochemical, and computational analysis to a soluble fragment of TDP-43 spanning residues 1–177, which lacks the second RRM domain (RRM2) and the amyloid-forming CTD. This limitation was imposed by the biochemically intractable nature of the CTD that readily aggregates in solution and requires a

high concentration of the ionic detergent sarkosyl to be solubilized (Wright et al., 2020). We found that sarkosyl disrupts the binding of importin $\alpha 1$ and β , as well as denatures Δ IBB-importin $\alpha 1$, making it impossible to study the association of these proteins with TDP-43 using high-resolution biophysical methods. However, we cannot exclude that the TDP-43 domains missing in our analysis, RRM2 and CTD, make additional contacts with importin $\alpha 1/\beta$ that cannot be detected using our minimal TDP-43 construct (NTD-NLS-RRM1). Further studies, both *in vitro* and in live cells, will have to test if there exist additional binding determinants for importin $\alpha 1/\beta$ in TDP-43 RRM2 and CTD capable of modulating the recognition of NLS described in this paper.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.111007>.

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AUTHOR CONTRIBUTIONS

S.G.D., R.K.L., K.G., F.L., R.E.G., Y.-H.K., and J.P. performed the experiments. S.G.D., R.K.L., and G.C. helped conceptualize the experiments. All

authors analyzed the data and provided expertise and feedback. S.G.D., J.P., and G.C. wrote the manuscript with help from all other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

- Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image processing with ImageJ. *Biophot. Int.* 11, 36–42.
- Acerbo, A.S., Cook, M.J., and Gillilan, R.E. (2015). Upgrade of MacCHES facility for X-ray scattering of biological macromolecules in solution. *J. Synchrotron Radiat.* 22, 180–186.
- Afroz, T., Hock, E.M., Ernst, P., Foglieni, C., Jambeau, M., Gilhespy, L.A.B., Laferriere, F., Maniecka, Z., Pluckthun, A., Mittl, P., et al. (2017). Functional and dynamic polymerization of the ALS-linked protein TDP-43 antagonizes its pathologic aggregation. *Nat. Commun.* 8, 45.
- Arseni, D., Hasegawa, M., Murzin, A.G., Kametani, F., Arai, M., Yoshida, M., and Ryskeldi-Falcon, B. (2022). Structure of pathological TDP-43 filaments from ALS with FTL. *Nature* 601, 139–143.
- Ayala, Y.M., Zago, P., D'Ambrogio, A., Xu, Y.F., Petrucelli, L., Buratti, E., and Baralle, F.E. (2008). Structural determinants of the cellular localization and shuttling of TDP-43. *J. Cell Sci.* 121, 3778–3785.
- Berning, B.A., and Walker, A.K. (2019). The pathobiology of TDP-43 C-terminal fragments in ALS and FTL. *Front. Neurosci.* 13, 335.
- Bhattacharya, S., and Lin, X. (2019). Recent advances in computational protocols addressing intrinsically disordered proteins. *Biomolecules* 9.
- Brautigam, C.A. (2015). Calculations and publication-quality illustrations for analytical ultracentrifugation data. *Methods Enzymol.* 562, 109–133.
- Cao, Q., Boyer, D.R., Sawaya, M.R., Ge, P., and Eisenberg, D.S. (2019). Cryo-EM structures of four polymorphic TDP-43 amyloid cores. *Nat. Struct. Mol. Biol.* 26, 619–627.
- Case, D.A., Ben-Shalom, I.Y., Brozell, S.R., Cerutti, D.S., Cheatham, T.E., III, Cruzeiro, V.W.D., Darden, T.A., Duke, R.E., Ghoreishi, D., Gilson, M.K., et al. (2018). AMBER 2018. University of California, San Francisco.
- Chang, C.K., Wu, T.H., Wu, C.Y., Chiang, M.H., Toh, E.K., Hsu, Y.C., Lin, K.F., Liao, Y.H., Huang, T.H., and Huang, J.J. (2012). The N-terminus of TDP-43 promotes its oligomerization and enhances DNA binding affinity. *Biochem. Biophys. Res. Commun.* 425, 219–224.
- Chou, C.C., Zhang, Y., Umoh, M.E., Vaughan, S.W., Lorenzini, I., Liu, F., Sayegh, M., Donlin-Asp, P.G., Chen, Y.H., Duong, D.M., et al. (2018). TDP-43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD. *Nat. Neurosci.* 21, 228–239.
- Cingolani, G., Petosa, C., Weis, K., and Muller, C.W. (1999). Structure of importin-beta bound to the IBB domain of importin-alpha. *Nature* 399, 221–229.
- Colombrita, C., Zennaro, E., Fallini, C., Weber, M., Sommacal, A., Buratti, E., Silani, V., and Ratti, A. (2009). TDP-43 is recruited to stress granules in conditions of oxidative insult. *J. Neurochem.* 111, 1051–1061.
