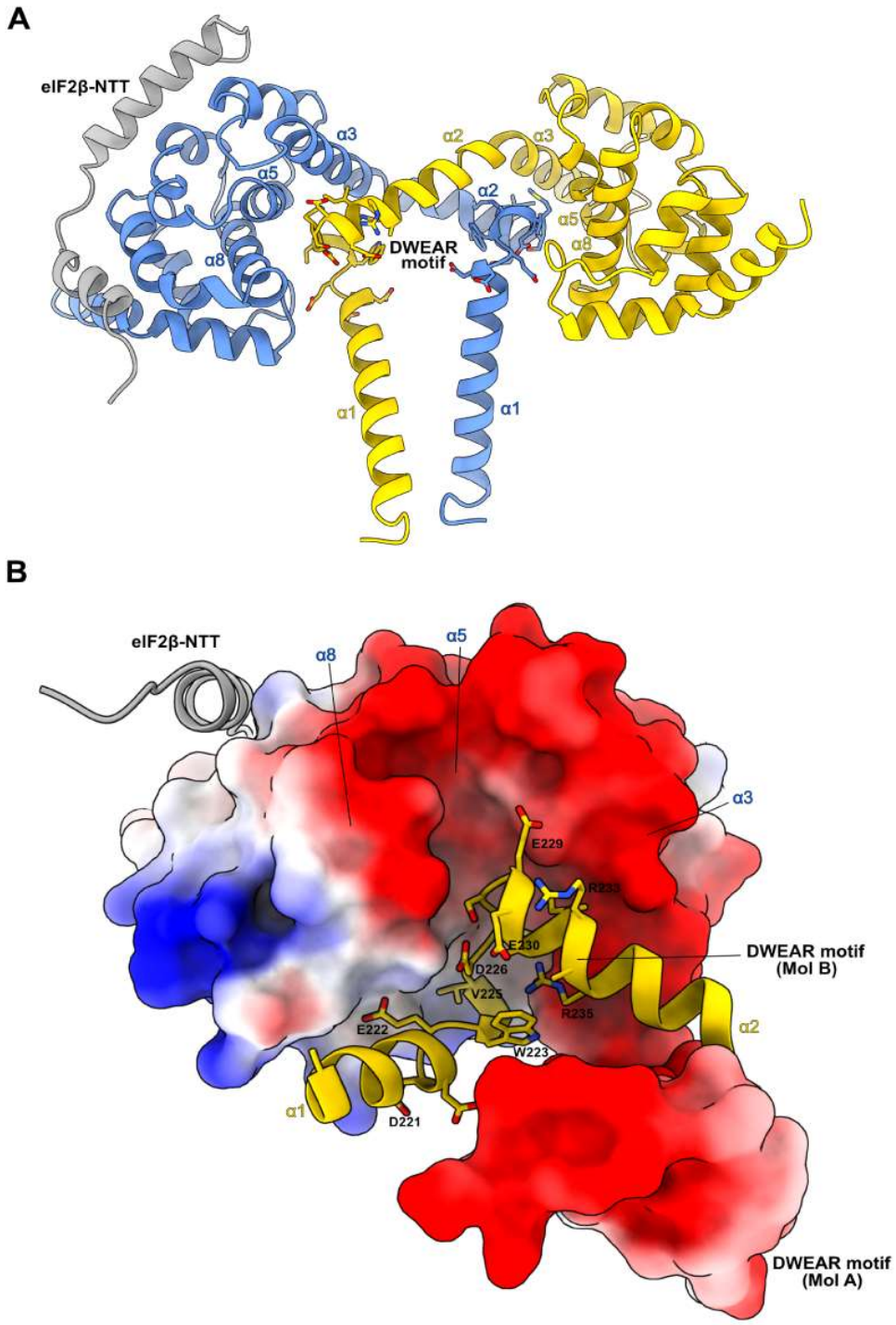
