

Molecular basis for the interactions of eIF2 β with eIF5, eIF2B, and 5MP1 and their regulation by CK2

Paul A. Wagner,^{1,6} Meimei Song,^{1,7} Paul Doran,¹ Ayşenur Şeker,¹ Ralf Ficner,² Bernhard Kuhle,^{3,4,5,*} and Assen Marintchev^{1,*}

¹Department of Pharmacology, Physiology, & Biophysics, Boston University Chobanian & Avedisian School of Medicine, Boston, MA, USA

²Department of Molecular Structural Biology, GZMB, University of Göttingen, 37077 Göttingen, Germany

³Department of Molecular Biology, University Medical Center Göttingen, 37073 Göttingen, Germany

⁴Research Group Structure and Function of Molecular Machines, Max Planck Institute for Multidisciplinary Sciences, 37077 Göttingen, Germany

⁵Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA

⁶Present address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, USA

⁷Present address: Department of Chemistry, University of North Carolina, Chapel Hill, NC, USA

*To whom correspondence should be addressed: bernhard.kuhle@med.uni-goettingen.de,
amarint@bu.edu

Keywords

translation initiation; integrated stress response; protein structure; X-ray crystallography; NMR; CK2

Running Title: Interactions of eIF2 β with eIF5, eIF2B, and 5MP1

Abstract

The heterotrimeric GTPase eukaryotic translation initiation factor 2 (eIF2) delivers the initiator Met-tRNA_i to the ribosomal translation preinitiation complex (PIC). eIF2 β has three lysine-rich repeats (K-boxes), important for binding to the GTPase-activating protein eIF5, the guanine nucleotide exchange factor eIF2B, and the regulator eIF5-mimic protein (5MP). Here, we combine X-ray crystallography with NMR to understand the molecular basis and dynamics of these interactions. The crystal structure of yeast eIF5-CTD in complex with eIF2 β K-box 3 reveals an extended binding site on eIF2 β , far beyond the K-box. We show that eIF2 β contains three distinct binding sites, centered on each of the K-boxes, and that human eIF5, eIF2B ϵ , and 5MP1 can bind to all three sites. Our results reveal how eIF2B speeds up the dissociation of eIF5 from eIF2-GDP to promote nucleotide exchange; and how 5MP1 can destabilize eIF5 binding to eIF2 and the PIC, to promote stringent start codon selection. All these affinities are increased by CK2 phosphomimetic mutations, highlighting the role of CK2 in both remodeling and stabilizing the translation apparatus.

Introduction

Translation initiation in eukaryotes relies on a complex network of interactions that are continuously reorganized throughout the process. As more information becomes available about the structure of the ribosomal preinitiation complex (PIC) at various points in translation initiation, new questions arise about which interactions occur when, their roles, and regulation. The eukaryotic translation initiation factor 2 (eIF2) is a heterotrimeric GTPase playing a central role in the regulation of translation initiation and the integrated stress response (ISR). In its active GTP-bound form, eIF2 binds the initiator Met-tRNA_i to form the eIF2-GTP•Met-tRNA_i ternary complex (TC). The TC binds to the PIC, either directly or as part of the multifactor complex (MFC), which also includes eIFs 1, 3, and 5. eIF5 is the GTPase-activating protein (GAP) for eIF2. eIF1 serves as a gate-keeper promoting stringency of start codon selection. eIF3 is a large protein complex with multiple roles in translation initiation. Mammalian eIF3 has 13 subunits, while *Saccharomyces cerevisiae* (*S. cerevisiae*) eIF3 has six (five core subunits and the sub-stoichiometric HCR1/eIF3j). Most of eIF3 resides on the back, solvent-exposed surface of the 40S ribosomal subunit, while some of its subunits reach the interface with the 60S ribosomal subunit. One of them, the N-terminus of eIF3c wraps around the 40S and interacts with eIFs 1, 2, and 5. The interactions among the MFC components are not fully understood but it has become clear that they are remodeled upon binding to the PIC, where the MFC components also interact with the 40S ribosomal subunit, the mRNA, and eIF1A. The complex is further continually remodeled at every step of translation initiation, including release of eIF1 upon start codon selection to form the so-called “closed” PIC, arrested at the start codon, and the eIF5 N-terminal domain (NTD) occupying the site vacated by eIF1 following its release (Brito Querido et al., 2024, Dever et al., 2023, Jackson et al., 2010, Marintchev & Wagner, 2004, Petrychenko et al., 2024, Weisser & Ban, 2019).

Following GTP hydrolysis and the release of eIF2-GDP from the PIC, its reactivation to eIF2-GTP and regeneration of the TC require the guanine nucleotide exchange factor (GEF) eIF2B (a decamer with two copies of five different subunits), which promotes nucleotide exchange and subsequent Met-tRNA_i binding to eIF2-GTP. At least in *S. cerevisiae*, eIF2-GDP is released from the PIC in a stable complex with eIF5, which serves as GDP dissociation inhibitor (GDI). eIF2B has to displace eIF5 before it can promote GDP dissociation from eIF2, thus acting as GDI dissociating factor (GDF) (Jennings & Pavitt, 2010, Jennings et al., 2013, Singh et al., 2006). As a result, eIF2 is channeled from the PIC, through eIF5, to eIF2B (Jennings & Pavitt, 2010, Jennings et al., 2013, Singh et al., 2006), and could also be directly transferred from eIF2B back to eIF5 and the MFC at the end of the cycle (Bogorad et al., 2018).

eIF2B is one of the main targets in the regulation of protein synthesis in the cell (reviewed in (Bogorad et al., 2018, Brito Querido et al., 2024, Hinnebusch, 2014, Marintchev & Ito, 2020)). The activity of eIF2B is regulated by phosphorylation of its substrate eIF2, by binding of nucleotides and cofactors to eIF2B, and by phosphorylation of eIF2B itself by at least two kinases: casein kinase 2 (CK2) stimulates eIF2B activity, while GSK-3 β inhibits it. In human, several kinases phosphorylate eIF2 α at Serine 51 (S51) in response to various types of stress, including viral infection (PKR), unfolded proteins in the ER (PERK), amino acid starvation (GCN2), and heme deficiency (HRI), in what is collectively known as the Integrated Stress Response (ISR). Phosphorylated eIF2-GDP (eIF2(α -P)-GDP) is a competitive inhibitor of eIF2B. Inhibition of eIF2B activity causes downregulation of global protein synthesis and triggers the ISR by inducing the production of a set of transcription factors and other proteins. The result is the activation of both pro-apoptotic and pro-survival pathways aimed at restoring homeostasis. Since the stressors themselves can cause cell death, either an insufficient or overly aggressive stress response can lead to apoptosis, the latter being the desired outcome in the case of viral infection. Dysregulated ISR is a causative factor in the pathology of a number of

neurodegenerative disorders, including Alzheimer's disease. Mutations in all five subunits of eIF2B cause the genetic disease leukoencephalopathy with vanishing white matter (VWM) (reviewed in (Bogorad et al., 2018, Dever et al., 2007, Hinnebusch, 2005, Hinnebusch et al., 2007, Marintchev & Ito, 2020, Marintchev & Wagner, 2004, Ron & Harding, 2007, Wek et al., 2006).

The eIF5 mimic protein (5MP), also known as BZW, is a regulator that competes for binding to eIF2 with eIF5, leading to increased stringency of start codon selection, and with eIF2B, leading to modulation of nucleotide exchange on eIF2 (Hiraishi et al., 2014, Kozel et al., 2016, Singh et al., 2021, Tang et al., 2017). 5MP also binds to at least one of the other eIF5 partners, eIF3c. While there are two 5MP isoforms in human, 5MP1/BZW2 and 5MP2/BZW1, most of the studies have been performed on 5MP1 (Hiraishi et al., 2014, Kozel et al., 2016, Singh et al., 2021, Tang et al., 2017).

The C-terminal domains (CTD) of eIF5, eIF2B ϵ , and 5MP are homologous to each other and mediate binding to the intrinsically disordered N-terminal tail of the β subunit of eIF2 (eIF2 β -NTT). The interaction is mediated by two acidic/aromatic (AA) boxes in the CTD of eIF5, eIF2B ϵ , and 5MP and three lysine-rich segments (K-boxes) in eIF2 β -NTT (Asano et al., 1999, Das et al., 1997). The contact interfaces of eIF5-CTD with eIF1, 1A, and possibly also 3c, appear to overlap with the eIF2 β -NTT binding surface; therefore, eIF2 β -NTT is presumed to be at least partially displaced from eIF5 within the PIC (Luna et al., 2013, Luna et al., 2012, Obayashi et al., 2017). eIF5 and eIF2B ϵ also interact with eIF2 γ , with eIF2B ϵ having two binding sites, one in the NTD and another in the CTD (Alone & Dever, 2006, Kashiwagi et al., 2019, Kenner et al., 2019). While 5MP likely also has additional binding sites (Singh et al., 2021), interactions between 5MP and eIF2 outside the eIF2 β -NTT have not yet been reported.

Mammalian eIF5, eIF2B ϵ , and both 5MP isoforms all have conserved Casein Kinase 2 (CK2) phosphorylation sites in AA-box 2. CK2 phosphorylation of eIF5 leads to stimulation of

protein synthesis and cell proliferation (Homma et al., 2005). Phosphorylation of eIF2B ϵ stimulates eIF2B activity (Singh et al., 1996). The effect of phosphorylation is likely mediated by adding negative charges to AA-box-2, which interacts with the positively charged K-boxes (Gamble et al., 2022, Homma et al., 2005, Paul et al., 2022). The effect of phosphorylation of 5MP1 or 5MP2 has not been investigated. CK2 has hundreds of substrates, including many eIFs, and regulates protein synthesis in a number of ways (Borgo et al., 2017, Franchin et al., 2018, Gandin et al., 2016, Homma et al., 2005, Llorens et al., 2003, Paytubi et al., 2008, Turowec et al., 2010). CK2, together with mTOR, also phosphorylates eIF2 β , which helps recruit the PP1 phosphatase that dephosphorylates eIF2 α and suppresses the ISR (Gandin et al., 2016). While CK2 is constitutively active in most cells, it is inactivated during chronic stress, leading to dephosphorylation of its substrates and translational reprogramming of the cell (Gandin et al., 2016, Lamper et al., 2020, Lee et al., 2016, Mukhopadhyay et al., 2023).

Despite their importance for translation initiation, the molecular basis for the interactions of eIF2 β -NTT with eIF5, eIF2B ϵ and 5MP, their dynamics and regulation remain poorly understood. The presence of three K-boxes suggests that there could be three binding sites in eIF2 β , but this possibility has received little attention because of the 1:1 stoichiometry of the relevant complexes. The first article describing the role of the K-boxes, from the Maitra lab, claimed based on co-IP that a small segment corresponding to K-box 2 is necessary and sufficient for binding to mammalian eIF5 (Das et al., 1997). Asano and co-authors reported the importance of the K-boxes in *S. cerevisiae* eIF2 β for binding to both eIF5 and eIF2B ϵ . While they found that the effects of the K-boxes are largely additive, they concluded based on co-IP that binding of eIF5 and eIF2B is mutually exclusive (Asano et al., 1999). Later, the Asano group found that eIF5 and eIF2B can in fact bind to eIF2 simultaneously but maintained that binding to eIF2 β is mutually exclusive and the simultaneous binding is due to additional interactions elsewhere on eIF2 (Singh et al., 2006). Subsequent publications from a number of labs,

including ours, as well as reports on 5MP from the Asano lab, also maintained the notion of competition for eIF2 β binding (see e.g., (Bogorad et al., 2018, Hiraishi et al., 2014, Jennings et al., 2016, Jennings & Pavitt, 2010, Jennings et al., 2013, Kozel et al., 2016, Luna et al., 2013, Luna et al., 2012, Obayashi et al., 2017, Sato et al., 2019, Singh et al., 2011, Tang et al., 2017)). We recently reported that human eIF2 β -NTT can bind at least two, and most likely three eIF5-CTD molecules simultaneously (Paul et al., 2022), and that phosphomimetic mutations in eIF5-CTD, mimicking CK2 phosphorylation, increase its affinity for eIF2 β (Paul et al., 2022) and eIF1A (Gamble et al., 2022).

Here, we report the crystal structure of the complex of *S. cerevisiae* eIF5-CTD with K-box 3 of eIF2 β (eIF2 β -K3), which shows an extended binding site on eIF2 β that involves segments on both sides of the actual K-box. We go on to show that this binding mode is conserved with human eIF5, as well as eIF2B ϵ and 5MP1, and that all three proteins can bind to each of the three K-boxes independently and with similar affinities. Finally, phosphomimetic mutations in all three proteins differentially increase their affinities for each K-box and the intact eIF2 β -NTT by up to an order of magnitude. Taken together, our results provide both a structural snapshot of the eIF5-eIF2 β interactions as well as a plausible model for the dynamic reorganization and regulation of eIF2 β -NTT interactions during the translation initiation cycle.

Results

Crystal structure of eIF2 β -NTT bound to eIF5-CTD

To gain insight into the structural basis for eIF2 β -eIF5 interactions, we determined the X-ray crystal structure of *S. cerevisiae* eIF5-CTD bound to the eIF2 β -NTT. Among different combinations of eIF5 and eIF2 β constructs used for initial crystallization trials, only the complex between eIF5₂₀₁₋₄₀₅ and eIF2 β ₃₉₋₁₀₆, which contains K2 and K3 and flanking regions, produced

well-diffracting single crystals in space group $P3_212$ that were suitable for structure determination.