- Colombrita, C., Onesto, E., Megiorni, F., Pizzuti, A., Baralle, F.E., Buratti, E., Silani, V., and Ratti, A. (2012). TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells. *J. Biol. Chem.* 287, 15635–15647.
- Conti, E., and Kuriyan, J. (2000). Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha. *Structure* 8, 329–338.
- Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. *Cell* 94, 193–204.
- Coyne, A.N., Yamada, S.B., Siddegowda, B.B., Estes, P.S., Zaepfel, B.L., Johannesmeyer, J.S., Lockwood, D.B., Pham, L.T., Hart, M.P., Cassel, J.A., et al. (2015). Fragile X protein mitigates TDP-43 toxicity by remodeling RNA granules and restoring translation. *Hum. Mol. Genet.* 24, 6886–6898.
- Darden, T., York, D., and Pedersen, L. (1993). Particle mesh Ewald: an N·log(N) method for Ewald sums in large systems. *J. Chem. Phys.* 98, 10089.
- DeLano, W.L. (2002). The PyMOL Molecular Graphics System (Schrödinger, LLC). Version 1.8.
- Eck, R.J., Kraemer, B.C., and Liachko, N.F. (2021). Regulation of TDP-43 phosphorylation in aging and disease. *Geroscience* 43, 1605–1614.
- Elber, R., Ruymgaart, A.P., and Hess, B. (2011). SHAKE parallelization. *Eur. Phys. J. Spec. Top.* 200, 211–223.
- Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132.
- Fanara, P., Hodel, M.R., Corbett, A.H., and Hodel, A.E. (2000). Quantitative analysis of nuclear localization signal (NLS)-importin alpha interaction through fluorescence depolarization. Evidence for auto-inhibitory regulation of NLS binding. *J. Biol. Chem.* 275, 21218–21223.
- Fontes, M.R., Teh, T., and Kobe, B. (2000). Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin-alpha. *J. Mol. Biol.* 297, 1183–1194.
- Franke, D., Petoukhov, M.V., Konarev, P.V., Panjkovich, A., Tuukkanen, A., Mertens, H.D.T., Kikhney, A.G., Hajizadeh, N.R., Franklin, J.M., Jeffries, C.M., et al. (2017). ATSAS 2.8: a comprehensive data analysis suite for small-angle scattering from macromolecular solutions. *J. Appl. Crystallogr.* 50, 1212–1225.
- Garcia Morato, J., Hans, F., von Zweydford, F., Feederle, R., Elsasser, S.J., Skodras, A.A., Gloeckner, C.J., Buratti, E., Neumann, M., and Kahle, P.J. (2022). Sirtuin-1 sensitive lysine-136 acetylation drives phase separation and pathological aggregation of TDP-43. *Nat. Commun.* 13, 1223.
- Gasset-Rosa, F., Lu, S., Yu, H., Chen, C., Melamed, Z., Guo, L., Shorter, J., Da Cruz, S., and Cleveland, D.W. (2019). Cytoplasmic TDP-43 de-mixing independent of stress granules drives inhibition of nuclear import, loss of nuclear TDP-43, and cell death. *Neuron* 102, 339–357.e7.
- Grant, T.D. (2018). Ab initio electron density determination directly from solution scattering data. *Nat. Methods* 15, 191–193.
- Grujic da Silva, L.A., Simonetti, F., Hutten, S., Riemenschneider, H., Sternburg, E.L., Pietrek, L.M., Gebel, J., Dotsch, V., Edbauer, D., Hummer, G., et al. (2022). Disease-linked TDP-43 hyperphosphorylation suppresses TDP-43 condensation and aggregation. *EMBO J.* 41, e108443.
- Gull, S., and Daniell, G. (1978). Image reconstruction from incomplete and noisy data. *Nature* 272, 686–690.
- Guo, L., Kim, H.J., Wang, H., Monaghan, J., Freyermuth, F., Sung, J.C., O'Donovan, K., Fare, C.M., Diaz, Z., Singh, N., et al. (2018). Nuclear-import receptors reverse aberrant phase transitions of RNA-binding proteins with prion-like domains. *Cell* 173, 677–692.e20.
- Hasegawa, M., Arai, T., Nonaka, T., Kametani, F., Yoshida, M., Hashizume, Y., Beach, T.G., Buratti, E., Baralle, F., Morita, M., et al. (2008). Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Ann. Neurol.* 64, 60–70.
- Hofweber, M., Hutten, S., Bourgeois, B., Spreitzer, E., Niedner-Boblitz, A., Schifferer, M., Ruepp, M.D., Simons, M., Niessing, D., Madl, T., et al. (2018). Phase separation of FUS is suppressed by its nuclear import receptor and arginine methylation. *Cell* 173, 706–719.e13.
- Homeyer, N., and Gohlke, H. (2012). Free energy calculations by the molecular Mechanics Poisson-Boltzmann surface area method. *Mol. Inform.* 31, 114–122.
- Hopkins, J.B., Gillilan, R.E., and Skou, S. (2017). BioXTAS RAW: improvements to a free open-source program for small-angle X-ray scattering data reduction and analysis. *J. Appl. Crystallogr.* 50, 1545–1553.