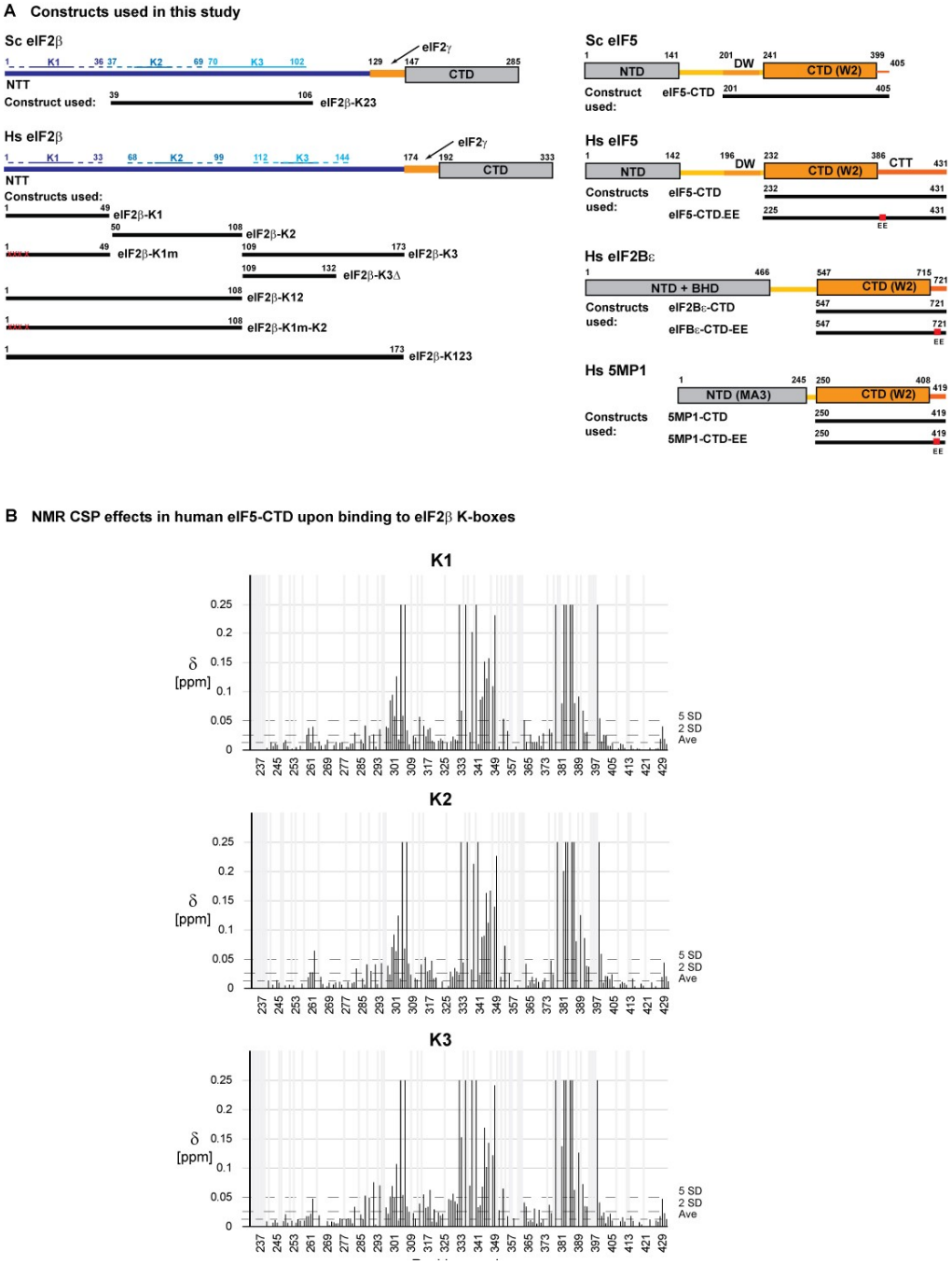