The final atomic model of the eIF5-eIF2 β complex, refined at a resolution of 2.0 Å, contains two copies of eIF5₂₀₁₋₄₀₅ per asymmetric unit (AU), each including residues 201 to 399 and additional vector-encoded residues at the N-terminus (₅PGLGS₁) (details of data collection and refinement statistics are summarized in **Table 1**). Residues 241-399 of both molecules adopt a compact fold composed of four antiparallel helical repeats (R_I-R_{IV}) that is very similar to the previously reported yeast eIF5-CTD structure (Wei et al., 2006): r.m.s.d of 0.8 Å over 133 C α atoms (**Fig. 1A**). The N-terminal extension (amino acids 201-240), which is part of the linker between the N- and C-terminal domains of full-length eIF5, folds into two additional α -helices (α 1 and α 2) that protrude away from the globular CTD and form extensive contacts with symmetry related molecules in the crystal packing. Interestingly, the DWEAR motif, a highly conserved and functionally important region of eIF5, forms the loop between helices α 1 and α 2 and is inserted into a hydrophobic cleft between α -helices α 3, α 5, and α 8 of another eIF5-CTD molecule in the crystal packing (**Supplemental Fig. S1**). Notably, although this interaction occurs *in trans*, likely as a crystallographic artifact (**Supplemental Fig. S1**), a similar interaction could be formed *in cis*, which would allow the DWEAR-motif to associate with the CTD. Such intramolecular contacts between the DWEAR motif and the CTD are supported by NMR chemical shift perturbation (CSP) and deletion analysis (Paul et al., 2022).

A single eIF2 β molecule is bound by only one of the two eIF5-CTDs in the AU. This eIF2 β fragment includes residues 66-105 and forms two amphipathic α -helices (α N and α C) flanking an unstructured loop containing K-box 3 (**Fig. 1A, B**). Residues 39-65 of eIF2 β , including K2, are not resolved and thus appear to be flexible. Helices α N and α C are both well-defined in the electron density and form extensive hydrophobic as well as polar interactions with eIF5. Helix α N (residues 69-79) lies parallel to eIF5 helices α 9 and α 11 with which it forms a

mostly hydrophobic interface (**Fig. 1C**). Helix α C (residues 89-103) binds on top of and orthogonal to eIF5 helices α 4 and α 7, with Phe96 and Leu100 inserted into a hydrophobic pocket on eIF5 and eIF2 β -Glu97 forming a salt bridge with eIF5-Lys313 (**Fig. 1D**). Between helices α N and α C lies the K3-loop, which is positioned in the immediate vicinity to the negatively charged AA-boxes of eIF5 (area I). Notably, only two of the seven lysine residues, Lys83 and Lys89, form direct interactions with AA-box 1 (**Fig. 1E**). The remaining lysine residues of K-box 3 are poorly resolved in the electron density, indicating that they do not participate in stable interactions with eIF5 under the given conditions.

Taken together, the eIF5-bound eIF2 β -NTT adopts an elongated conformation, containing secondary, but no tertiary structural features. This allows eIF2 β to form an extended interaction interface with eIF5, centered on the K-box and wrapping half around the eIF5-CTD (**Fig. 1**). In the following text, 'K-box' will refer to this extended binding site, including the adjoining segments α N and α C. eIF2 β thereby contacts only the periphery of the negatively charged area I of eIF5, with α N and α C lying between the negatively charged areas I and III and the positively charged area II (**Fig. 1B**). Most of area I remains solvent-exposed in the complex, including the highly negatively charged C-terminus (residues 393-405), Glu358, and Glu359, which are critical for high affinity binding of eIF2 β -NTT (Das & Maitra, 2000, Yamamoto et al., 2005).

Human eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD form similar complexes with each of the three eIF2 β -NTT K-boxes.

Multiple-sequence alignment of eIF2 β -NTT sequences shows that the segments surrounding the K-boxes are conserved both among species and among the three K-boxes, enriched in acidic and hydrophobic amino acids (**Fig. 2A**). If the binding sites centered around K1 and K2 are similar in size to that observed with K3 in the crystal structure (**Fig. 1**), then in *S.*

cerevisiae eIF2 β -NTT, the three binding sites are immediately adjacent to each other, with helix α N of one binding site starting where helix α C of the previous binding site ends (**Fig. 2A**). In contrast, in human eIF2 β -NTT, there are about 30 amino acids between the predicted K1 and K2 binding site, and about 10 amino acids between K2 and K3 (**Fig. 2A**). The spacer between the end of helix α C after K3 and the eIF2 γ -binding helix appears to be about 20 amino acids in both human and *S. cerevisiae* eIF2 β -NTT.

We used NMR Chemical Shift Perturbation (CSP) assay to compare the contact surfaces of human eIF5-CTD for each of the three K-boxes and the complete eIF2 β -NTT (eIF2 β -K123). All further experiments described below use human proteins (constructs used in this work are shown in **Supplemental Fig. S2A**). Comparison of the ^{15}N -transverse relaxation optimized spectroscopy – heteronuclear single quantum coherence (TROSY-HSQC) NMR spectra of free $^{15}\text{N}/^2\text{H}$ -labeled eIF5-CTD and of eIF5-CTD in complex with unlabeled individual K-boxes and eIF2 β -K123 shows that the vast majority of the peaks affected by binding are the same in all four complexes, with even the magnitude of the peak movement being similar as well (**Fig. 2B and Supplemental Fig. S2B**). Mapping the affected amino acids on the surface of eIF5-CTD (**Fig. 2C**) shows that the contact interface is similar to that observed in the crystal structure of the yeast eIF5-CTD•eIF2 β -K3 complex (**Fig. 1**) and also extends into the intrinsically disordered eIF5 C-terminal tail (**Fig. 2C**). Note that in **Fig. 2C**, the position of eIF5-CTT is from the crystal structure of human eIF5-CTD (2iu1.pdb) (Bieniossek et al., 2006). However, we have previously shown that the CTT is intrinsically disordered and remains disordered while contacting eIF2 β (Paul et al., 2022). Accordingly, the corresponding region of *S. cerevisiae* eIF5 (residues 400-405), which is not resolved in the crystal structure (**Fig. 1**), could also be involved in dynamic interactions with eIF2 β . Consistent with the crystal structure of the *S. cerevisiae* complex, mutating hydrophobic residues N-terminal from K1, corresponding to helix α N (eIF2 β -K1m, carrying the M6S/I7G/F8S/M12G point mutations), or deleting the aa.

133-173 segment C-terminal from the lysines in K3, including the C-terminal portion of helix α C, aa. 133-144 (eIF2 β -K3 Δ), both weaken binding to eIF5-CTD by at least an order of magnitude (see **Fig. 3A** below). Therefore, human eIF5-CTD forms very similar complexes with all K-boxes, each likely similar to the complex of *S. cerevisiae* eIF5-CTD with eIF2 β -K3 (**Fig. 1**).

NMR backbone resonance assignments are not available for eIF2B ϵ -CTD and 5MP1-CTD, meaning that we do not know which peak in the TROSY-HSQC spectra corresponds to which residue in the protein. However, as with eIF5-CTD, most peaks in the eIF2B ϵ -CTD and 5MP1-CTD spectra, affected by binding of the three individual K-boxes or the entire eIF2 β -NTT, are the same in all four complexes, with even the magnitude of the peak movement being similar (**Supplemental Fig. S3A, B**). Therefore, as for eIF5-CTD, the same surfaces of eIF2B ϵ -CTD and 5MP1-CTD are affected in all four complexes. Thus, all three proteins form similar complexes with all three K-boxes, both in isolation and when part of the three-K-box eIF2 β -NTT (**Fig. 2, Supplemental Fig. S2, and S3A, B**).

Human eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD have distinct binding affinities for eIF2 β -NTT and individual K-boxes.

We used NMR CSP assay titrations to obtain the K_D s of eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD binding to each of the three K-boxes and the intact eIF2 β -K123 (**Fig. 3** and **Table 2**). Binding is in fast to intermediate exchange on the NMR time scale, depending on the degree of change in peak position (**Supplemental Fig. S3C**). Peaks that move by > 100 Hz (0.2 ppm for ^1H on a 500 MHz instrument) undergo exchange broadening; therefore, under the experimental conditions (150 mM KCl and 25 °C), k_{off} is in the order of 100 s $^{-1}$ (reviewed in (Marintchev et al., 2007)). The effect of eIF2 β -K123 binding is the weighted average of the binding of the three individual K-boxes. The presence of one average peak for the bound state

indicates that each molecule of the labeled protein constantly samples all three K-boxes on a millisecond time scale (**Fig. 2B** and **Supplemental Fig. S3**).

We found that eIF5-CTD binds to all three eIF2 β -NTT K-boxes with similar affinities (**Fig. 3A**). In contrast, eIF2B ϵ -CTD has greater affinity for K2 and K1 than for K3 (**Fig. 3B**). As a result, eIF5-CTD has a much higher affinity for K3 than eIF2B ϵ -CTD, whereas their affinities for K1 and K2 are comparable (compare **Fig. 3A** and **B**). 5MP1-CTD binds with similar affinities to all three eIF2 β -NTT K-boxes (**Fig. 3C**), a few-fold weaker than eIF5-CTD and eIF2B ϵ -CTD (**Fig. 3A, B, C, Table 2**). The relative affinities for a given K-box ranged from similar (eIF5-CTD and eIF2B ϵ -CTD for K2) to different by an order of magnitude (eIF5-CTD and 5MP1-CTD for K3). Overall, K3 shows clear preference for eIF5-CTD, whereas the K_D s of the proteins for K1 and 2 are much closer (**Fig. 3A, B, C, Table 2**).

Surprisingly, the affinities for eIF2 β -K123 (containing K-boxes 1, 2, and 3) are lower than expected from the K_D s for the individual K-boxes. In Fig. 3, the concentration of eIF2 β -K123 is shown in K-boxes, allowing direct comparison of binding between individual K-boxes and eIF2 β -K123. For example, 50 μ M eIF2 β -K123, which contains 50 μ M each of K1, K2, and K3, binds a smaller fraction of the labeled protein than 150 μ M of any of the three K-boxes alone, including the weakest-binding one, or the same fraction as 150 μ M K3 in the case of eIF2B ϵ -CTD-EE (**Fig. 3E**). Because eIF2 β -K123 has three non-equivalent binding sites, obtaining all three K_D s reliably is only possible if they differ by at least 5-fold (and the three binding sites are occupied mostly consecutively). However, at least the average K_D (assuming three binding sites with identical affinities) can be obtained. For eIF2B ϵ -CTD and 5MP1, the average K_D , calculated assuming three binding sites with equal affinities, is not only higher than the average of the K_D s for the three individual K-boxes, but 2-fold higher than the highest K_D . For eIF5-CTD, this difference is even greater: the average K_D for eIF2 β -K123 is 6-fold higher than the highest individual K_D , that for K2 (**Fig. 3A, B, C, Table 2**). As a result, the overall affinities of all three

proteins for eIF2 β -K123 end up being similar to each other (**Table 2**). As described above, the peak positions in the presence of eIF2 β -K123 are the weighted average of their positions in the presence of the three individual K-boxes. This weighted average when eIF2 β -K123 is in excess and there is on average only one occupied K-box (**Fig. 2B** and **Supplemental Fig. S3A, B**) appears similar to the relative affinities for the three K-boxes measured in isolation. While in the presence of excess binding partner, all three K-boxes have high occupancy, at lower ligand concentrations the occupancy of the binding partners would be expected to be higher at K-boxes with higher affinity. We compared the CSP effects of peaks belonging to K-boxes 1, 2, or 3 in the NMR spectra of 50 μ M 15 N-eIF2 β -K123 (150 μ M K-boxes) in the presence of 50 μ M eIF2B ϵ -CTD, and 20 μ M 15 N-eIF2 β -K123 (60 μ M K-boxes) in the presence of 150 μ M eIF2B ϵ -CTD (**Supplemental Fig. S4A**). The changes in peak positions in the NMR spectra with 50 μ M eIF2B ϵ -CTD as a percentage of the changes with 150 μ M eIF2B ϵ -CTD were: 28 ± 1 % for K1 and 30 ± 3 % for K2 (not statistically significant difference ($p = 0.47$)). In contrast, the changes for K3, which has lower affinity in isolation (**Table 2**) were 19 ± 1 % ($p < 0.01$). Therefore, it appears that the relative affinities for the three K-boxes are at least qualitatively similar in isolation and when part of the eIF2 β -NTT.

Similarly, NMR spectra of eIF2 β -K123 in the presence of equimolar amounts of eIF5-CTD, 5MP1-CTD, and eIF2B ϵ -CTD showed that the positions of the peaks affected by binding were the weighted average of their positions in the presence of each of the three individual proteins (**Supplemental Fig. S4B**). Therefore, each K-box binds to each of the proteins on a millisecond time scale, and each eIF2 β -K123 molecule binds simultaneously to any combination of one, two, or all three proteins on a millisecond time scale.