- Huang, J., Rauscher, S., Nawrocki, G., Ran, T., Feig, M., de Groot, B.L., Grubmüller, H., and MacKerell, A.D., Jr. (2017). CHARMM36m: an improved force field for folded and intrinsically disordered proteins. *Nat. Methods* 14, 71–73.
- Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. *J. Mol. Graph.* 14, 27–38.
- Hutten, S., Usluer, S., Bourgeois, B., Simonetti, F., Odeh, H.M., Fare, C.M., Czuppa, M., Hruska-Plochan, M., Hofweber, M., Polymenidou, M., et al. (2020). Nuclear import receptors directly bind to arginine-rich dipeptide repeat proteins and suppress their pathological interactions. *Cell Rep.* 33, 108538.
- Jiang, L.L., Xue, W., Hong, J.Y., Zhang, J.T., Li, M.J., Yu, S.N., He, J.H., and Hu, H.Y. (2017). The N-terminal dimerization is required for TDP-43 splicing activity. *Sci. Rep.* 7, 6196.
- Johnson, B.S., McCaffery, J.M., Lindquist, S., and Gitler, A.D. (2008). A yeast TDP-43 proteinopathy model: exploring the molecular determinants of TDP-43 aggregation and cellular toxicity. *Proc. Natl. Acad. Sci. USA* 105, 6439–6444.
- Kametani, F., Nonaka, T., Suzuki, T., Arai, T., Dohmae, N., Akiyama, H., and Hasegawa, M. (2009). Identification of casein kinase-1 phosphorylation sites on TDP-43. *Biochem. Biophys. Res. Commun.* 382, 405–409.
- Kametani, F., Obi, T., Shishido, T., Akatsu, H., Murayama, S., Saito, Y., Yoshida, M., and Hasegawa, M. (2016). Mass spectrometric analysis of accumulated TDP-43 in amyotrophic lateral sclerosis brains. *Sci. Rep.* 6, 23281.
- Khosravi, B., LaClair, K.D., Riemenschneider, H., Zhou, Q., Frotin, F., Mareljic, N., Czuppa, M., Famy, D., Hartmann, H., Michaelsen, M., et al. (2020). Cell-to-cell transmission of C9orf72 poly-(Gly-Ala) triggers key features of ALS/FTD. *EMBO J.* 39, e102811.
- Kobe, B. (1999). Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha. *Nat. Struct. Biol.* 6, 388–397.
- Kralt, A., Jagalur, N.B., van den Boom, V., Lokareddy, R.K., Steen, A., Cingolani, G., Fornerod, M., and Veenhoff, L.M. (2015). Conservation of inner nuclear membrane targeting sequences in mammalian Pom121 and yeast Heh2 membrane proteins. *Mol. Biol. Cell* 26, 3301–3312.
- Krissinel, E., and Henrick, K. (2007). Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797.
- Kuo, P.H., Chiang, C.H., Wang, Y.T., Doudeva, L.G., and Yuan, H.S. (2014). The crystal structure of TDP-43 RRM1-DNA complex reveals the specific recognition for UG- and TG-rich nucleic acids. *Nucleic Acids Res.* 42, 4712–4722.
- Kurischko, C., and Broach, J.R. (2017). Phosphorylation and nuclear transit modulate the balance between normal function and terminal aggregation of the yeast RNA-binding protein Ssd1. *Mol. Biol. Cell* 28, 3057–3069.
- Laskowski, R.A. (2009). PDBsum new things. *Nucleic Acids Res.* 37, D355–D359.
- Laue, T.M., Shah, B., Ridgeway, T.M., and Pelletier, S.L. (1992). Computer-aided interpretation of analytical sedimentation data for proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, S. Harding, A. Rowe, and J. Horton, eds. (Royal Society of Chemistry), pp. 90–125.
- Lee, B.J., Cansizoglu, A.E., Suel, K.E., Louis, T.H., Zhang, Z., and Chook, Y.M. (2006). Rules for nuclear localization sequence recognition by karyopherin beta 2. *Cell* 126, 543–558.
- Li, Q., Babinchak, W.M., and Surewicz, W.K. (2021). Cryo-EM structure of amyloid fibrils formed by the entire low complexity domain of TDP-43. *Nat. Commun.* 12, 1620.
- Liebschner, D., Afonine, P.V., Moriarty, N.W., Poon, B.K., Sobolev, O.V., Terwilliger, T.C., and Adams, P.D. (2017). Polder maps: improving OMIT maps by excluding bulk solvent. *Acta Crystallogr. D Struct. Biol.* 73, 148–157.
- Liebschner, D., Afonine, P.V., Baker, M.L., Bunkóczi, G., Chen, V.B., Croll, T.I., Hintze, B., Hung, L.W., Jain, S., McCoy, A.J., et al. (2019). Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. *Acta Crystallogr. D Struct. Biol.* 75, 861–877.
- Liu, E.A., Mori, E., Hamasaki, F., and Lieberman, A.P. (2021). TDP-43 proteinopathy occurs independently of autophagic substrate accumulation and underlies nuclear defects in Niemann-Pick C disease. *Neuropathol. Appl. Neurobiol.* 7, 1019–1032. <https://doi.org/10.1111/nan.12738>.
- Lokareddy, R.K., Hapsari, R.A., van Rheenen, M., Pumroy, R.A., Bhardwaj, A., Steen, A., Veenhoff, L.M., and Cingolani, G. (2015). Distinctive properties of the nuclear localization signals of inner nuclear membrane proteins Heh1 and Heh2. *Structure* 23, 1305–1316.
- Lott, K., and Cingolani, G. (2011). The importin beta binding domain as a master regulator of nucleocytoplasmic transport. *Biochim. Biophys. Acta* 1813, 1578–1592.
- Majumder, P., Chu, J.F., Chatterjee, B., Swamy, K.B., and Shen, C.J. (2016). Co-regulation of mRNA translation by TDP-43 and Fragile X Syndrome protein FMRP. *Acta Neuropathol.* 132, 721–738.
- Marfori, M., Mynott, A., Ellis, J.J., Mehdi, A.M., Saunders, N.F., Curmi, P.M., Forwood, J.K., Boden, M., and Kobe, B. (2011). Molecular basis for specificity of nuclear import and prediction of nuclear localization. *Biochim. Biophys. Acta* 1813, 1562–1577.
- Maris, C., Dominguez, C., and Allain, F.H. (2005). The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. *FEBS J.* 272, 2118–2131.
- McCoy, A.J. (2007). Solving structures of protein complexes by molecular replacement with Phaser. *Acta Crystallogr. D Biol. Crystallogr.* 63, 32–41.
- Miller, B.R., 3rd, McGee, T.D., Jr., Swails, J.M., Homeyer, N., Gohlke, H., and Roitberg, A.E. (2012). MMPBSA.py: an efficient program for end-state free energy calculations. *J. Chem. Theor. Comput.* 8, 3314–3321.
- Miyatake, H., Sanjoh, A., Unzai, S., Matsuda, G., Tatsumi, Y., Miyamoto, Y., Dohmae, N., and Aida, Y. (2015). Crystal structure of human importin-alpha1 (Rch1), revealing a potential autoinhibition mode involving homodimerization. *PLoS One* 10, e0115995.
- Mompean, M., Romano, V., Pantoja-Uceda, D., Stuaní, C., Baralle, F.E., Buratti, E., and Laurents, D.V. (2016). The TDP-43 N-terminal domain structure at high resolution. *FEBS J.* 283, 1242–1260.
- Mompean, M., Romano, V., Pantoja-Uceda, D., Stuaní, C., Baralle, F.E., Buratti, E., and Laurents, D.V. (2017). Point mutations in the N-terminal domain of transactive response DNA-binding protein 43 kDa (TDP-43) compromise its stability, dimerization, and functions. *J. Biol. Chem.* 292, 11992–12006.
- Nakada, R., Hirano, H., and Matsuura, Y. (2015). Structure of importin-alpha bound to a non-classical nuclear localization signal of the influenza A virus nucleoprotein. *Sci. Rep.* 5, 15055.
- Nardozi, J.D., Lott, K., and Cingolani, G. (2010). Phosphorylation meets nuclear import: a review. *Cell Commun. Signal.* 8, 32.
- Nishimura, A.L., Zupunski, V., Troakes, C., Kathe, C., Fratta, P., Howell, M., Gallo, J.M., Hortobágyi, T., Shaw, C.E., and Rogelj, B. (2010). Nuclear import impairment causes cytoplasmic trans-activation response DNA-binding protein accumulation and is associated with frontotemporal lobar degeneration. *Brain* 133, 1763–1771.
- Nonaka, T., Suzuki, G., Tanaka, Y., Kametani, F., Hirai, S., Okado, H., Miyashita, T., Saitoe, M., Akiyama, H., Masai, H., et al. (2016). Phosphorylation of TAR DNA-binding protein of 43 kDa (TDP-43) by truncated casein kinase 1delta triggers mislocalization and accumulation of TDP-43. *J. Biol. Chem.* 291, 5473–5483.
- Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 276, 307–326. *Macromolecular Crystallography*.
- Perez-Hernandez, G., Paul, F., Giorgino, T., De Fabritiis, G., and Noe, F. (2013). Identification of slow molecular order parameters for Markov model construction. *J. Chem. Phys.* 139, 015102.
- Petersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612.
- Pinarbasi, E.S., Çağatay, T., Fung, H.Y.J., Li, Y.C., Chook, Y.M., and Thomas, P.J. (2018). Active nuclear import and passive nuclear export are the primary determinants of TDP-43 localization. *Sci. Rep.* 8, 7083.