Supplemental Figure S1. The eIF5 DWEAR motif contacts the eIF5-CTD.

(A) The asymmetric unit of the crystal structure of eIF5-CTD•eIF2β-K23 complex. The two eIF5-CTD molecules are colored in yellow and blue, respectively, and the eIF2β-K23 is shown in gray. In both eIF5-CTDs, the DWEAR-motif overlaps with the loop connecting helices α1 and α2 in the N-terminal extension and engages in crystallographic contacts with a groove formed by helices α3, α5, and α8 on the neighboring eIF5-CTD molecule.

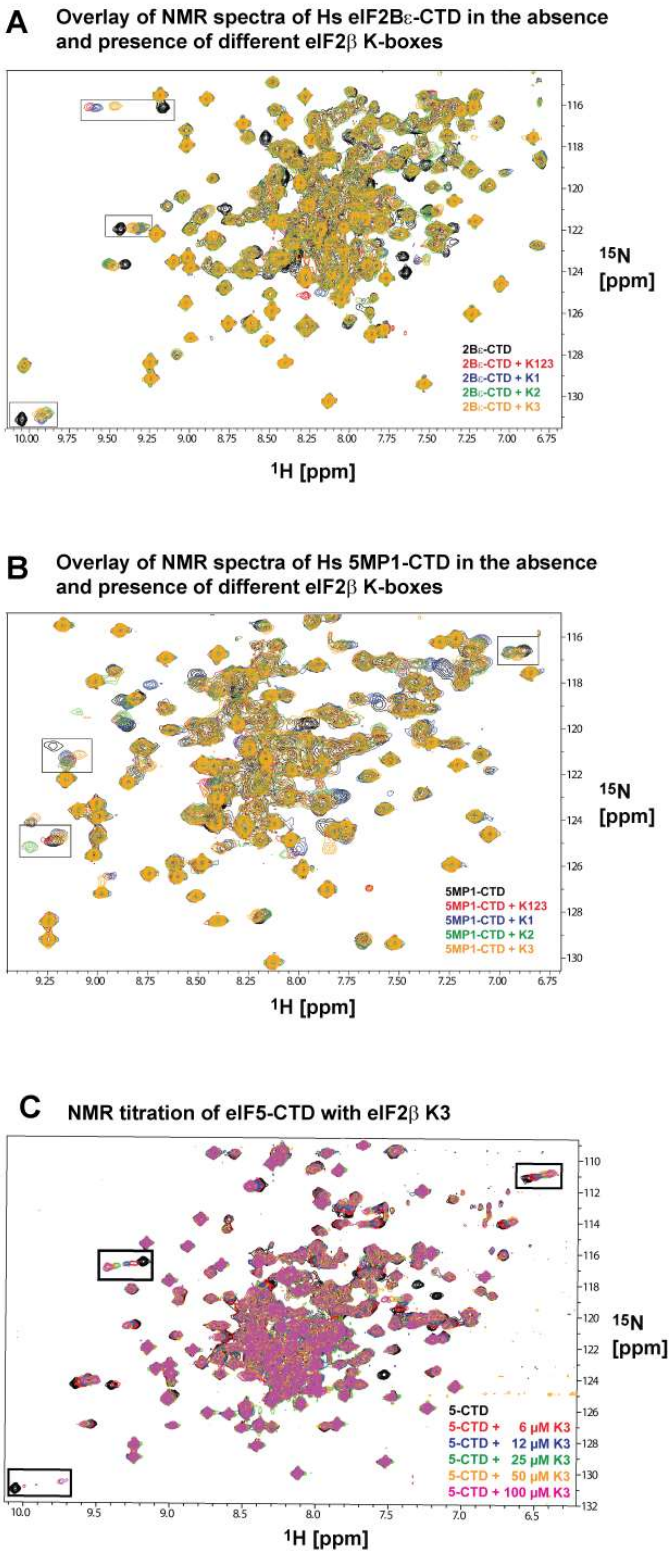
(B) Details of the interactions between the DWEAR-motif of one eIF5-CTD molecule (Mol B) and the binding pocket on the neighboring eIF5-CTD molecule (Mol A). The DWEAR-motif of Mol A points away from its globular domain and inserts into the corresponding binding pocket on Mol B. Mol B is shown in cartoon representation; Mol A is shown in surface representation with electrostatic surface charge distribution.



Supplemental Figure S2. NMR CSP effects in human eIF5-CTD upon binding to eIF2β K-boxes.