We used Isothermal Titration Calorimetry (ITC) experiments to measure a subset of the binding affinities in **Table 2**. The affinities of eIF5-CTD for eIF2 β -K3 (4.7 μ M) and eIF2 β -K123 (33 μ M) were similar to those obtained by NMR and showed similar trends between the single

K-box and eIF2 β -K123 (**Supplemental Fig. S5A, B**). Likewise, the stoichiometry of binding was 1:1 for eIF2 β -K3 and 1:3 for eIF2 β -K123 - fit value of 3.46 for N (a measure of the stoichiometry).

We used size exclusion chromatography (SEC) to visualize directly the formation of a complex of eIF2 β -K12 (containing K-boxes 1 and 2) with eIF5-CTD and eIF2B ϵ -CTD. As expected, when mixed at equimolar concentrations, eIF5-CTD and eIF2B ϵ -CTD comigrate with eIF2 β -K12 in a trimeric complex, which demonstrates that eIF5-CTD and eIF2B ϵ -CTD can bind simultaneously to different K-boxes on the same eIF2 β molecule (**Fig. 4A**). We used equimolar mix of eIF2 β -K1m-K2 (with mutated K-box 1) and eIF2B ϵ -CTD as a marker for a binary complex. Mixing eIF2 β -K1m-K2 with eIF5-CTD and eIF2B ϵ -CTD in a 1:1:1 ratio failed to yield a trimeric complex, indicating that the interaction with the mutant K1 is not stable enough to withstand the SEC run (**Fig. 4A**). A SEC experiment using eIF2 β -K123 with equimolar concentrations of eIF5-CTD, eIF2B ϵ -CTD, 5MP1-CTD yielded similar results, but with partial dissociation of 5MP1-CTD (**Fig. 4B**). To visually compare the relative affinities of eIF5-CTD and eIF2B ϵ -CTD, we repeated the SEC experiment with excess of both proteins over eIF2 β -K123. SDS-PAGE analysis of the SEC fractions indicates that eIF5-CTD and eIF2B ϵ -CTD have similar affinities (**Fig. 4B**), qualitatively consistent with the binding affinities (**Fig. 3** and **Table 2**).

Phosphomimetic mutations in human eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD differentially enhance binding to eIF2 β -NTT and individual K-boxes.

Mammalian eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD all have conserved CK2 phosphorylation sites in their AA-box 2, which is important for eIF2 β binding. Residues S389 and S390 in the disordered C-terminal tail of human eIF5, which are phosphorylated by CK2 (Homma et al., 2005), are shown in **Fig. 2C** and labeled. While phosphorylation of eIF5-CTD (Homma et al., 2005) and eIF2B ϵ -CTD (Singh et al., 1996) by CK2 and its biological effects

have been studied *in vitro*, phosphorylation of the 5MPs has only been observed in high-throughput studies (see e.g. (Mertins et al., 2016, Yi et al., 2014)). Therefore, we first confirmed that 5MP1-CTD is readily phosphorylated by CK2 *in vitro* (**Fig. 4C**). Phosphomimetic mutations mimicking CK2 phosphorylation in all three proteins increased their affinities for eIF2 β -NTT and the individual K-boxes (**Fig. 3D, E, F, Table 2**). However, the degree of increase varied significantly, from 50% (5MP1-CTD-EE with K3) to an order of magnitude (eIF5-CTD-EE with K2). The increase in average affinity for eIF2 β -NTT also ranged from 50% (5MP1-CTD-EE) to an order of magnitude (eIF5-CTD-EE) (**Table 2**). The overall pattern observed was that the phosphomimetic mutations in eIF5-CTD increased its affinity to a greater extent than those in eIF2B ϵ -CTD or 5MP1-CTD. Among the different eIF2 β constructs tested, the phosphomimetic mutations increased the affinities for K2 the most (three-fold or greater change in K_D). Notably, the affinity of eIF2B ϵ -CTD-EE for K123 was equal to its affinity for K3, while still being much weaker than the average of its affinities for the three individual K-boxes. The phosphomimetic mutations changed the relative affinities among the three proteins for a given K-box by up to three-fold, in favor of eIF5-CTD (**Table 2**).

The affinities of eIF5-CTD-EE for eIF2 β -K3 (0.86 μ M) and eIF2 β -K123 (6.2 μ M), obtained from ITC (**Supplemental Fig. S5C, D**), were similar to those obtained by NMR (**Table 2**) and showed similar trends between the single K-box and eIF2 β -K123, and between the WT and phosphomimetic proteins. Likewise, the stoichiometry of binding was 1:1 for eIF2 β -K3 and 1:3 for eIF2 β -K123 - fit value of 2.76 for N (a measure of the stoichiometry).

The change in relative affinities between eIF5-CTD-EE and eIF2B ϵ -CTD-EE is visually illustrated by the SEC experiments shown in **Fig. 4B**. In the presence of excess eIF5-CTD and eIF2B ϵ -CTD over eIF2 β -K123, comparable fractions of each protein are bound to eIF2 β -K123; thus, the proteins appear to have similar affinities. In contrast, the experiment with excess eIF5-CTD-EE, eIF2B ϵ -CTD-EE, and 5MP1-CTD-EE yields predominantly eIF5-CTD-EE bound to

eIF2 β -K123 (**Fig. 4B**). Therefore, in addition to increasing all their affinities, phosphorylation by CK2 modulates the relative affinities of the individual proteins for eIF2 β -NTT.

Discussion

In the crystal structure of the *S. cerevisiae* eIF2 β -K3•eIF5-CTD complex (**Fig. 1**), eIF2 β -K3 and the adjoining sequences wrap half-around eIF5-CTD and fold upon binding, forming two helices, α N and α C. The K-box itself forms the loop between the two helices and the N-terminal portion of α C (**Fig. 1**). Notably, helix α C ends just above the first of the four antiparallel helical repeats (R_i) of eIF5-CTD (**Fig. 1**), thus supporting the role of eIF2 β -NTT in recruiting and positioning the eIF5-CTD and the preceding DWEAR motif relative to the eIF2 $\alpha\beta\gamma$ complex. The interaction of eIF5-CTD with a portion of the eIF2 β -NTT was recently modeled in the context of the 43S PIC from *Trypanosoma cruzi* (*T. cruzi*). The low local resolution and absence of side chain information in the corresponding region allowed only a tentative placement of two α -helices, which were interpreted as belonging to K-box 3 of eIF2 β (chain **n** in 7ASE.pdb, labeled as “Translation initiation factor, putative”) (Bochler et al., 2020). Notwithstanding the lack of molecular details, this interpretation is confirmed by our crystal structure of the yeast eIF5-CTD•eIF2 β complex, which would suggest that the interactions between eIF5-CTD and the eIF2 β K-boxes are conserved throughout eukaryotes. Interestingly, K3 is the least conserved K-box in kinetoplastids, with only 2-3 positive charges in *Trypanosoma* eIF2 β and no positive charges in *Leishmania* eIF2 β (see Fig. S2B in ref. (Bochler et al., 2020)). Thus, should *T. cruzi* eIF5-CTD indeed bind to K3 as proposed in (Bochler et al., 2020), this would further highlight the importance of the regions flanking the K-box itself. Our NMR data (**Fig. 2** and **Supplemental Fig. S3 and S4**) show that each of the three K-boxes in human eIF2 β forms similar complexes with eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD, suggesting a common interface similar to that observed in the *S. cerevisiae* eIF2 β -K3•eIF5-CTD complex (**Fig. 1**).

The affinities of individual proteins to different K-boxes vary by up to an order of magnitude, indicating defined preferences of proteins for one K-box over another, and preferences of K-boxes for one protein over another, respectively. At the same time, most of the individual affinities are high enough and similar enough to be physiologically relevant (**Table 2**). In *S. cerevisiae*, GCN2 phosphorylates eIF2 β on Ser80 in K-box 3, which increases its affinity for eIF5, but has no effect on its affinity for eIF2B ϵ (Dokladal et al., 2021). This finding provides indirect evidence that in *S. cerevisiae*, eIF5 may prefer K3, while eIF2B ϵ could bind preferentially to K1 or 2, which is consistent with the binding affinities we measured for the human proteins (**Table 2**). In the *S. cerevisiae* eIF2 β -K3•eIF5-CTD complex (**Fig. 1**), S80 faces away from the interface with eIF5-CTD (**Supplemental Fig. S6**). Thus, its phosphorylation is unlikely to directly affect the interaction. Instead, it could serve to stabilize K82, whose side chain is also facing away from eIF5-CTD. While the respective surfaces of *S. cerevisiae* eIF5 and eIF2B ϵ are highly conserved, a notable difference is the nearby D664 in eIF2B ϵ (N352 in eIF5), which could stabilize K82 and/or repulse each other with phosphorylated S80 (**Supplemental Fig. S6**). Thus, the reason why phosphorylation of S80 does not affect the affinity for eIF2B could be that K-box 3 is not as important for the overall affinity and/or that S80 phosphorylation is not as favorable for the interaction with eIF2B ϵ as it is for the interaction with eIF5.

The binding affinities for the intact eIF2 β -NTT (eIF2 β -K123) and individual K-boxes (**Table 2**) indicate that the three K-boxes interfere with each other's binding, especially to eIF5. While in *S. cerevisiae*, the three binding sites are adjacent to each other, in human eIF2 β -NTT, the K-boxes are farther apart (**Fig. 2A**); thus, steric interference was not expected *a priori*. It is not clear whether this difference between human and yeast eIF2 β has any functional significance. However, it should be pointed out that in humans, eIF2 β -NTT has additional regulatory roles: it has a binding site for raptor (and thus mTORC1) and is phosphorylated by

both mTORC1 and CK2, which promotes binding of NCK1, an adaptor of the PP1 phosphatase, leading to eIF2 α dephosphorylation and blunting of the ISR (Gandin et al., 2016). It is possible that a protein bound to one K-box and the surrounding segments could transiently contact another K-box. Such contacts could interfere with a second protein binding to the second K-box. Unfortunately, we were unable to obtain evidence for or against a second K-box simultaneously contacting eIF5-CTD, and there are alternative possible explanations: the lower affinities for the K-boxes within the intact eIF2 β -NTT could for instance be caused by the rest of the NTT interfering with binding, instead of being mediated by another protein bound to another K-box. In view of the greater proximity of the individual K-boxes in *S. cerevisiae* (**Fig. 2A**), it is possible that the degree of interference between proteins bound simultaneously to adjacent K-boxes could be even greater than observed here for the human proteins.

What is the stoichiometry *in vivo*? Extensive proteomics data in paxdb.org (Huang et al., 2023) shows that in human cells, the combined number of eIF5, eIF2B, 5MP1, and 5MP2 molecules is greater than that of eIF2, typically 1.5-fold, and up to twice as large. The same is true in *S. cerevisiae*, which does not have a 5MP homolog, but the combined concentrations of eIF5 and eIF2B are greater than that of eIF2, again about 1.5-fold. Therefore, *in vivo*, the majority of eIF2 β -NTT would typically be bound to one or two of these proteins. As to why, the most likely answer is regulation. Specifically, formation of a transient complex, where two proteins are bound simultaneously to eIF2 β -NTT, likely allows fast displacement of one protein bound to the trimeric eIF2 by the other. For example, this could be involved in eIF2B displacing eIF5 from eIF2-GDP in order to initiate nucleotide exchange on eIF2 (Jennings & Pavitt, 2010, Jennings et al., 2013, Singh et al., 2006). Such speeding up of the interchange between eIF2-bound proteins would be even more efficient if simultaneous binding of two proteins to eIF2 β -NTT weakens their affinities (**Table 2**). Consistent with this idea, mutating any of the three K-boxes in *S. cerevisiae* eIF2 β causes a general control derepressed (Gcd⁻) phenotype, indicating

lower activity of eIF2B that in turn leads to lower than normal levels of the eIF2-GTP•Met-tRNA_i ternary complex (Asano et al., 1999). At the same time, the mutant strains are viable and grow at WT rates in rich medium; and at least K-boxes 1 and 3 alone are sufficient for viability (Asano et al., 1999). Therefore, the presence of multiple K-boxes serves mostly regulatory roles, at least in *S. cerevisiae*.

How is eIF2 handed off between eIF5 and eIF2B, and how do the 5MPs modulate eIF5 and eIF2B binding to eIF2? Both eIF2B and eIF5 contact eIF2 at two or more separate surfaces synergistically, yielding a stable overall interaction; and the same is probably the case also for the 5MPs (Singh et al., 2021). Binding to each individual site is weaker, with faster off-rates, but after transient loss of one interaction, the other contact is sufficient to maintain the integrity of the complex and keep the protein in the vicinity of the other site, increasing the rate of re-binding (see e.g., (Fersht, 1999)). Our results show that eIF5, eIF2B ϵ , and 5MP1 bind each of the three K-boxes multiple times per second (**Fig. 2B** and **Supplemental Fig. S3A, B**), and each K-box binds eIF5, eIF2B ϵ , and 5MP1 multiple times per second (**Supplemental Fig. S4B**). Therefore, the proteins modulate each other's binding to eIF2 both by competing for the same K-boxes and by binding simultaneously to different K-boxes. Simultaneous binding to eIF2 β -NTT has two complementary effects (**Fig. 5**):

- Faster, unimpeded binding to eIF2 β -NTT. If there is only one functional K-box (**Fig. 5A**), as in the mutant *S. cerevisiae* strains in (Asano et al., 1999), eIF2B has to be in the vicinity of the K-box exactly when eIF5 transiently dissociates from it, in order to bind and replace it. If there are two or more functional K-boxes (**Fig. 5B**), at least one of them will be open for eIF2B to bind, even when eIF5 is already bound to eIF2.
- Increased effective concentration in the vicinity of their other binding sites on eIF2. For example, eIF2B ϵ -CTD binding to a different K-box on eIF2 β , simultaneously with eIF5-CTD, would increase the probability of eIF2B binding to eIF2 γ when eIF5

transiently vacates its binding site there, which in turn would accelerate eIF5 dissociation from eIF2 (**Fig. 5B**). The possible destabilization of each other's binding to eIF2 β -NTT (**Table 2**) (illustrated with red T-shaped lines labeled with “?” in the model in **Fig. 5B**), could further accelerate dissociation.