- Polizzi, N.F., Therien, M.J., and Beratan, D.N. (2016). Mean first-passage times in Biology. *Isr. J. Chem.* 56, 816–824.
- Prasad, A., Bharathi, V., Sivalingam, V., Girdhar, A., and Patel, B.K. (2019). Molecular mechanisms of TDP-43 misfolding and pathology in amyotrophic lateral sclerosis. *Front. Mol. Neurosci.* 12, 25.
- Pumroy, R.A., and Cingolani, G. (2015). Diversification of importin- α isoforms in cellular trafficking and disease states. *Biochem. J.* 466, 13–28.
- Pumroy, R.A., Ke, S., Hart, D.J., Zachariae, U., and Cingolani, G. (2015). Molecular determinants for nuclear import of influenza A PB2 by importin α isoforms 3 and 7. *Structure* 23, 374–384.
- Qamar, S., Wang, G., Randle, S.J., Ruggeri, F.S., Varela, J.A., Lin, J.Q., Phillips, E.C., Miyashita, A., Williams, D., Strohl, F., et al. (2018). FUS phase separation is modulated by a molecular chaperone and methylation of arginine cation- π interactions. *Cell* 173, 720–734.e15.
- Riddick, G., and Macara, I.G. (2005). A systems analysis of importin- $\{\alpha\}$ - $\{\beta\}$ mediated nuclear protein import. *J. Cell Biol.* 168, 1027–1038.
- Roblitz, S., Stotzel, C., Deuffhard, P., Jones, H.M., Azulay, D.O., van der Graaf, P.H., and Martin, S.W. (2013). A mathematical model of the human menstrual cycle for the administration of GnRH analogues. *J. Theor. Biol.* 321, 8–27.
- Sankhala, R.S., Lokareddy, R.K., Begum, S., Pumroy, R.A., Gillilan, R.E., and Cingolani, G. (2017). Three-dimensional context rather than NLS amino acid sequence determines importin α subtype specificity for RCC1. *Nat. Commun.* 8, 979.
- Scherer, M.K., Trendelkamp-Schroer, B., Paul, F., Pérez-Hernández, G., Hoffmann, M., Plattner, N., Wehmeyer, C., Prinz, J.H., and Noé, F. (2015). PyEMMA 2: a software package for estimation, validation, and analysis of Markov models. *J. Chem. Theor. Comput.* 11, 5525–5542.
- Schneidman-Duhovny, D., Hammel, M., and Sali, A. (2010). FoXS: a web server for rapid computation and fitting of SAXS profiles. *Nucleic Acids Res.* 38, W540–W544.
- Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys. J.* 78, 1606–1619.
- Sephton, C.F., Cenik, C., Kucukural, A., Dammer, E.B., Cenik, B., Han, Y., Dewey, C.M., Roth, F.P., Herz, J., Peng, J., et al. (2011). Identification of neuronal RNA targets of TDP-43-containing ribonucleoprotein complexes. *J. Biol. Chem.* 286, 1204–1215.
- Shiina, Y., Arima, K., Tabunoki, H., and Satoh, J. (2010). TDP-43 dimerizes in human cells in culture. *Cell. Mol. Neurobiol.* 30, 641–652.
- Tian, C., Kasavajhala, K., Belfon, K.A.A., Raguette, L., Huang, H., Miguez, A.N., Bickel, J., Wang, Y., Pincay, J., Wu, Q., et al. (2020). ff19SB: amino-acid-specific protein backbone parameters trained against quantum Mechanics energy surfaces in solution. *J. Chem. Theor. Comput.* 16, 528–552.
- Tollervey, J.R., Curk, T., Rogelj, B., Briesse, M., Cereda, M., Kayikci, M., König, J., Hortobágyi, T., Nishimura, A.L., Zupunski, V., et al. (2011). Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. *Nat. Neurosci.* 14, 452–458.
- Tsoi, P.S., Choi, K.J., Leonard, P.G., Sizovs, A., Moosa, M.M., MacKenzie, K.R., Ferreón, J.C., and Ferreón, A.C.M. (2017). The N-terminal domain of ALS-linked TDP-43 assembles without misfolding. *Angew. Chem. Int. Ed. Engl.* 56, 12590–12593.
- Wang, Y.T., Kuo, P.H., Chiang, C.H., Liang, J.R., Chen, Y.R., Wang, S., Shen, J.C., and Yuan, H.S. (2013). The truncated C-terminal RNA recognition motif of TDP-43 protein plays a key role in forming proteinaceous aggregates. *J. Biol. Chem.* 288, 9049–9057.
- Wang, A., Conicella, A.E., Schmidt, H.B., Martin, E.W., Rhoads, S.N., Reeb, A.N., Nourse, A., Ramirez Montero, D., Ryan, V.H., Rohatgi, R., et al. (2018). A single N-terminal phosphomimic disrupts TDP-43 polymerization, phase separation, and RNA splicing. *EMBO J.* 37.
- Winton, M.J., Igaz, L.M., Wong, M.M., Kwong, L.K., Trojanowski, J.Q., and Lee, V.M. (2008a). Disturbance of nuclear and cytoplasmic TAR DNA-binding protein (TDP-43) induces disease-like redistribution, sequestration, and aggregate formation. *J. Biol. Chem.* 283, 13302–13309.
- Winton, M.J., Van Deerlin, V.M., Kwong, L.K., Yuan, W., Wood, E.M., Yu, C.E., Schellenberg, G.D., Rademakers, R., Caselli, R., Karydas, A., et al. (2008b). A90V TDP-43 variant results in the aberrant localization of TDP-43 in vitro. *FEBS Lett.* 582, 2252–2256.
- Wright, G.S.A., Watanabe, T.F., Ampornpanai, K., Plotkin, S.S., Cashman, N.R., Antonyuk, S.V., and Hasnain, S.S. (2020). Purification and structural characterization of aggregation-prone human TDP-43 involved in neurodegenerative diseases. *iScience* 23, 101159.
- Yang, C., Tan, W., Whittle, C., Qiu, L., Cao, L., Akbarian, S., and Xu, Z. (2010). The C-terminal TDP-43 fragments have a high aggregation propensity and harm neurons by a dominant-negative mechanism. *PLoS One* 5, e15878.
- Yoshizawa, T., Ali, R., Jiou, J., Fung, H.Y.J., Burke, K.A., Kim, S.J., Lin, Y., Peebles, W.B., Saltzberg, D., Soniat, M., et al. (2018). Nuclear import receptor inhibits phase separation of FUS through binding to multiple sites. *Cell* 173, 693–705.e22.
- Zhang, Y.J., Xu, Y.F., Cook, C., Gendron, T.F., Roettges, P., Link, C.D., Lin, W.L., Tong, J., Castaneda-Casey, M., Ash, P., et al. (2009). Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. *Proc. Natl. Acad. Sci. USA* 106, 7607–7612.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HRP conjugated anti-GFP polyclonal primary antibody	Millipore Sigma	Cat#GERPN1236; RRID:AB_771429
Bacterial and virus strains		
BL21-DE3	Millipore Sigma	Cat#CMC0016
BL21-DE3 LOBSTR	Kerafast	Cat#EC1002
Chemicals, peptides, and recombinant proteins		
Δ IBB-importin α 1 construct	This paper	Cingolani_001
Importin α 1/ β heterodimer	This paper	Cingolani_002
GST-TDP-43 NTD-NLS-RRM1 wild-type	This paper	Cingolani_003
GST-TDP-43 NTD-NLS-RRM1 T88E (pT88)	This paper	Cingolani_004
GST-TDP-43 NTD-NLS-RRM1 S91E (pS91)	This paper	Cingolani_005
GST-TDP-43 NTD-NLS-RRM1 S92E (pS92)	This paper	Cingolani_006
GST-TDP-43 NTD-NLS-RRM1 T88E, S91E, S92E (pT88+pS91+pS92)	This paper	Cingolani_007
GST-TDP-43 NTD-NLS	This paper	Cingolani_008
GST-TDP-43 NLS-RRM1	This paper	Cingolani_009
Ampicillin	ThermoFisher Scientific	CAS#69-53-4 Cat#11593027
Kanamycin sulfate	ThermoFisher Scientific	CAS#25389-94-0 Cat#15160054
Chloramphenicol	ThermoFisher Scientific	CAS#56-75-7 Cat#227925000
3,3',5,5'-Tetramethylbenzidine (TMB)	abcam	CAS#54827-17-7 Cat#ab171523
Isopropylthio- β -galactoside (IPTG), >99%	ThermoFisher Scientific	CAS#367-93-1 Cat#15529019
Sodium Chloride (NaCl), >99%	ThermoFisher Scientific	CAS#7647-14-5 Cat#A12313.0I
Tris base, >98%	ThermoFisher Scientific	CAS#77-86-1 Cat#J22675.36
2-mercaptoethanol (BME), 14.3 M, >99%	Millipore Sigma	CAS#60-24-2 Cat#M6250
Tween-20	Millipore Sigma	CAS#9005-64-5 Cat# P1379
Hydrochloric acid (HCl), 12 N	ThermoFisher Scientific	CAS#7647-01-0 Cat#NC004378
Dulbecco's Phosphate buffered saline (PBS), 10x	Millipore Sigma	CAS#7647-14-5 Cat#D1408
Ethylenedinitrotetraacetic acid disodium salt (EDTA), >99%,	Millipore Sigma	CAS#6381-92-6 Cat#E5134
Imidazole, >99%	Millipore Sigma	CAS#288-32-4 Cat#814223
High density nickel resin	GoldBio	Cat#H-320-5
GST agarose resin	GoldBio	Cat#G-250-5
Critical commercial assays		
Pierce BCA Protein Assay Kit	ThermoFisher Scientific	Cat#23227
Natrix Crystallization Screen	Hampton Research	HR2-116