(A) Constructs used in this work. Folded domains are shown as boxes. Intrinsically disordered segments are shown as lines. K-boxes K1, K2, and K3 are marked and colored dark blue, blue, and light blue, respectively. The K3 segment of *S. cerevisiae* eIF2β visible in the crystal structure, including the αN and αC helices (**Fig. 1**) is marked with a solid line. The corresponding segments surrounding the other K-boxes, corresponding to the αN and αC helices are marked with dashed lines. The four point mutations in eIF2β-K1m and eIF2β-K1m2: M6S, I7G, F8S, M12G in the putative αN helix are shown with red "X". eIF2β-NTT is blue. The eIF2γ-binding helix is orange. The homologous C-terminal HEAT (W2) domains of eIF5, eIF2Bε, and 5MP1 are orange. The DWEAR motif in eIF5 is labeled with "DW". BHD, β-helix domain. The positions of the phosphomimetic mutations in the disordered C-termini are shown as red boxes and labeled "EE".

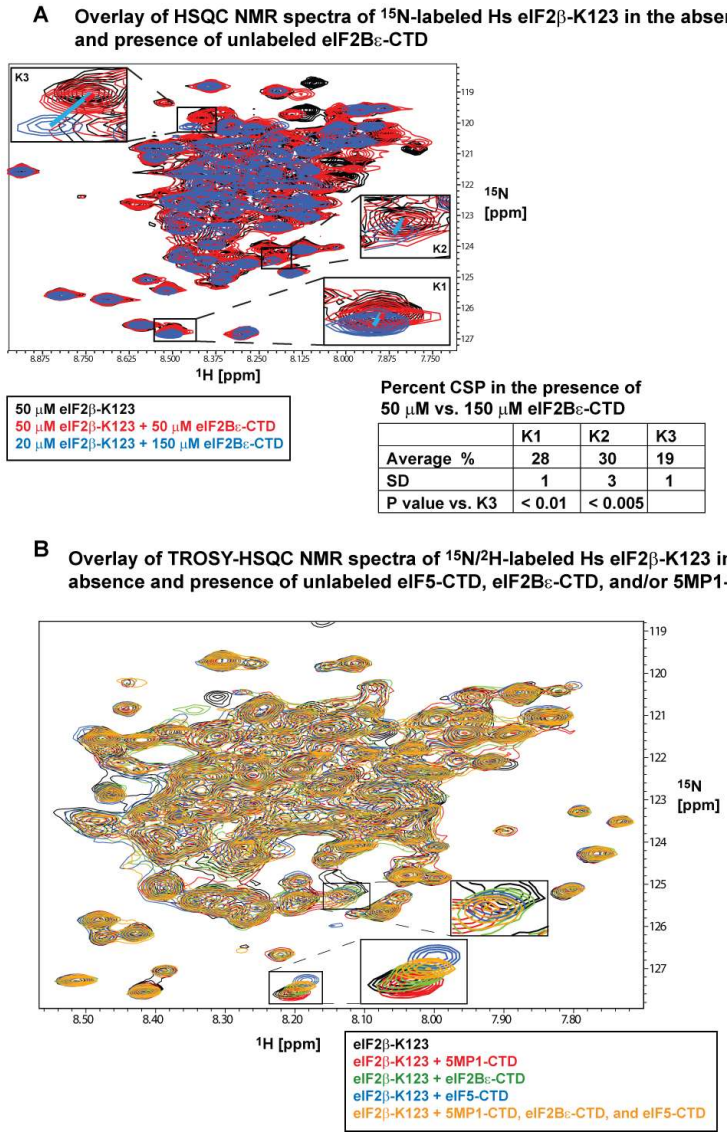
(B) NMR chemical shift perturbation (CSP) effects in 50 μM human $^{15}N/^{2}H$-eIF5-CTD upon binding to 100 μM eIF2β K-boxes 1, 2, and 3. Amino-acids that could not be analyzed are shown as light grey bars. Average CSP effect, average plus two standard deviations (SD), and average plus five SD are marked with dashed lines and labeled.



Supplemental Figure S3. eIF2Bε-CTD and 5MP1-CTD form similar complexes with each of the three K-boxes of eIF2β.