All of this applies equally to eIF5 displacing eIF2B, presumably from the TC, if TC dissociation from eIF2B is too slow and/or the equilibrium is shifted too far toward the eIF2B•TC complex (Bogorad et al., 2018). In the eIF2B•TC complex, eIF2 α -NTD would likely contact the Met-tRNA_i, and not eIF2B, which would presumably weaken the eIF2B – eIF2 interaction, but not the eIF5 – eIF2 interaction. As a result, by simultaneously binding to eIF2, eIF5 and eIF2B weaken each other's affinities, leading to accelerated dissociation rates and exchange. Likewise, simultaneous binding would allow the 5MPs to modulate the eIF2 affinity for eIF5 and eIF2B without the need to win a direct competition.

The stringency of start codon selection is controlled by the interplay among eIF1, eIF5, and the 5MPs; and these proteins regulate the translation of their own and each other's mRNAs through their effects on the stringency (Ivanov et al., 2010, Loughran et al., 2018, Loughran et al., 2012). If the stringency of start codon selection depends on the concentrations of eIF1, eIF5 and the 5MPs, their binding to the PIC must be reversible and in equilibrium with the free proteins (occupancy must be at least slightly below 100%). Our results show how the 5MPs and eIF5 can co-exist on the PIC, weaken each other's binding affinity for the PIC, and thus lower each other's occupancy on eIF2 and the PIC. A similar mechanism should be in place for eIF1 and eIF5. Grosely and coauthors recently reported that in an *in vitro* reconstituted system, eIF1 and eIF5 binding to the PIC is indeed dynamic (Grosely et al., 2024). Here, we show that CK2 not only shifts the equilibrium among multiple competing interactions, but also stabilizes all of them (**Table 2**), potentially stabilizing the PIC and making the entire process more efficient. As described above, the stringency of start codon selection is controlled by the interplay among

eIF1, eIF5, and the 5MPs (Ivanov et al., 2010, Loughran et al., 2018, Loughran et al., 2012). Therefore, translation initiation at suboptimal start codons will likely also be modulated by eIF5 and 5MP phosphorylation. Dephosphorylation of eIF5 under chronic stress would lower its affinities for eIF1A (Gamble et al., 2022) and eIF2; and the resulting destabilization of the PICs would coincide with a drop in overall protein synthesis, and thus cellular concentrations of ribosomes and eIFs, which are proportional to the rates of protein synthesis (Milo et al., 2010). This raises the possibility for the formation of PICs with alternative properties under these conditions. In view of all these considerations, it would be very intriguing to elucidate how the translation apparatus operates under chronic stress. The potential effects on the GEF activity of eIF2B would be difficult to predict, as at lower translation rates, there would be lower demand for TC formation. Furthermore, CK2 phosphorylation appears to have a smaller effect on eIF2B than on eIF5; therefore, the lower affinity of eIF2-GDP for eIF2B may also be compensated by a more favorable relative affinity for eIF2B vs. eIF5.

The results presented here indicate that the differences in translation under various conditions, e.g., under chronic stress vs. actively dividing cells, may not be as simple as having different numbers of ribosomes and translation factors, but likely include kinetic and possibly even structural differences in the translational apparatus. This reinforces the need to always be mindful of the posttranslational modifications present under the specific conditions studied, especially when performing quantitative analysis or modeling.

Materials and Methods

Protein expression and purification

All *S. cerevisiae* constructs were expressed in *E. coli* BL21(DE3) Rosetta II cells (Novagene). Cells transformed with the respective plasmid were grown in 2xYT (containing the appropriate antibiotics) at 37 °C with shaking at 220 rpm until they reached an OD₆₀₀ of ~0.8

and were subsequently transferred to 16 °C. After allowing the cultures to cool down for 20 min, the expression was induced by the addition of isopropyl- β -D-thiogalactopyranosid (IPTG) to a final concentration of 0.5 mM. The cells were harvested after 16 hours at 16 °C. All human constructs were expressed in *E. coli* BL21(DE3) (Novagene), grown in LB at 37 °C. Expression was induced at 20 °C O/N with 1 mM IPTG. $^{15}\text{N}/^2\text{H}$ -labeled proteins were expressed in minimal medium in $^2\text{H}_2\text{O}$, with $^{15}\text{N}\text{-NH}_4\text{Cl}$ as the only nitrogen source, using the same procedure. $^{15}\text{N}^2\text{H}$ -labeled eIF2B ϵ -CTD-EE had poor solubility. Therefore, ^{15}N -labeled eIF2B ϵ -CTD-EE was used, instead, expressed in minimal medium with $^{15}\text{N}\text{-NH}_4\text{Cl}$ as the only nitrogen source, using the same procedure.

All yeast eIF2 β variants were cloned into the pGEX-6P1 vector and expressed as N-terminal GST-fusion proteins. Cells were resuspended in lysis buffer (500 mM NaCl, 20 mM HEPES (pH 7.5), 5% glycerol, 0.1 mM EDTA, 4 mM β -mercaptoethanol (BME)) supplied with a mixture of protease inhibitors including aprotinin, leupeptin, pepstatin (ALP), and PMSF. The cells were lysed in a microfluidizer (Microfluidics, USA) and cell debris was removed by centrifugation for 30 min at 30,000 $\times g$. After centrifugation, the supernatant was loaded onto a GSTrap column (GE Healthcare) equilibrated in lysis buffer. The column was washed with 2 column volumes of lysis buffer and bound fusion protein was eluted lysis buffer containing 30 mM of reduced glutathione. Fractions containing target protein were pooled and incubated over night at 4°C with PreScission protease (GE Healthcare) at a ratio of 1:100 (w/w) of protease to fusion protein to remove the GST-tag. After a desalting step in 200 mM NaCl, 20 mM HEPES pH 7.5, 5% glycerol, 4 mM BME, the cleaved GST, PreScission protease, and uncleaved protein were removed by a second GSH-Sepharose step. The flow-through containing the cleaved eIF2 β was pooled and applied to a Superdex S200 column (GE Healthcare) equilibrated in a buffer containing 150 mM KCl, 10 mM HEPES (pH 7.5), 5% glycerol, 2 mM DTT.

All yeast eIF5-CTD constructs were cloned into pGEX-6P1 for expression as N-terminal GST-fusions and purified similar to eIF2 β with minor modifications. Following lysis and sample application in lysis buffer, the GSTrap column was equilibrated in low salt buffer (100 mM NaCl, 10 mM HEPES pH 8, 5% glycerol, 4 mM BME), followed by elution in low salt buffer containing 30 mM reduced glutathione. After removal of the GST-tag by PreScission protease treatment, the protein was loaded onto a Source 30Q column (GE Healthcare) equilibrated in low salt buffer. Bound eIF5-CTD was eluted with a linear gradient into high salt buffer (1 M NaCl, 10 mM HEPES pH 7.5, 5% glycerol, 4 mM BME). Fractions containing the target protein were pooled and loaded onto a Superdex S75 column (GE Healthcare) equilibrated in buffer containing 150 mM KCl, 10 mM HEPES pH 7.5, 5% glycerol, 2 mM DTT. Fractions containing pure target protein were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at -80 °C.

Expression and purification of recombinant His₆-tagged human eIF2 β -NTT (residues 1-191) (Luna et al., 2012), eIF5-CTD (residues 232-431) and its phosphomimetic mutant (Paul et al., 2022) was as described previously. All other human constructs were cloned in pET21a with an N-terminal GB1 tag, a His₆-tag and a TEV protease cleavage site. Phosphomimetic point mutations were generated by site-directed mutagenesis. The proteins were purified on a TALON Cell-Thru His-tag affinity column (Clontech) in a buffer containing 10 mM Na Phosphate (pH 7.0), 300 mM KCl, 7 mM BME, and 0.1 mM AEBSF, followed by ion exchange chromatography on a Uno Q column, using a 100 mM to 1 M NaCl gradient. Where necessary, the proteins were further purified by SEC on an Enrich SEC70 column in buffer containing 10 mM Na Phosphate (pH 7.0), 150 mM KCl, 1 mM EDTA, 0.02% NaN₃, 1 mM DTT, and 0.1 mM AEBSF. If necessary, the GB1 tag was cleaved using TEV protease. Ion exchange chromatography on a Uno Q column was used to remove the GB1 tag. Proteins were exchanged into buffer containing 10 mM Na Phosphate (pH 7.0), 150 mM KCl, 1 mM EDTA, 0.02% NaN₃, 1 mM DTT, and 0.1 mM AEBSF.

Reconstitution, crystallization, and structure determination of the eIF5-eIF2 β complex

The individual yeast proteins, eIF2 β ₃₉₋₁₀₆ and eIF5₂₀₁₋₄₀₅, were purified as described above. The complex was reconstituted in binding buffer (150 mM KCl, 10 mM HEPES pH 7.5, 5% glycerol, 2 mM DTT) by mixing eIF5₂₀₁₋₄₀₅ with a 1.5-fold molar excess of eIF2 β ₃₉₋₁₀₆. After incubation for 30 min at 20 °C, the complex was separated from excess eIF2 β ₃₉₋₁₀₆ by size exclusion chromatography on a Superdex S75 equilibrated in binding buffer. The purified complex was concentrated to ~20 mg/ml and directly used for crystallization trials.

Crystallization trials were performed using the sitting drop vapor diffusion method. Initial crystals of the eIF5₂₀₁₋₄₀₅/eIF2 β ₃₉₋₁₀₆ complex grew within one day at 20 °C in a condition containing 0.4 M (NH₄)₂SO₄ and 0.05 M Li₂SO₄. After optimization, high-quality crystals that were used for structure determination were obtained with 12 mg/ml protein in 0.4 M (NH₄)₂SO₄ and 0.08 M Li₂SO₄ at 10 °C.

X-ray diffraction data were collected at BL 14.1 (HZB, BESSY, Berlin) (Mueller et al., 2012). The obtained data were processed in space group P3₂12 using XDS (Kabsch, 2010) and scaled to a final resolution of 2.0 Å. The phase problem was solved by molecular replacement using the program PHASER (McCoy et al., 2007) with the atomic coordinates of yeast eIF5₂₄₁₋₃₉₆ (PDB 2FUL) as search model. The initial structural model comprised two eIF5₂₄₁₋₃₉₆ molecules per asymmetric unit. Missing regions of the eIF5 construct (residues 201-240) and eIF2 β were built manually in Coot (Emsley et al., 2010). The final model was obtained gradually by iterative rounds of refinement in PHENIX (Adams et al., 2010), followed by manual model building.

Nuclear Magnetic Resonance (NMR)

NMR experiments were performed in buffer containing 10 mM Na Phosphate, pH 7.0, 150 mM KCl, 1 mM EDTA, 0.02% NaN₃, 1 mM DTT and 0.1 mM AEBSF, with 5% ²H₂O. NMR data were collected on a 500 MHz Bruker spectrometer (Boston University School of Medicine)

equipped with a cryoprobe. NMR resonance assignments for eIF5-CTD were available (Paul et al., 2022).

NMR Chemical Shift Perturbation (CSP) assay

The CSP assay was performed using Heteronuclear single-quantum coherence (HSQC) experiments on ^{15}N -labeled proteins. ^{15}N Transverse relaxation optimized spectroscopy (TROSY) HSQC experiments were run on $^{15}\text{N}/^2\text{H}$ -labeled proteins. Unless otherwise stated, the concentration of the labeled protein was 50 μM . The titrations of $^{15}\text{N}/^2\text{H}$ -labeled eIF5-CTD-EE with eIF2 β K-boxes (**Fig. 3D**) were performed with 20 μM $^{15}\text{N}/^2\text{H}$ -eIF5-CTD-EE, to keep the labeled protein concentration closer to the K_D . Some of the experiments with $^{15}\text{N}/^2\text{H}$ -labeled or ^{15}N -labeled eIF2 β -K123 (**Supplemental Fig. S4A**) were performed with 20 μM labeled eIF2 β -K123, to ensure sufficient excess of the unlabeled ligand.