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Natrix 2 Crystalization Screen	Hampton Research	HR2-117
Crystal Screen	Hampton Research	HR2-110
Index Screen	Hampton Research	HR2-144
Deposited data		
Raw and analyzed X-ray data for TDP-43 NTD-NLS+ΔIBB-importin α1	This paper	Mendely Data: https://data.mendeley.com/datasets/mpkdx4hsj/
TDP-43 NTD-NLS+ΔIBB-importin α1 PDB	This paper	PDB: 7N9H
Recombinant DNA		
Vector: pET-30a	EMD biosciences/Millipore Sigma	Cat#69909
Vector: pGEX-6p-1	Amersham/Millipore Sigma	Cat# GE28-9546-48
Vector: pETduet-1	EDM biosciences/Millipore Sigma	Cat#71146
Software and algorithms		
ImageJ	(Abramoff et al., 2004)	N/A
PyMol	(DeLano, 2002)	N/A
HKL2000	(Otwinowski and Minor, 1997)	N/A
Phaser	(McCoy, 2007)	N/A
Phenix.refine	(Liebschner et al., 2019)	Version 1.19.2
Phenix.polder	(Liebschner et al., 2017)	N/A
COOT	(Emsley and Cowtan, 2004)	N/A
ATSAS	(Franke et al., 2017)	N/A
RAW	(Hopkins et al., 2017)	Version 2.1.1
DENSS	(Grant, 2018)	N/A
Chimera	(Pettersen et al., 2004)	N/A
FoXS Server	(Schneidman-Duhovny et al., 2010)	N/A
GraphPad Prism	Non-linear regression: one-site specific binding curve	Version 9.0.0
Chromlab	Bio-Rad	N/A
SEDFIT	(Schuck, 2000)	Center for Information Technology, NIH
SEDNTERP	(Laue et al., 1992)	N/A
GUSSI	UTSouthwestern Medical Center	N/A
PDBsum	(Laskowski, 2009)	N/A
PISA	(Krissinel and Henrick, 2007)	N/A
Pyemma	(Scherer et al., 2015)	Version 2.5.7
Amber	(D.A. Case et al., 2018)	Version 18
SHAKE algorithm	(Elber et al., 2011)	N/A
PCCA++ algorithm	(Roblitz et al., 2013)	N/A
VMD	(Humphrey et al., 1996)	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Gino Cingolani (gino.cingolani@jefferson.edu).