(A, B) NMR spectra overlay of 50 μM $^{15}N/^{2}H$-labeled human eIF2Bε-CTD (A) and 5MP1-CTD (B), free (black) and in the presence of eIF2β-K123 (red), K1 (blue), K2 (green), and K3 (orange). Examples of peaks that are in similar or different positions in the different complexes are boxed.

(C) NMR spectra overlay of titration of 50 μM $^{15}N/^{2}H$-labeled eIF5-CTD with K1, with insets for a couple of moving peaks in fast and intermediate exchange.

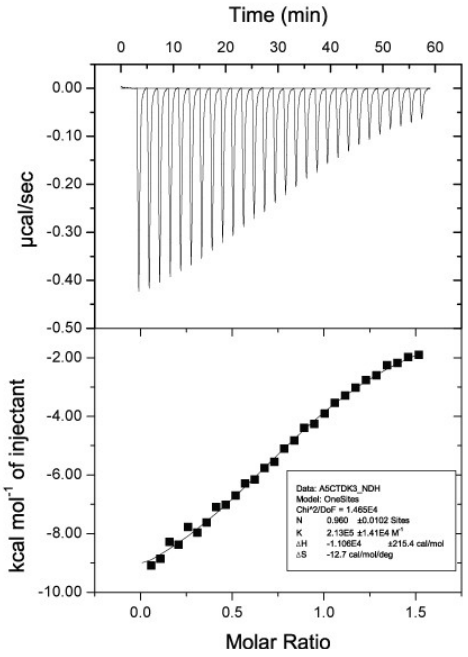


Supplemental Figure S4. Protein binding to eIF2β-K123

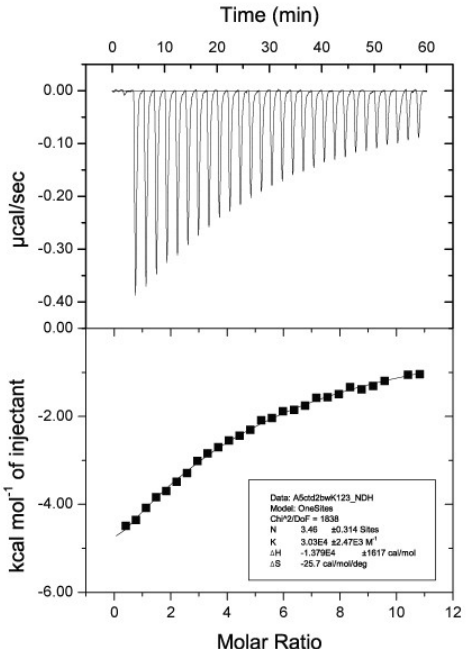
(A) HSQC NMR spectra overlay of 50 μM ^{15}N-labeled human GB1-tagged eIF2β-K123, free (black) and in the presence of 50 μM eIF2Bε-CTD (red), and 20 μM eIF2β-K123 in the presence of 150 μM eIF2Bε-CTD (blue). One representative peak belonging to each of the three K-boxes are zoomed in the inset boxes. CSP effects (peak movement) in the presence of 50 μM eIF2Bε-CTD is shown with a red bar from the center of the peak in free eIF2β-K123 to the center of the peak in partially bound eIF2β-K123. CSP effects in the presence of 150 μM eIF2Bε-CTD is shown with a blue bar from the center of the peak in free eIF2β-K123 to the center of the peak in nearly fully bound eIF2β-K123. Only the relevant portion of the spectra is shown.
Analysis of the CSP effects caused by 50 μM eIF2Bε-CTD as a percent of the CSP effects caused by 150 μM eIF2Bε-CTD is shown in the table below the spectra. The difference in percent binding indicates that at subsoichiometric concentrations of eIF2Bε-CTD, the occupancy of K-box 3 is lower that those of K-boxes 1 and 2.