Chemical shift changes were calculated according to the formula $\delta = ((\delta_H)^2 + (\delta_N/5)^2)^{1/2}$. For statistical analysis, average chemical shift changes and standard deviations were calculated in Excel. Affected residues were mapped on the surface of eIF5-CTD, for which the backbone NMR resonance assignments were available (Paul et al., 2022)

For K_D determination, $^{15}\text{N}/^2\text{H}$ -labeled or ^{15}N -labeled protein samples were titrated with increasing concentrations of an unlabeled binding partner, until saturation or until solubility limit was reached. Data analysis was done in SigmaPlot, as described previously (Paul et al., 2022). In **Fig. 3**, the concentration of eIF2 β -K123 (which has three K-boxes) is displayed in K-box concentration – to allow direct visual comparisons with titrations using single K-boxes. For example, 10 μM eIF2 β -K123 is displayed as 30 μM . When measuring binding to eIF2 β -K123, it was not possible to extract all three binding affinities to the three K-boxes. Instead, we calculated an average K_D for all three K-boxes within eIF2 β -K123. An additional challenge presented by the three K-boxes in eIF2 β -K123 is that when eIF2 β -K123 is in excess, the relative occupancy at the individual K-boxes is proportional to the relative affinities, whereas

when the binding partners are in excess, binding to all three K-boxes approaches saturation. If the different interactions induce different chemical shift changes, that could affect the titration curve. To minimize these possible effects, we selected for analysis peaks that moved similarly in the presence of each of the three K-boxes and eIF2 β -K123, which was the case for most affected peaks in eIF5-CTD (**Fig. 2B**), eIF2B ϵ -CTD (**Supplemental Fig. S3A**), and 5MP1-CTD (**Supplemental Fig. S3B**).

k_{off} estimation from NMR data was based on its effect on exchange broadening (reviewed in (Marintchev et al., 2007)). In **Supplemental Fig. S3C**, peaks that move by up to 50 Hz (0.1 ppm for ^1H on a 500 MHz NMR spectrometer) are in fast exchange (where peaks move from their position in the free labeled protein to their position in the complex with the ligand as a function of the fraction of bound protein), with no significant exchange broadening. Peaks that move by > 100 Hz (0.2 ppm for ^1H) undergo exchange broadening, sometimes beyond detection, at ligand concentrations where a fraction of the protein is bound. Under fast exchange conditions, the line broadening $\delta\nu$, due to exchange is dependent on the chemical shift change between the free and bound state, Δ [s $^{-1}$]. At equal populations of free and bound states, $\delta\nu$ is approximately equal to $(\pi\Delta^2)/4k_{\text{off}}$. Therefore, under the experimental conditions (150 mM KCl and 25 °C), if exchange broadening becomes significant for $\Delta > 100$ s $^{-1}$, k_{off} is in the order of 100 s $^{-1}$.

Size exclusion chromatography (SEC)

SEC experiments were performed on a Bio-Rad NGC chromatography system in buffer containing 10 mM Na Phosphate, pH 7.0, 150 mM KCl, 1 mM EDTA, 0.02% NaN $_3$, 1 mM DTT and 0.1 mM AEBSF. SEC molecular weight standards (Bio-Rad) and BSA (Sigma) were used for calibration. Sample volume was 100 μL . Experiments with eIF2 β -K12 and eIF2 β -K1m-K2 were performed using an Enrich SEC70 column. Experiments with eIF2 β -K123 were performed

using an Enrich SEC650 column. Unless otherwise stated, the protein concentrations were 30 μ M for free proteins and for 1:1 (or 1:1:1, etc.) mixes and 30 and 90 μ M for 1:3 (or 1:3:3, etc.) mixes.

Isothermal Titration Calorimetry (ITC)

ITC experiments were performed on a MicroCal VP-ITC instrument (Malvern Panalytical) at 26 °C, in buffer containing 10 mM Na Phosphate, pH 7.0, 150 mM KCl, 1 mM EDTA, 0.02% NaN₃, 7 mM BME and 0.1 mM AEBSF. eIF5-CTD or eIF5-CTD-EE was injected into a cell containing eIF2 β -K123 or eIF2 β -K3. Where the solubilities of the individual proteins allowed, the concentration (expressed in binding sites) of the protein in the cell was $\geq$ 10-fold higher than the expected K_D , and the concentration of the protein in the syringe was $\geq$ 7-fold higher than the concentration (expressed in binding sites) of the protein in the cell. The stoichiometry parameter, N was kept free during fitting, to compensate for possible concentration errors. The values of N resulting from the fit were between 0.96 and 1.04 for experiments with eIF2 β -K3 (one binding site, N = 1) and between 2.76 and 3.46 for experiments with eIF2 β -K123 (three binding sites, N = 3).

Sequence and structure analysis

Protein BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to obtain initial sequence alignment between human and *S. cerevisiae* eIF2 β . Aligning all six K-boxes was done using Clustal O from the Max-Planck Institute for Developmental Biology Bioinformatics Toolkit (<http://toolkit.tuebingen.mpg.de/>), followed by manual editing. Instead of by conservation, the aminoacids were colored by hydrophobicity (green), negative charge (red), and positive charge

(blue). MOLMOL (Koradi et al., 1996) was used to align the structures of human and *S. cerevisiae* eIF5-CTD, and for structure visualization.

Data Availability

The atomic model generated in this study has been deposited in the Protein Data Bank (PDB: 9F79). The data that support this study are available from corresponding authors upon reasonable request.

Acknowledgments

The authors thank Anvesh Sharma for assistance with NMR data analysis. This work was supported by the National Institute of Health [GM134113 to A.M.].

Author Contributions

Conceptualization, B.K, R.F., and A.M.; Methodology, B.K, R.F., and A.M.; Investigation, P.A.W., M.S., P.D., A.S., B.K., R.F., and A.M.; Writing – Original Draft, P.A.W., B.K., R.F., and A.M.; Writing – Review & Editing, P.A.W., B.K., R.F., and A.M.; Funding Acquisition, B.K, R.F., and A.M.; Supervision, B.K, R.F., and A.M.

Disclosure and competing interests statement

The authors declare no competing interest.

References

- Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66: 213-21
- Alone PV, Dever TE (2006) Direct binding of translation initiation factor eIF2gamma-G domain to its GTPase-activating and GDP-GTP exchange factors eIF5 and eIF2B epsilon. *The Journal of biological chemistry* 281: 12636-44
- Asano K, Krishnamoorthy T, Phan L, Pavitt GD, Hinnebusch AG (1999) Conserved bipartite motifs in yeast eIF5 and eIF2Bepsilon, GTPase-activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2. *The EMBO journal* 18: 1673-88
- Bieniossek C, Schutz P, Bumann M, Limacher A, Uson I, Baumann U (2006) The crystal structure of the carboxy-terminal domain of human translation initiation factor eIF5. *J Mol Biol* 360: 457-65
- Bochler A, Querido JB, Prilepskaja T, Soufari H, Simonetti A, Del Cistia ML, Kuhn L, Ribeiro AR, Valasek LS, Hashem Y (2020) Structural Differences in Translation Initiation between Pathogenic Trypanosomatids and Their Mammalian Hosts. *Cell Rep* 33: 108534
- Bogorad AM, Lin KY, Marintchev A (2018) eIF2B Mechanisms of Action and Regulation: A Thermodynamic View. *Biochemistry* 57: 1426-1435
- Borgo C, Franchin C, Scalco S, Bosello-Travain V, Donella-Deana A, Arrigoni G, Salvi M, Pinna LA (2017) Generation and quantitative proteomics analysis of CK2alpha/alpha'((-/-)) cells. *Sci Rep* 7: 42409
- Brito Querido J, Diaz-Lopez I, Ramakrishnan V (2024) The molecular basis of translation initiation and its regulation in eukaryotes. *Nat Rev Mol Cell Biol* 25: 168-186
- Das S, Maiti T, Das K, Maitra U (1997) Specific interaction of eukaryotic translation initiation factor 5 (eIF5) with the beta-subunit of eIF2. *The Journal of biological chemistry* 272: 31712-8
- Das S, Maitra U (2000) Mutational analysis of mammalian translation initiation factor 5 (eIF5): role of interaction between the beta subunit of eIF2 and eIF5 in eIF5 function in vitro and in vivo. *Mol Cell Biol* 20: 3942-50
- Dever TE, Dar AC, Sicheri F (2007) The eIF2 α kinases. In *Translational Control in Biology and Medicine*, Mathews MB, Sonenberg, N., Hershey, J. W. B. (ed) pp 319-44. Cold Spring harbor, NY: Cold Spring Harbor Laboratory Press
- Dever TE, Ivanov IP, Hinnebusch AG (2023) Translational regulation by uORFs and start codon selection stringency. *Genes & development* 37: 474-489
- Dokladal L, Stumpe M, Pillet B, Hu Z, Garcia Osuna GM, Kressler D, Dengjel J, De Virgilio C (2021) Global phosphoproteomics pinpoints uncharted Gcn2-mediated mechanisms of translational control. *Molecular cell* 81: 1879-1889 e6
- Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66: 486-501
- Fersht A (1999) *Structure and Mechanism in Protein Science*. W. H. Freeman and Company,
- Franchin C, Borgo C, Cesaro L, Zaramella S, Vilardell J, Salvi M, Arrigoni G, Pinna LA (2018) Re-evaluation of protein kinase CK2 pleiotropy: new insights provided by a phosphoproteomics analysis of CK2 knockout cells. *Cell Mol Life Sci* 75: 2011-2026
- Gamble N, Paul EE, Anand B, Marintchev A (2022) Regulation of the interactions between human eIF5 and eIF1A by the CK2 kinase. *Curr Res Struct Biol* 4: 308-319
- Gandin V, Masvidal L, Cargnello M, Gyenis L, McLaughlan S, Cai Y, Tenkerian C, Morita M, Balanathan P, Jean-Jean O, Stambolic V, Trost M, Furic L, Larose L, Koromilas AE, Asano K,

- Litchfield D, Larsson O, Topisirovic I (2016) mTORC1 and CK2 coordinate ternary and eIF4F complex assembly. *Nat Commun* 7: 11127
- Grosely R, Alvarado C, Ivanov IP, Nicholson OB, Puglisi JD, Dever TE, Lapointe CP (2024) eIF1 and eIF5 dynamically control translation start site fidelity. *bioRxiv*
- Hinnebusch AG (2005) Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* 59: 407-50
- Hinnebusch AG (2014) The scanning mechanism of eukaryotic translation initiation. *Annu Rev Biochem* 83: 779-812
- Hinnebusch AG, Dever TE, Asano K (2007) Mechanism of translation initiation in the yeast *Saccharomyces cerevisiae*. In *Translational Control in Biology and Medicine*, Mathews MB, Sonenberg, N., Hershey, J. W. B. (ed) pp 225-68. Cold Spring harbor, NY: Cold Spring Harbor Laboratory Press
- Hiraishi H, Oatman J, Haller SL, Blunk L, McGivern B, Morris J, Papadopoulos E, Gutierrez W, Gordon M, Bokhari W, Ikeda Y, Miles D, Fellers J, Asano M, Wagner G, Tazi L, Rothenburg S, Brown SJ, Asano K (2014) Essential role of eIF5-mimic protein in animal development is linked to control of ATF4 expression. *Nucleic acids research* 42: 10321-30
- Homma MK, Wada I, Suzuki T, Yamaki J, Krebs EG, Homma Y (2005) CK2 phosphorylation of eukaryotic translation initiation factor 5 potentiates cell cycle progression. *Proceedings of the National Academy of Sciences of the United States of America* 102: 15688-93
- Huang Q, Szklarczyk D, Wang M, Simonovic M, von Mering C (2023) PaxDb 5.0: Curated Protein Quantification Data Suggests Adaptive Proteome Changes in Yeasts. *Mol Cell Proteomics* 22: 100640
- Ivanov IP, Loughran G, Sachs MS, Atkins JF (2010) Initiation context modulates autoregulation of eukaryotic translation initiation factor 1 (eIF1). *Proceedings of the National Academy of Sciences of the United States of America* 107: 18056-60
- Jackson RJ, Hellen CU, Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* 11: 113-27
- Jennings MD, Kershaw CJ, White C, Hoyle D, Richardson JP, Costello JL, Donaldson IJ, Zhou Y, Pavitt GD (2016) eIF2beta is critical for eIF5-mediated GDP-dissociation inhibitor activity and translational control. *Nucleic acids research*
- Jennings MD, Pavitt GD (2010) eIF5 has GDI activity necessary for translational control by eIF2 phosphorylation. *Nature* 465: 378-81
- Jennings MD, Zhou Y, Mohammad-Qureshi SS, Bennett D, Pavitt GD (2013) eIF2B promotes eIF5 dissociation from eIF2*GDP to facilitate guanine nucleotide exchange for translation initiation. *Genes & development* 27: 2696-707
- Kabsch W (2010) Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr D Biol Crystallogr* 66: 133-44
- Kashiwagi K, Yokoyama T, Nishimoto M, Takahashi M, Sakamoto A, Yonemochi M, Shirouzu M, Ito T (2019) Structural basis for eIF2B inhibition in integrated stress response. *Science* 364: 495-499
- Kenner LR, Anand AA, Nguyen HC, Myasnikov AG, Klose CJ, McGeever LA, Tsai JC, Miller-Vedam LE, Walter P, Frost A (2019) eIF2B-catalyzed nucleotide exchange and phosphoregulation by the integrated stress response. *Science* 364: 491-495
- Koradi R, Billeter M, Wuthrich K (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J Mol Graph* 14: 51-5, 29-32
- Kozel C, Thompson B, Hustak S, Moore C, Nakashima A, Singh CR, Reid M, Cox C, Papadopoulos E, Luna RE, Anderson A, Tagami H, Hiraishi H, Slone EA, Yoshino KI, Asano M, Gillaspie S, Nietfeld J, Perchellet JP, Rothenburg S et al. (2016) Overexpression of eIF5 or its protein mimic 5MP perturbs eIF2 function and induces ATF4 translation through delayed re-initiation. *Nucleic acids research* 44: 8704-8713