Materials availability

All unique/stable reagents generated in this study are available from the [lead contact](#) with a completed materials transfer agreement.

Data and code availability

- The crystallographic coordinates for the TDP-43 NTD-NLS+ΔIBB-importin α1 complex have been deposited in the Protein Data Bank and raw X-ray diffraction data are posted on Mendeley Data and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#).

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We made use of an *E. coli* expression system for the synthesis of recombinant proteins. *E. coli* of the BL21-DE3 strain or derivations therein were transformed with one or more constructs and exposed to the corresponding selection conditions.

METHOD DETAILS

Molecular biology

Fragments of human TDP-43 (Figure 1A) were synthesized by GeneWiz and inserted into a pGEX-6p-1 vector. In the case of the TDP-43 NTD-NLS-RRM1 phosphomimetics, glutamic acid was individually substituted for each of the three residues within the NLS linker (T88, S91, and S92). An additional construct in which all of these residues were mutated to glutamic acid was also generated. Using the TDP-43 NLS:ΔIBB-importin α 1 structure, we designed a second set of TDP-43 NTD-NLS-RRM1 point mutants in which the residues at the P₂ (K97) and P₂' (R83) positions within the NLS were replaced with alanine; either individually or in concert. The gene encoding mouse importin α 1 lacking the IBB domain was cloned in a pET30a vector (Novagen) (Sankhala et al., 2017). Human FL importin α 1 and importin β were cloned in tandem into a pETDuet-1 vector for co-expression.

Protein expression and purification

The pGEX-6p-1-TDP-43 NTD-NLS and pGEX-6p-1-TDP-43 NLS-RRM1 constructs were each co-transformed with pET30a-ΔIBB importin α 1 into *E. coli* BL21-DE3 expression strains. Protein was expressed overnight at 18°C with 400 μ M IPTG. Complexes consisting of GST-TDP-43 fragments bound to ΔIBB-importin α 1 were purified in a low-salt buffer (150 mM NaCl, 20 mM Tris pH 8, 5 mM BME, 1 mM EDTA, and 0.1% Tween 20) and bound to glutathione agarose resin. The GST tag was removed through overnight PreScission Protease (PPase) cleavage at 4°C on a gravity flow column, and untagged TDP-43 fragments bound to ΔIBB-importin α 1 were collected in the column flowthrough. These complexes were further purified through size-exclusion chromatography using a Superdex 200 16:600 preparative column. Fractions corresponding to mono-disperse TDP-43:ΔIBB-importin α 1 at a 1:1 ratio were pooled and concentrated in a 30 kDa concentrator (Vivaspin) and subjected to downstream applications.

For crystallization of the pGEX-6p-1-TDP-43 NTD-NLS-RRM1 complex with ΔIBB-importin α 1, both proteins were individually transformed into BL21-DE3 *E. coli* expression strains. The pGEX-6-1-TDP-43 NTD-NLS-RRM1 was expressed overnight at 18°C with 400 μ M IPTG. The pET30a-ΔIBB importin α 1 was expressed for 5 h at 30°C with 400 μ M IPTG. TDP-43 NTD-NLS-RRM1 was purified in a low-salt buffer (150 mM NaCl, 20 mM Tris pH 8, 5 mM BME, 1 mM EDTA, and 0.1% Tween 20) and bound to glutathione agarose resin. The GST tag was removed through overnight PPase cleavage at 4°C on a gravity flow column, and untagged TDP-43 NTD-NLS-RRM1 was collected in the column flowthrough. The protein was further purified by SEC using a Superdex 200 10:300 analytical column. Fractions corresponding to mono-disperse TDP-43 were used in the formation of TDP-43:ΔIBB-importin α 1 complex. The ΔIBB-importin α 1 was purified in a high salt buffer (600 mM NaCl, 20 mM Tris pH 8.5, 3 mM BME) and bound to high-density nickel resin. The protein was eluted in 150 mM imidazole and immediately subjected to SEC using a Superdex 200 10:300 analytical column. Fractions corresponding to mono-disperse, monomeric importin α 1 were concentrated in a 30 kDa concentrator (Vivaspin) and quantified. Purified TDP-43 NTD-NLS-RRM1 was concentrated in a 10 kDa concentrator and quantified in tandem. The two proteins were pooled at a ratio in which TDP-43 NTD-NLS-RRM1 was in excess. Complex formation proceeded at 25°C for 30 min, after which the sample was applied to a Superdex 200 10:300 column and subjected to a further round of SEC. Fractions corresponding to the monodisperse complex at an apparent 1:1 ratio, as determined by elution profile, were used in the downstream application, specifically hanging drop vapor diffusion.

Similar to the above, the pGEX-6p-1-TDP-43 NTD-NLS-RRM1 constructs (WT P₂, P₂', and P₂+P₂' mutants) were co-transformed with pETDuet-1 importin α 1/β into BL21-DE3 *E. coli* expression strain. Proteins were expressed for 1.5 h at 30°C with 500 μ M IPTG. Heterotrimeric complex consisting of TDP-43 NTD-NLS-RRM1 and importin α 1/β was purified in a low-salt buffer (75 mM NaCl, 20 mM Tris pH 8, and 3 mM BME) and bound to low-density nickel beads. Complex was eluted using a low-imidazole elution buffer (75 mM imidazole, 50 mM NaCl, 20 mM NaCl, and 3 mM BME).

The TDP-43 NTD-NLS, NLS-RRM1, and NTD-NLS-RRM1 constructs could be expressed and purified individually using the same conditions employed for the TDP-43:ΔIBB-importin α 1 complex. Likewise, the importin α 1/β heterodimer could be expressed purified as described for the TDP-43:α1:β heterotrimeric complex.

All SEC in this paper was conducted using a BioRad NGC single wavelength FPLC unit. The Chromlab software was provided by BioRad to facilitate visualization and analysis of chromatograms. The chromatograms presented here are derivative of Chromlab.

Crystallographic methods

Crystals of ΔIBB-importin α 1 bound to TDP-43 NTD-NLS were obtained using the hanging drop vapor diffusion method. A droplet containing 1 μ L of gel filtration-purified complex at 5 mg mL⁻¹ and was mixed with an equal volume of 0.1 M sodium citrate tribasic

dihydrate pH 5.6, 0.7 M sodium citrate tribasic dihydrate, 10 mM BME, and equilibrated against 600 μ L of the precipitant, at 18°C. Crystals were harvested in nylon cryo-loops, cryo-protected with 27% ethylene glycol, and flash-frozen in liquid nitrogen. Crystals were diffracted at the Stanford Synchrotron Radiation Lightsources (SSRL) using beamline 9-2. Data were indexed, integrated, and scaled using HKL2000 (Otwinowski and Minor, 1997). Initial phases were obtained by molecular replacement using Phaser (McCoy, 2007) and PDB 1Y2A as a search model. The TDP-43 NLS was built in Fo-Fc electron density difference maps using Coot (Emsley and Cowtan, 2004), and complete atomic models were refined using *phenix.refine* (Liebschner et al., 2019) using positional and isotropic B-factor refinement cycles with six Translation:Libration:Screw groups. Polder omit maps were calculated using *phenix.polder* (Liebschner et al., 2017). Data collection and refinement statistics are summarized in Table S1. All ribbon diagrams and surface representations were prepared using PyMol (DeLano, 2002).