(B) TROSY-HSQC NMR spectra overlay of ^{15}N/^{2}H-labeled human GB1-tagged eIF2β-K123, 40 μM, free (black) and in the presence of 150 μM 5MP1-CTD (red), 150 μM eIF5-CTD (blue), 150 μM eIF2Bε-CTD (green), or in the presence of all three proteins at 70 μM each (orange). Two of the peaks affected by binding are zoomed in the inset boxes. Note that in the presence of all three binding partners, the positions of the affected eIF2β peaks are the weighted average of their positions in the presence of the individual proteins. Therefore, binding to all three proteins is in fast exchange on the NMR time scale (each binding site on eIF2β binds to, and dissociates from each of the three proteins multiple times per second). If the dissociation rates of the individual interactions were slower, the affected eIF2β peaks would have been split into three peaks, corresponding to the three individual complexes.

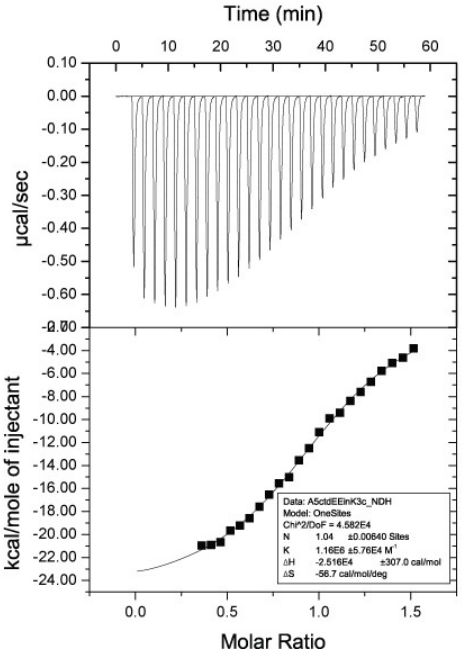
A Binding of eIF5-CTD to eIF2β-K3



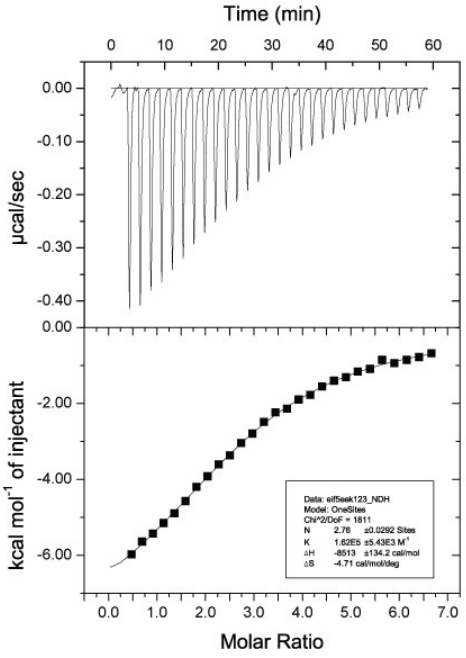
B Binding of eIF5-CTD to eIF2β-K123



C Binding of eIF5-CTD-EE to eIF2β-K3



D Binding of eIF5-CTD-EE to eIF2β-K123



Supplemental Figure S5. Binding affinities of eIF5-CTD and eIF5-CTD-EE to eIF2β-K3 and eIF2β-K123 measured by Isothermal Titration Calorimetry (ITC)

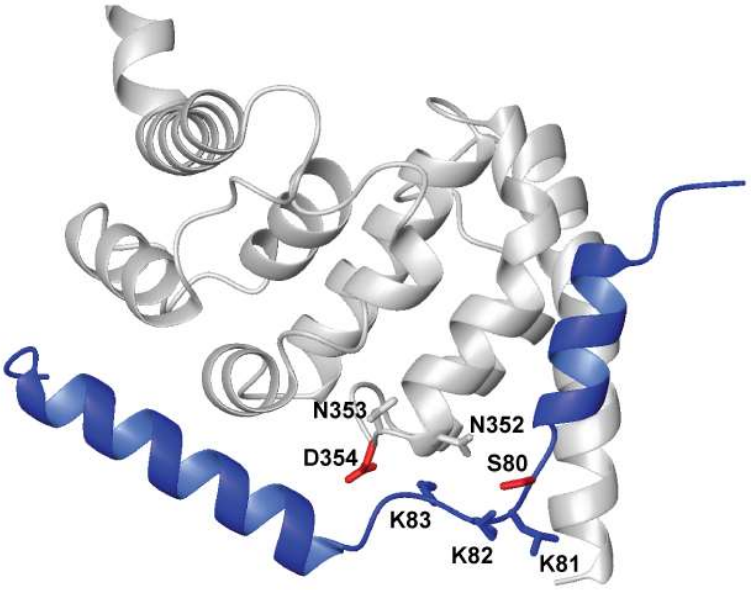
(A) eIF5-CTD (150 µM) injected in eIF2β-K3 (21 µM). KD = 4.7 µM, using one-site model. N = 0.96.