- Lamper AM, Fleming RH, Ladd KM, Lee ASY (2020) A phosphorylation-regulated eIF3d translation switch mediates cellular adaptation to metabolic stress. *Science* 370: 853-856
- Lee AS, Kranszus PJ, Doudna JA, Cate JH (2016) eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation. *Nature* 536: 96-9
- Llorens F, Roher N, Miro FA, Sarno S, Ruiz FX, Meggio F, Plana M, Pinna LA, Itarte E (2003) Eukaryotic translation-initiation factor eIF2beta binds to protein kinase CK2: effects on CK2alpha activity. *Biochem J* 375: 623-31
- Loughran G, Firth AE, Atkins JF, Ivanov IP (2018) Translational autoregulation of BZW1 and BZW2 expression by modulating the stringency of start codon selection. *PLoS One* 13: e0192648
- Loughran G, Sachs MS, Atkins JF, Ivanov IP (2012) Stringency of start codon selection modulates autoregulation of translation initiation factor eIF5. *Nucleic acids research* 40: 2898-906
- Luna RE, Arthanari H, Hiraishi H, Akabayov B, Tang L, Cox C, Markus MA, Luna LE, Ikeda Y, Watanabe R, Bedoya E, Yu C, Alikhan S, Wagner G, Asano K (2013) The interaction between eukaryotic initiation factor 1A and eIF5 retains eIF1 within scanning preinitiation complexes. *Biochemistry* 52: 9510-8
- Luna RE, Arthanari H, Hiraishi H, Nanda J, Martin-Marcos P, Markus MA, Akabayov B, Milbradt AG, Luna LE, Seo HC, Hyberts SG, Fahmy A, Reibarkh M, Miles D, Hagner PR, O'Day EM, Yi T, Marintchev A, Hinnebusch AG, Lorsch JR et al. (2012) The C-terminal domain of eukaryotic initiation factor 5 promotes start codon recognition by its dynamic interplay with eIF1 and eIF2beta. *Cell Rep* 1: 689-702
- Marintchev A, Frueh D, Wagner G (2007) NMR methods for studying protein-protein interactions involved in translation initiation. *Methods Enzymol* 430: 283-331
- Marintchev A, Ito T (2020) eIF2B and the Integrated Stress Response: A Structural and Mechanistic View. *Biochemistry* 59: 1299-1308
- Marintchev A, Wagner G (2004) Translation initiation: structures, mechanisms and evolution. *Q Rev Biophys* 37: 197-284
- McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ (2007) Phaser crystallographic software. *J Appl Crystallogr* 40: 658-674
- Mertins P, Mani DR, Ruggles KV, Gillette MA, Clauser KR, Wang P, Wang X, Qiao JW, Cao S, Petralia F, Kawaler E, Mundt F, Krug K, Tu Z, Lei JT, Gatza ML, Wilkerson M, Perou CM, Yellapantula V, Huang KL et al. (2016) Proteogenomics connects somatic mutations to signalling in breast cancer. *Nature* 534: 55-62
- Milo R, Jorgensen P, Moran U, Weber G, Springer M (2010) BioNumbers--the database of key numbers in molecular and cell biology. *Nucleic acids research* 38: D750-3
- Mueller U, Darowski N, Fuchs MR, Forster R, Hellmig M, Paithankar KS, Puhlinger S, Steffien M, Zocher G, Weiss MS (2012) Facilities for macromolecular crystallography at the Helmholtz-Zentrum Berlin. *J Synchrotron Radiat* 19: 442-9
- Mukhopadhyay S, Amodeo ME, Lee ASY (2023) eIF3d controls the persistent integrated stress response. *Molecular cell* 83: 3303-3313 e6
- Obayashi E, Luna RE, Nagata T, Martin-Marcos P, Hiraishi H, Singh CR, Erzberger JP, Zhang F, Arthanari H, Morris J, Pellarin R, Moore C, Harmon I, Papadopoulos E, Yoshida H, Nasr ML, Unzai S, Thompson B, Aube E, Hustak S et al. (2017) Molecular Landscape of the Ribosome Pre-initiation Complex during mRNA Scanning: Structural Role for eIF3c and Its Control by eIF5. *Cell Rep* 18: 2651-2663
- Paul EE, Lin KY, Gamble N, Tsai AW, Swan SHK, Yang Y, Doran M, Marintchev A (2022) Dynamic interaction network involving the conserved intrinsically disordered regions in human eIF5. *Biophys Chem* 281: 106740

- Paytubi S, Morrice NA, Boudeau J, Proud CG (2008) The N-terminal region of ABC50 interacts with eukaryotic initiation factor eIF2 and is a target for regulatory phosphorylation by CK2. *Biochem J* 409: 223-31
- Petrychenko V, Yi SH, Liedtke D, Peng BZ, Rodnina MV, Fischer N (2024) Structural basis for translational control by the human 48S initiation complex. *Nat Struct Mol Biol*
- Ron D, Harding HP (2007) eIF2 α phosphorylation in cellular stress responses and disease. In *Translational Control in Biology and Medicine*, Mathews MB, Sonenberg, N., Hershey, J. W. B. (ed) pp 345-68. Cold Spring harbor, NY: Cold Spring Harbor Laboratory Press
- Sato K, Masuda T, Hu Q, Tobo T, Gillaspie S, Niida A, Thornton M, Kuroda Y, Eguchi H, Nakagawa T, Asano K, Mimori K (2019) Novel oncogene 5MP1 reprograms c-Myc translation initiation to drive malignant phenotypes in colorectal cancer. *EBioMedicine* 44: 387-402
- Singh CR, Glineburg MR, Moore C, Tani N, Jaiswal R, Zou Y, Aube E, Gillaspie S, Thornton M, Cecil A, Hilgers M, Takasu A, Asano I, Asano M, Escalante CR, Nakamura A, Todd PK, Asano K (2021) Human oncoprotein 5MP suppresses general and repeat-associated non-AUG translation via eIF3 by a common mechanism. *Cell Rep* 36: 109376
- Singh CR, Lee B, Udagawa T, Mohammad-Qureshi SS, Yamamoto Y, Pavitt GD, Asano K (2006) An eIF5/eIF2 complex antagonizes guanine nucleotide exchange by eIF2B during translation initiation. *The EMBO journal* 25: 4537-46
- Singh CR, Watanabe R, Zhou D, Jennings MD, Fukao A, Lee B, Ikeda Y, Chiorini JA, Campbell SG, Ashe MP, Fujiwara T, Wek RC, Pavitt GD, Asano K (2011) Mechanisms of translational regulation by a human eIF5-mimic protein. *Nucleic acids research* 39: 8314-28
- Singh LP, Denslow ND, Wahba AJ (1996) The interaction of rabbit reticulocyte guanine nucleotide exchange factor eIF-2B with chain initiation factor 2: studies with N-ethylmaleimide and trypsin. *Biochem Biophys Res Commun* 223: 604-11
- Tang L, Morris J, Wan J, Moore C, Fujita Y, Gillaspie S, Aube E, Nanda J, Marques M, Jangal M, Anderson A, Cox C, Hiraishi H, Dong L, Saito H, Singh CR, Witcher M, Topisirovic I, Qian SB, Asano K (2017) Competition between translation initiation factor eIF5 and its mimic protein 5MP determines non-AUG initiation rate genome-wide. *Nucleic acids research* 45: 11941-11953
- Turowec JP, Duncan JS, French AC, Gyenis L, St Denis NA, Vilk G, Litchfield DW (2010) Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. *Methods in enzymology* 484: 471-93
- Wei Z, Xue Y, Xu H, Gong W (2006) Crystal structure of the C-terminal domain of *S.cerevisiae* eIF5. *J Mol Biol* 359: 1-9
- Weisser M, Ban N (2019) Extensions, Extra Factors, and Extreme Complexity: Ribosomal Structures Provide Insights into Eukaryotic Translation. *Cold Spring Harb Perspect Biol* 11
- Wek RC, Jiang HY, Anthony TG (2006) Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans* 34: 7-11
- Yamamoto Y, Singh CR, Marintchev A, Hall NS, Hannig EM, Wagner G, Asano K (2005) The eukaryotic initiation factor (eIF) 5 HEAT domain mediates multifactor assembly and scanning with distinct interfaces to eIF1, eIF2, eIF3, and eIF4G. *Proceedings of the National Academy of Sciences of the United States of America* 102: 16164-9
- Yi T, Zhai B, Yu Y, Kiyotsugu Y, Raschle T, Etzkorn M, Seo HC, Nagiec M, Luna RE, Reinherz EL, Blenis J, Gygi SP, Wagner G (2014) Quantitative phosphoproteomic analysis reveals system-wide signaling pathways downstream of SDF-1/CXCR4 in breast cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 111: E2182-90

Tables

Table 1. Data collection and refinement statistics for the structure of the *S. cerevisiae* eIF5₂₀₁₋₄₀₅•eIF2 β ₃₉₋₁₀₆ complex

Crystallization	
Condition	0.8 M (NH ₄)SO ₄ , 0.3 M LiSO ₄
Temperature (°C)	10
Data Collection	
Space Group	<i>P</i> 3 ₂ 12
Unit Cell	<i>a</i> = 74.3 Å
	<i>b</i> = 74.3 Å
	<i>c</i> = 124.8 Å
	α = 90°
	β = 90°
	γ = 120°
Resolution (Å)	2.0 (2.2-2.0)
Observed reflections	359597 (87743)
Unique reflections	48223 (11831)
Redundancy	7.45
Completeness (%)	99.9 (99.8)
$\langle I \rangle / \sigma$	22.3 (3.9)
R _{merge} (%)	8.9 (42.8)
Refinement	
R _{work} /R _{free} (%)	20.7/22.8
Wilson B-factor (Å ²)	30.4
Total number of atoms	3824
Average B-factor, all atoms (Å ²)	42.0
Rmsd from Strd. Stereochemistry	
Bond length (Å)	0.015
Bond angles (°)	1.65
Ramachandran Plot Statistics	
Most favored (%)	98.9
Allowed regions (%)	1.1
Disallowed regions (%)	0
Values in parentheses refer to the highest resolution shell. R _{work} and R _{free} factors are calculated using the formula $R = \frac{\sum_{hkl} F(obs)_{hkl} - F(calc)_{hkl} }{\sum_{hkl} F(obs)_{hkl} }$, where $F(obs)_{hkl}$ and $F(calc)_{hkl}$ are observed and measured structure factors, respectively. R _{work} and R _{free} differ in the set of reflections they are calculated from: R _{free} is calculated for the test set, whereas R _{work} is calculated for the working set.	

Table 2. Binding affinities of WT and phosphomimetic eIF5, eIF2Bε, and 5MP1 for the eIF2β K-boxes

	K_D [μ M]			
	K1	K2	K3	K123
eIF5-CTD	3 ± 1	6 ± 1	4 ± 1	(3x) 37 ± 6^1
eIF2Bε-CTD	8 ± 1	9 ± 3	24 ± 3	(3x) 44 ± 3
5MP1-CTD	20 ± 7	18 ± 3	37 ± 5	(3x) 63 ± 5
eIF5-CTD_EE	0.2 ± 0.2^2	0.7 ± 0.2^2	0.3 ± 0.3^2	(3x) 2.3 ± 0.5
eIF2Bε-CTD_EE	4 ± 1	2.3 ± 0.5	14 ± 1	(3x) 14 ± 4
5MP1-CTD_EE	11 ± 3	5.6 ± 0.9	23 ± 3	(3x) 45 ± 6

¹ The K_D s for K123 are averages, assuming three binding sites with identical affinities.

² K_D values below 1 μ M are only approximate because the concentration of the labeled protein (20–50 μ M) becomes much larger than the K_D , making accurate fitting difficult.

Figure legends

Figure 1. Crystal Structure of the *S. cerevisiae* eIF5-CTD•eIF2 β -K23 complex.

(A) Crystal structure of the complex between eIF5-CTD (blue) and eIF2 β -K23 (yellow). Residues 39-65 of eIF2 β , including K2, are not resolved in the electron density, and are therefore not included in the model.

The second eIF5-CTD molecule in the asymmetric unit is not shown.

(B) View of the eIF5-CTD•eIF2 β -K23 complex centered on the loop containing K3. The eIF5-CTD is shown in surface representation with electrostatic surface charge distribution.

(C) Details of the interaction interface between the α N helix of eIF2 β -K3 and the surface of eIF5-CTD.

(D) Details of the interactions of the α C helix of eIF2 β -K3 with eIF5-CTD.

(E) Details of the interactions of the K3-loop of eIF2 β with eIF5-CTD.

Figure 2. Human eIF5-CTD forms similar complexes with each of the three K-boxes of eIF2 β .