Size exclusion chromatography coupled to small-angle X-ray diffraction (SEC-SAXS)

Heterodimeric complexes consisting of TDP-43 NTD-NLS: Δ IBB-importin α 1 and TDP-43 NLS-RRM1: Δ IBB-importin α 1 were submitted to the Cornell High Energy Synchrotron Source (CHESS) and Cornell University in Ithaca, NY. The complex was first separated on a Superdex 200 10:300 analytical column and then subjected to X-ray diffraction. Small-angle diffraction data was collected and subsequently processed using the RAW software package (version 2.1.1) (Hopkins et al., 2017), including plug-ins from ATSAS (Franke et al., 2017). Sample peak intensities were normalized to a region of the scattering baseline that satisfied the following conditions: 1. The selected baseline did not include any individual frames that differed significantly from the average intensity and 2. The selected baseline produced a distribution of radii of gyration that included a Guinier region. From the Guinier region, the molecular mass of each complex was predicted, and a P(r) plot was generated. P(r) plots corresponding to each complex were submitted to the DENSITY from Solution Scattering (DENSS) (Grant, 2018) plug-in to generate low-resolution electron densities. We modeled the TDP-43 NTD-NLS and NLS-RRM1 bound to Δ IBB-importin α 1 using the crystal structure of TDP-43 NLS: Δ IBB-importin α 1 complex determined in this paper and previously determined structures of the TDP-43 NTD (PDB: 5MDI) and TDP-43 RRM1 (PDB: 4BS2). These complexes were fit into the DENSS density and refined against the low-resolution SAXS density using the *fit-into-map* command in Chimera (Pettersen et al., 2004). The scattering profiles of these models were compared to the empirically observed scattering data using the FoXS server (Schneidman-Duhovny et al., 2010), which generated a predicted-to-observed χ^2 value in each case.

ELISA-based microtiter binding assay

GST-TDP-43 NTD-NLS-RRM1 WT and phosphomimetic constructs were individually purified and dialyzed overnight against 150 mM NaCl, 20 mM Tris pH 8, and 0.05% Tween 20. The importin α 1/ β heterodimer was purified and isolated by SEC using a Superdex 200 16:60 preparative column. The heterodimer was diluted to 2 μ g/ml in adsorption buffer (approximately 50 mM sodium carbonate:sodium bicarbonate) and bound to MaxiSorp® (Nunc) polystyrene plates at a final concentration of 200 ng per well overnight at 4°C. Plates were then blocked at 25°C for at least two hours in blocking buffer (0.1% PBS-Tween with 2% BSA). TDP-43 NTD-NLS-RRM1 ligands were prepared in dilution gradients from 0 to 500 nM in blocking buffer. Ligand dilutions were applied to the ELISA plate in 100 μ L aliquots and incubated for 1 h at 25°C. Unbound ligand was removed through washing, and plates were then incubated with an HRP-conjugated anti-GST polyclonal antibody (Sigma) at a 1:5000 dilution for 1 h at 37°C. Plates were then washed to remove excess antibodies, followed by the application of 100 μ L of TMB substrate. The colorimetric reaction was allowed to proceed for 15 min at 25°C before being stopped through the addition of 2 N HCl. Plates were read at 450 nm using a Tecan microplate reader (Life Sciences). Each ELISA-based assay was run in duplicate and independently reproduced at least two times. Binding isotherms were plotted in GraphPad Prism and fit by a one-site specific binding non-linear regression. Errors are given as the standard deviation across independent experiments.

Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

SV-AUC experiments on TDP-43 NTD-NLS: Δ IBB-importin α 1 heterodimer was performed at concentrations ranging 1–2 mg/mL in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 3 mM β -ME. Centrifugation was performed at 40,000 rpm at 6°C using a Beckman XLA Analytical Ultracentrifuge (Beckman-Coulter, Brea, CA). SV-AUC experiments on TDP-43 NTD-NLS and Importin α / β were performed at 20°C with an Optima analytical ultracentrifuge (Beckman-Coulter, Brea, CA) and a TiAn50 rotor with two-channel charcoal-filled Epon centerpieces and sapphire windows, using both absorbance and interference optics. Data were collected with detection at 260, 280, & 295 nm, as well as interference optics. TDP-43 NTD-NLS analyses were performed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 3 mM β -ME, and Importin α / β -NTD-NLS analyses were performed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, and 2 mM DTT. Complete sedimentation velocity profiles were recorded every 30 s at 40,000 rpm. Data were fit using the c(S) implementations of the Lamm equation as implemented in the program SEDFIT (Schuck, 2000). The partial specific volume (\bar{v}), solvent density (ρ), and viscosity (η) were derived from chemical composition by SEDNTERP (Laue et al., 1992). All S values calculated were corrected to $s_{20,w}$ values. Figures were created using the program GUSI (Brautigam, 2015).

In cellulo pull-down assays

Plasmids encoding pGEX-6p-1-TDP-43 NTD-NLS-RRM1 (WT, K97A, R83A, K97A/R83A) mutants were co-expressed in *E. coli* BL21-DE3 expression strain with the pETDuet-1 plasmid expressing importin α / β . Protein was expressed for 1.5 h at 30°C with 500 μ M IPTG. Pre- and post-induction optical densities were measured for each co-transformant to ensure a similar rate of growth

across all cultures. Trimeric complexes were purified as previously described for the importin $\alpha 1/\beta$ complex and eluted from equal volumes of low-density nickel beads. Eluates were gently concentrated in 10 kDa concentrators (Vivaspin) to facilitate analysis by SDS-PAGE gel. Volumes corresponding to an equal mass of importin $\alpha 1$ were loaded onto a 13.7% SDS-PAGE gel alongside molecular weight marker. SDS-PAGE gels were run in triplicate ($n = 3$), and the average ratio of GST-TDP-43 NTD-NLS-RRM1 to importin $\alpha 1$ band intensity was calculated for each construct through densitometry using ImageJ (Abramoff et al., 2004). To determine statistical significance between the means of the control and experimental pull-downs, we employed a two-tailed Student's T test assuming unequal variance. The alpha parameter had to be outside the 99th percentile for statistical significance ($p < 0.01$, indicated by ***). Error bars indicate the standard deviation about the mean.