(B) eIF5-CTD (250 µM) injected in eIF2β-K123 (5 µM). KD = 33 µM, using one-site model. N= 3.46.

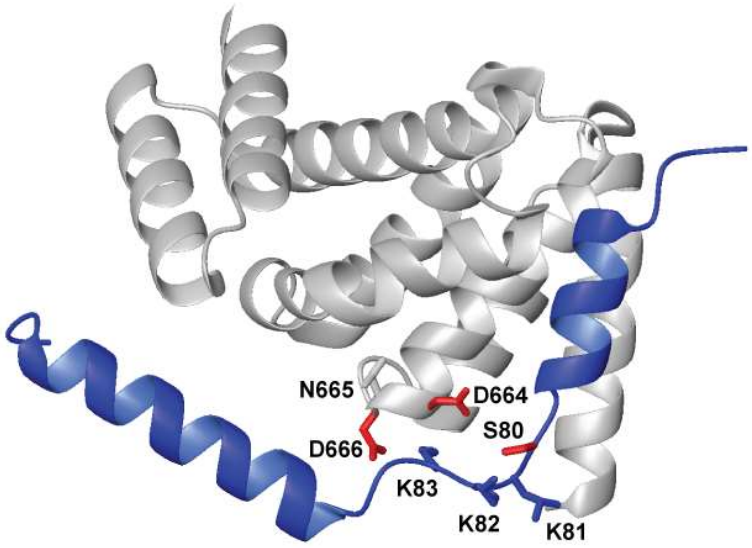
(C) eIF5-CTD-EE (70 µM) injected in eIF2β-K3 (10 µM). KD = 0.86 µM, using one-site model. N=1.04.

(D) eIF5-CTD-EE (200 µM) injected in eIF2β-K123 (6.5 µM). K_D = 6.2 µM, using one-site model. N= 2.76.

A **Sc. eIF5-CTD - eIF2β-K3 complex**



B **Model of the Sc. eIF2Bε-CTD - eIF2β-K3 complex**



Supplemental Figure S6. Comparison of the environment of S80 in the crystal structure of the *S. cerevisiae* eIF5-CTD•eIF2β-K3 complex and in a model for the *S. cerevisiae* eIF2Bε-CTD•eIF2β-K3 complex

(A) The crystal structure of the *S. cerevisiae* eIF5-CTD•eIF2β-K3 complex (**Fig. 1**). eIF2β is shown as a blue ribbon; eIF5-CTD is shown as grey ribbon. The side-chains of S80 and other relevant residues are shown as sticks and labeled. Note that S80 and K82 in eIF2β point away from the eIF5-CTD surface.

(B) The eIF2β-K3 segment from the crystal structure of the *S. cerevisiae* eIF5-CTD•eIF2β-K3 complex (**Fig. 1**) is overlayed on the structure of *S. cerevisiae* eIF2Bε-CTD (1paq.pdb (Boesen et al., 2004)). The overlay was obtained by aligning the structures of eIF5-CTD and eIF2Bε-CTD. eIF2β is shown as a blue ribbon; eIF2Bε-CTD is shown as grey ribbon. The side-chains of S80 and other relevant residues are shown as sticks and labeled.

Supplemental References

Boesen T, Mohammad SS, Pavitt GD, Andersen GR (2004) Structure of the catalytic fragment of translation initiation factor 2B and identification of a critically important catalytic residue. *The Journal of biological chemistry* 279: 10584-92