(A) Sequence alignment of the K-boxes and surrounding sequences in *S. cerevisiae* and human eIF2 β -NTT, (labeled “Sc 2 β ” and “Hs 2 β ”, respectively). The entire 2 β -NTT sequences are shown, up to the start of the eIF2 γ -binding helix. Residue numbers are shown at the beginning and end of each line. Positively charged amino acids are colored in blue; negatively charged amino acids are colored in red, and hydrophobic amino acids are colored in green. Helices α N and α C from the *S. cerevisiae* eIF5-CTD•eIF2 β -K3 complex (**Fig. 1**) are bold and underlined. The corresponding sequence segments in the other K-boxes in *S. cerevisiae* and human eIF2 β are underlined. Note that if the K1 and K2 in *S. cerevisiae* form helices of the same length as those observed for K3 (**Fig. 1**), then there are no linker amino acids between K1 and K2, and between K2 and K3 in *S. cerevisiae* eIF2 β . Diagrams of *S. cerevisiae* and human eIF2 β are shown below the alignment. 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* eIF2 β visible in the crystal

structure, including the α N and α C helices (**Fig. 1**) is marked with a solid line. The corresponding segments surrounding the other K-boxes, corresponding to the α N and α C helices are marked with dashed lines.

(B) NMR spectra overlay of $^{15}\text{N}/^2\text{H}$ -labeled human eIF5-CTD, free (black) and in the presence of eIF2 β -K123 (red), K1 (blue), K2 (green), and K3 (orange). Examples of peaks that are in similar positions in all complexes (top two boxes), or different positions in the different complexes (bottom left) are boxed.

(C) Comparison of eIF2 β K-box contacts in yeast and human, in cross-eyed stereo. The eIF2 β -K3 segment from the crystal structure of the *S. cerevisiae* eIF5-CTD•eIF2 β -K3 complex (**Fig. 1**) is overlayed on the structure of human eIF5-CTD (2iu1.pdb (Bieniossek et al., 2006)). The overlay was obtained by aligning the structures of human and yeast eIF5-CTD. eIF2 β is shown as a blue ribbon, with sidechains also shown. Human eIF5-CTD is shown in surface representation colored by CSP effects for K123 binding: from dark orange (large effects) to yellow (small effects). No significant changes are light grey; amino-acids that could not be analyzed are in dark grey. eIF5-CTT is disordered in solution (Paul et al., 2022) and shown as a ribbon. The sidechains of S389 and S390, which are phosphorylated by CK2 (Homma et al., 2005), are shown as sticks and labeled.

Figure 3. Binding affinities of WT and phosphomimetic human eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD for eIF2 β -NTT and its individual K-boxes.

(A) Overlay of titration curves of eIF5-CTD binding to eIF2 β -K1, eIF2 β -K2, eIF2 β -K3, eIF2 β -K123 (eIF2 β -NTT), eIF2 β -K1m, and eIF2 β -K3 Δ .

(B) Overlay of titration curves of eIF2B ϵ -CTD binding to eIF2 β -K1, eIF2 β -K2, eIF2 β -K3, and eIF2 β -K123 (eIF2 β -NTT).

(C) Overlay of titration curves of 5MP1-CTD binding to eIF2 β -K1, eIF2 β -K2, eIF2 β -K3, and eIF2 β -K123 (eIF2 β -NTT).

(D) Overlay of titration curves of eIF5-CTD-EE binding to eIF2 β -K1, eIF2 β -K2, eIF2 β -K3, and eIF2 β -K123 (eIF2 β -NTT).

(E) Overlay of titration curves of eIF2B ϵ -CTD-EE binding to eIF2 β -K1, eIF2 β -K2, eIF2 β -K3, and eIF2 β -K123 (eIF2 β -NTT).

(F) Overlay of titration curves of 5MP1-CTD-EE binding to eIF2 β -K1, eIF2 β -K2, eIF2 β -K3, and eIF2 β -K123 (eIF2 β -NTT). Note that the eIF2 β -K123 concentration is expressed in K-boxes. For example, 100 μ M eIF2 β -K123 is plotted as 300 μ M (= 100 μ M K1 + 100 μ M K2 + 100 μ M K3). Likewise, K_{Ds} for eIF2 β -K123 are averages, assuming three binding sites with identical affinities. Note that the same total concentration of K-boxes in eIF2 β -K123 binds smaller fraction of the respective binding partner than even the K-box with the lowest affinity alone, except in panel E, where eIF2 β -K123 binds with the same apparent affinity as eIF2 β -K3, the K-box with the weakest affinity. Standard deviations (SD) are shown as grey bars. The chemical shift change values were normalized to the point with the greatest chemical shift change (assigned a value of 1); therefore, the last point has no SD values. The calculated K_{Ds} and standard errors (SE) are shown in the insets.

Figure 4. Simultaneous binding of eIF5, eIF2B ϵ , and 5MP1 to eIF2 β visualized by SEC.

(A) Overlay of SEC traces on an Enrich SEC 70 column of free GB1-tagged eIF2 β -K12, eIF5-CTD, and eIF2B ϵ -CTD and the following mixes:

- eIF2 β -K12 (containing two K-boxes), eIF5-CTD, and eIF2B ϵ -CTD in a 1:1:1 ratio (orange trace). Most of the proteins migrate as a trimeric complex; therefore, most eIF2 β -K12 molecules must be bound to two eIF5-CTD and/or eIF2B ϵ -CTD molecules.
- eIF2 β -K1m-K2 (where K1 is mutated) and eIF2B ϵ -CTD in a 1:1 ratio (red trace) serves as a reference for the mobility of the heterodimeric complex.
- eIF2 β -K1m-K2, eIF5-CTD, and eIF2B ϵ -CTD in a 1:1:1 ratio (purple trace) does not show a trimeric complex, indicating that binding to the mutated K-box 1 is not stable under the conditions of the SEC.

The positions of molecular weight markers are shown with vertical dashed lines. Protein concentrations were 30 μ M; the sample volume was 100 μ L. Some of the chromatograms were scaled to make the peak heights comparable.

(B) Overlay of SEC traces on an Enrich SEC 650 column of free GB1-tagged eIF2 β -K123, eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD and the following mixes (SDS-PAGE gels of the fractions from each run are shown below the chromatograms, labeled, and color-coded):

- eIF2 β -K123, eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD in a 1:1:1:1 ratio (orange trace). The majority of the proteins migrate as a tetrameric complex; therefore, most eIF2 β -K123 molecules must be bound to three molecules, but lower molecular weight species are also present. The gel below shows that 5MP1-CTD (or possibly also eIF2B ϵ -CTD, which has a very similar mobility on the gel) is spread from the tetramer peak all the way to the free protein peak, indicating that it tends to dissociate during the SEC run.

- eIF2 β -K123, eIF5-CTD, and eIF2B ϵ -CTD in a 1:3:3 ratio (purple trace). Most of the proteins migrate either as a tetrameric complex or as free proteins, indicating that the tetrameric complex is more stable in the presence of excess binding partners and/or in the absence of the less stably bound 5MP1-CTD. Both eIF5-CTD and eIF2B ϵ -CTD are found in complexes and in the free protein peaks, qualitatively consistent with the similar affinities measured by NMR (**Table 2**).

- eIF2 β -K123, eIF5-CTD-EE, eIF2B ϵ -CTD-EE, and 5MP1-CTD-EE in a 1:3:3:3 ratio (grey trace). The majority of the proteins migrate as a tetrameric complex or as free proteins, similar to the mix with the WT proteins. The gel below shows that the majority of eIF5-CTD-EE is found in complexes, whereas little eIF2B ϵ -CTD-EE and no 5MP1-CTD-EE are found in complexes, qualitatively consistent with the affinities measured by NMR (**Table 2**).

The positions of molecular weight markers are shown with vertical dashed lines. Protein concentrations were 30 μ M for the free proteins and for equimolar mixes (1:1, 1:1:1, etc.), and 90 μ M for 1:3, 1:3:3, etc. mixes; the sample volume was 100 μ L. Some of the chromatograms were scaled to make the peak heights comparable.

(C) Phosphorylation of 5MP1-CTD by CK2 causes upward mobility shift in SDS-PAGE. The molecular weight markers are the same as in (B).

Figure 5. Model for the handoff of eIF2 between eIF5 and eIF2B.

(A) One functional K-box, as in the mutant *S. cerevisiae* strains in (Asano et al., 1999). eIF5 binds dynamically (on and off) to both the K-box and to eIF2 γ . eIF2B can bind first to either the K-box or to eIF2 γ when the respective binding site is transiently open, leading to a complex where both eIF5 and eIF2B are weakly bound to eIF2 (each to one binding site). This transition state will get resolved into a binary complex of eIF2 with only eIF5 (reverse reaction) or with only eIF2B (forward reaction).

(B) Two or more functional K-boxes. eIF5 binds dynamically (on and off) to both a K-box and to eIF2 γ . eIF2B can freely bind first to an open K-box, leading to a complex where eIF5 is still bound dynamically to both a K-box and to eIF2 γ , and eIF2B is weakly bound to a different K-box. This increases the effective concentration of eIF2B in the vicinity of eIF2 γ , increasing its chances to bind to eIF2 γ when eIF5 transiently dissociates from it. The result is a second transition complex where eIF2B is bound dynamically to both a K-box and to eIF2 γ (as well as eIF2 α), and eIF5 is weakly bound to a different K-box. This pair of transition states will get resolved by dissociation of eIF2B from the first complex on the left, leading to a binary complex of eIF2 with only eIF5 (reverse reaction) or dissociation of eIF5 from the second complex, leading to a binary complex of eIF2 with only eIF2B (forward reaction).

The same mechanism could work in the opposite direction in the handoff of the TC from eIF2B to eIF5, except there eIF2 α -NTD would be interaction with the Met-tRNA_i, instead of with eIF2B. Folded domains are shown as shapes. Intrinsically disordered regions are shown as lines. eIF2 is yellow; eIF5 is brown; eIF2B is blue. The shapes of the folded segments of eIF2 and eIF2B, their mutual orientations, and contacts are based on the eIF2B•eIF2 complex structure (6jlv.pdb (Kashiwagi et al., 2019)). For simplicity, only one eIF2 is shown bound to eIF2B. Interactions are shown with double arrows, instead of with physical contacts in the complex, to emphasize their dynamic nature. The possible mutual

destabilization between two proteins simultaneously bound to eIF2 β -NTT is illustrated by red T-shaped lines and marked with “?”.

Figure 1

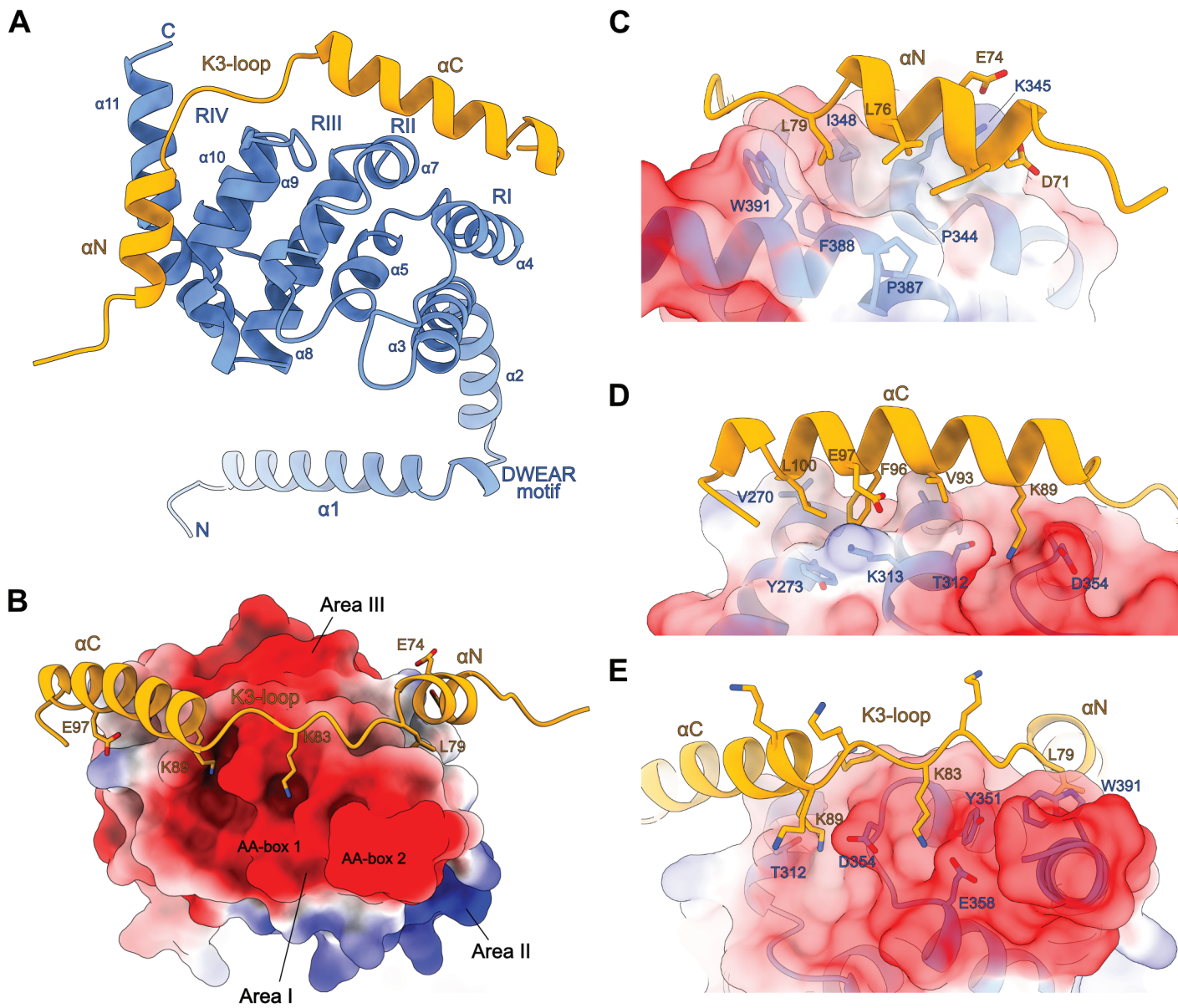
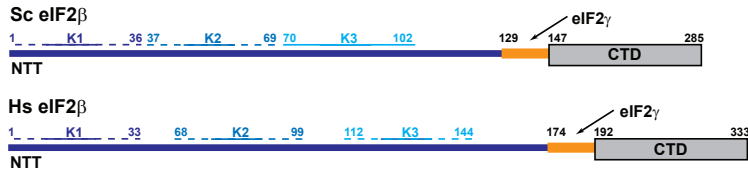