Molecular dynamics (MD) simulations

The atomic structure of TDP-43 NLS bound to Δ IBB-importin $\alpha 1$, explained in [Crystallographic methods](#), was used as the initial structure for MD simulations. Unphosphorylated TDP-43 NLS was utilized as the “wild-type” structure and was additionally utilized to prepare three single phosphorylated constructs, namely pT88, pS91, and pS92. An additional hyperphosphorylated construct was prepared, incorporating all three phosphorylations: pT88 + pS91 + pS92. The complex was placed in a solvent box with 12 Å of padding in positive and negative x, y, and z directions from the protein periphery. Systems employed the TIP3P water model, and all were charge neutralized and set to a NaCl concentration of 150 mM. The resulting models contained approximately 65,000 atoms, comprised of importin $\alpha 1$, TDP-43 NLS, ions, and water. The dimensions of each prepared system were $108 \times 108 \times 74 \text{ Å}^3$. After construction, all systems were initially subjected to a minimization and thermalization procedure. During thermalization, the system was heated to 310 K gradually over 50 ps, followed by a short 1 ns simulation performed while the protein atoms were positionally restrained using a force constant of 1.0 kcal/mol Å^2 . Subsequently, all systems were equilibrated over 20 ns using the NPT ensemble without any restraints on the solute atoms (Table S4). Consequently, the production simulations employed five replicas, where each was run for 100 ns to generate the trajectory and be used for the Molecular Mechanics Poisson–Boltzmann Surface Area (Homeyer and Gohlke, 2012; Miller et al., 2012) MM/PBSA calculation. For each frame, the MM/PBSA was estimated, and then the mean value and standard deviation as the error bar were computed and shown in Figure S8. Minimization, thermalization, equilibration, and production steps were completed in Amber 18 (D.A. Case et al., 2018) using the ff19SB force field (Tian et al., 2020), employing a time step of 2 fs. The temperature was maintained with a Langevin heat bath, with a collision frequency of 1.0 ps^{-1} . The SHAKE algorithm (Elber et al., 2011) was applied to restrain all covalent bonds involving hydrogen atoms. Long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method (T. Darden et al., 1993), and the nonbonded interactions were calculated with a cutoff distance of 8 Å. Our results indicated a significant difference between ΔG of WT and hyperphosphorylated structures in which the error bars from the multiple replicas of these two structures do not overlap. Therefore, WT and hyperphosphorylated TDP-43 NLS have the strongest and weakest binding affinity to Δ IBB-importin $\alpha 1$, respectively (Figure S8). On the other hand, the other three phosphorylated structures, pThr88, pSer91, and pSer92, have a ΔG that is placed between the ΔG values of WT and hyperphosphorylated complexes. This finding is also confirmed by the error bars definitions, which demonstrate a significant overlap between the pThr88, pSer91, and pSer92 complexes. Thus, the binding affinity of the single phosphorylated systems (pThr88, pSer91, and pSer92) are stronger than the hyperphosphorylated complex but weaker than the WT TDP-43 NLS complex. Additionally, for each of the complexes simulated in Figure 5, we performed cross-correlation analysis of the simulated structures against the experimental SAXS density (Figure S7). The MD trajectory of each system first was fitted to the experimental density map derived from SAXS data. The cross-correlation was calculated using the VMD Voltool package (Humphrey et al., 1996) to compare the SAXS data with the MD simulation trajectories, which suggested alternate conformations were not observed in our simulations.

Additionally, a second set of isolated TDP-43 NLS simulations, incorporating WT NLS and phosphorylated NLS systems, were performed. The resulting systems contained approximately 19,600 atoms each, including TDP-43 NLS, ions, and TIP3P water. Systems were neutralized, and the NaCl concentration was set to 150mM. The systems were minimized and equilibrated using Amber 18 (D.A. Case et al., 2018). We ran equilibrium simulations for 200 ns, for each phosphorylated NLS structure then selected structures at intervals of 33 ns to begin six independent production simulations for each construct. The six simulations per phosphorylated system were run for 1 μs each, totaling 6 μs for each TDP-43 variant (Scherer et al., 2015). First, the coordinates of isolated TDP-43 NLS phosphorylation simulation trajectories were transformed into features, including 1) radius of gyration, 2) root-mean-square distance, 3) distances between alpha carbon atoms ($C\alpha$) on the N- and C-terminus residues 4) distances between alpha carbon atoms on the N-terminus and Ala90 residues and 5) distances between alpha carbon atoms on the C-terminus and Ala90 residues. Subsequently, time-lagged independent component analysis (TICA) (Perez-Hernandez et al., 2013) was performed to decompose these features onto 100 slow independent components (ICs). The projected dataset was then discretized using the k -means clustering algorithm, resulting in 12 microstates. The transition probability matrix between these microstates was then computed at a lag time of 3 ns. Finally, a four-state MSM using the PCCA++ algorithm (Robitz et al., 2013) and the mean first-passage times (MFPT) (Polizzi et al., 2016) were measured between different states for each phosphorylated TDP-43 NLS structure.

QUANTIFICATION AND STATISTICAL ANALYSIS

Band intensities on SDS-PAGE gels were calculated using ImageJ (Abramoff et al., 2004). Details regarding the statistical analysis of these data can be found within the Figure 2 legend, as well as the Method details section. Significance was defined as $p < 0.01$.

Signal intensity from TMB substrate conversion was measured at 450 nm using a TECAN 96-well plate reader. Details regarding the processing of the data can be found within the [Method details](#) section. Data was plotted using GraphPad Prism software and in each case was fit to a non-linear regression designed to model specific binding. We used the 95% confidence interval of each predicted K_D to assess the quality of the fit and the likelihood that we had captured the true K_D . Error bars represent the standard deviation of the mean. For plotted points in which error bars do not appear to be present, the spread of the error was such that it did not exceed the dimensions of the data point marker.

For the plots of the free energies of binding shown in [Figure S8](#), error bars represent the standard deviation of the mean. Details regarding the statistical analysis of these data can be found within the [Method details](#) section.