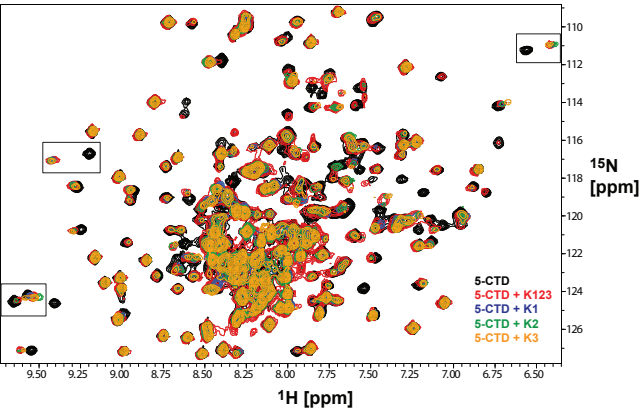
Figure 2

A eIF2β K-box sequence alignment

```
Sc 2β 1  MSSDLAALGFDPAKKKKKTKKVI PDDFDAVNGK----- 36
Hs 2β 1  MSGDEMIFDPTMSKKKKKKKPFMLDEGDTQTETQPSETKEVEPEPTEDKLEADEDTRK 64
Sc 2β 37  ---ENGSGDDLFAGLKKKKKKSKSVSADAEAEKEPT----- 69
Hs 2β 65  KDA SDDLDDLNFNQKKKKKKTKKIFDIDEAEGVKOLKIESDVQ----- 108
Sc 2β 70  ---DDIAEALGEISLKKKKKKTKKSSVDAPFEKELAKAGLDNVDAESKEGTPSANS---SIQQEVGL 129
Hs 2β 109 EPTEPEDDLDTIMLGNNKKKKKNVKFPDEDEILEKDEALEDEDNKKDGGISFSNQTPAWAGSERDY 174
```



B Overlay of NMR spectra of Hs eIF5-CTD in the absence and presence of different eIF2β K-boxes



C Comparison of eIF2β K-box contacts in yeast and human

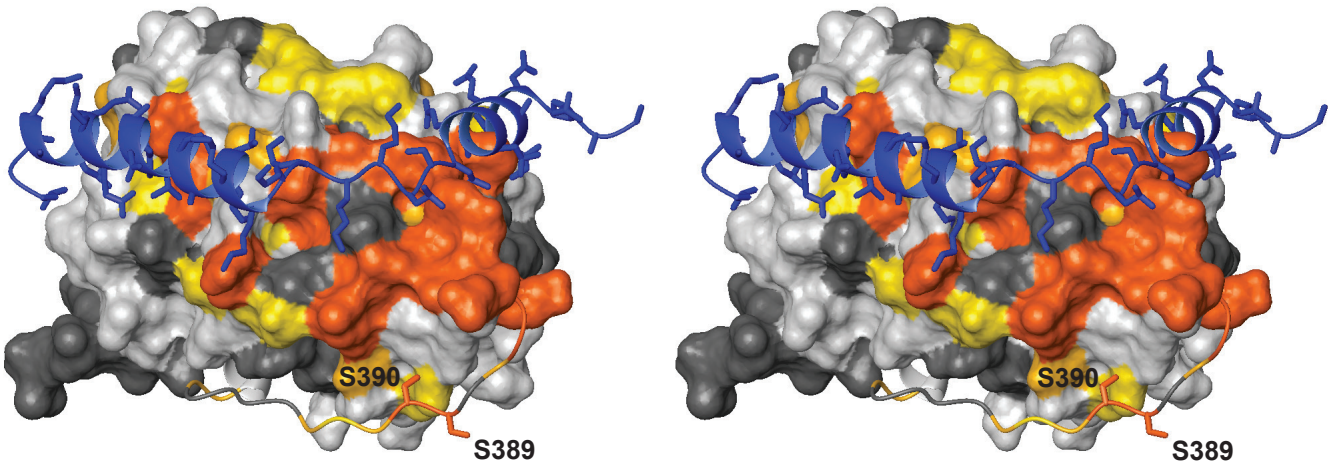


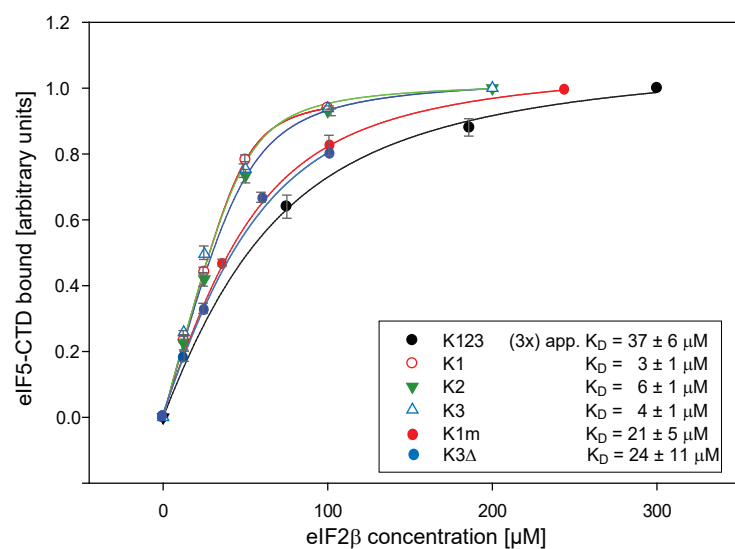
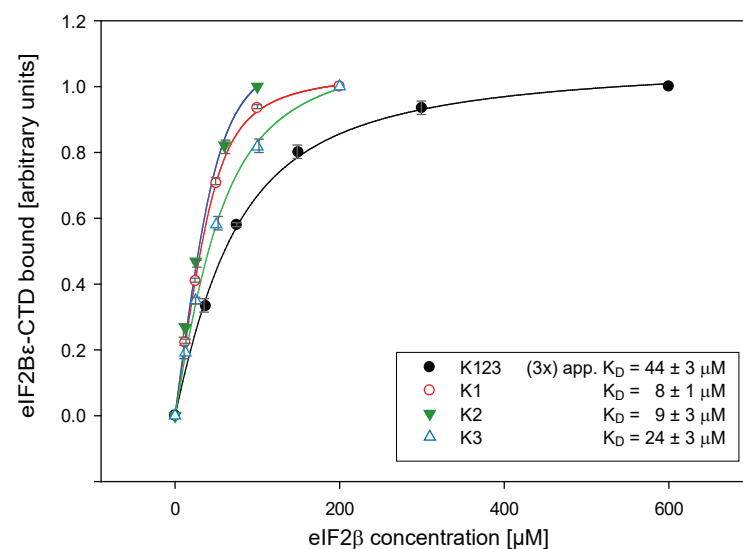
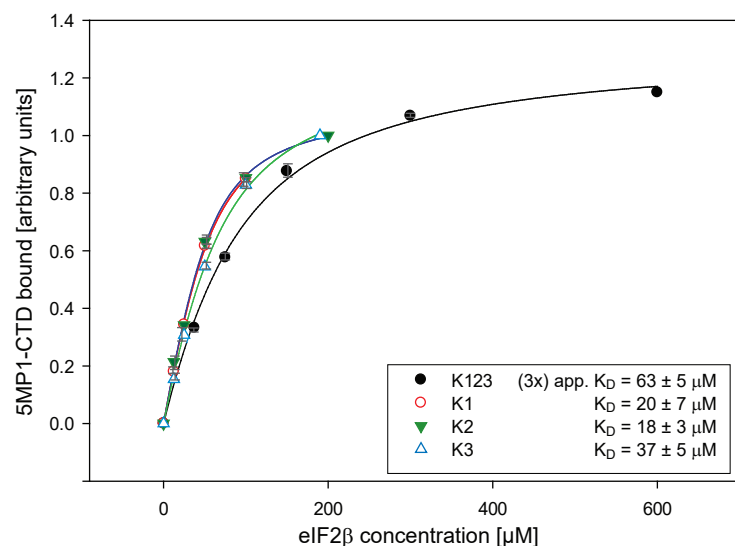
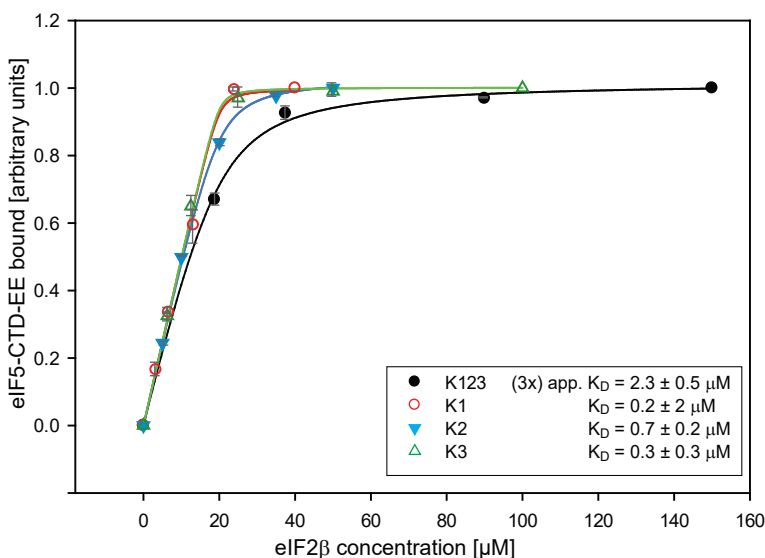
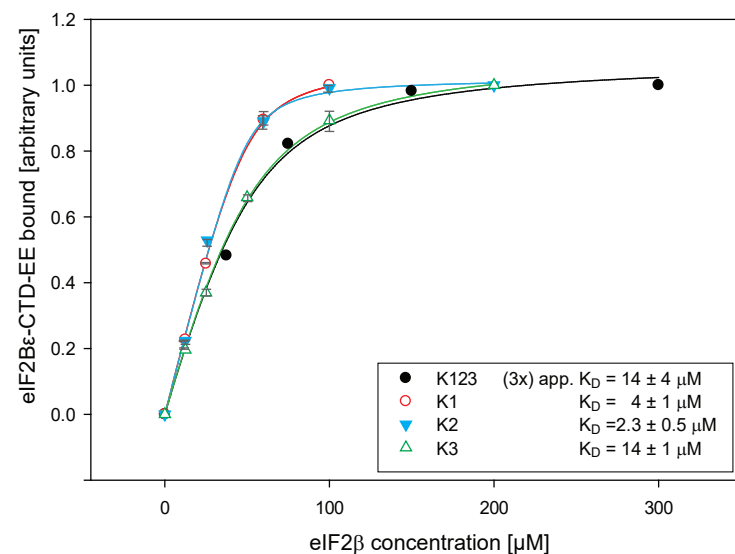
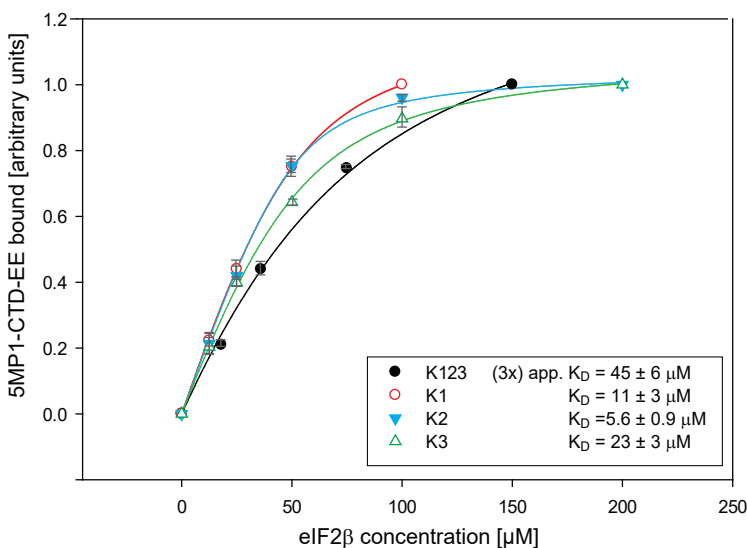
Figure 3**A Affinities of eIF2 β constructs for eIF5-CTD****B Affinities of eIF2 β constructs for eIF2B ϵ -CTD****C Affinities of eIF2 β constructs for 5MP1-CTD****D Affinities of eIF2 β constructs for eIF5-CTD-EE****E Affinities of eIF2 β constructs for eIF2B ϵ -CTD-EE****F Affinities of eIF2 β constructs for 5MP1-CTD-EE**

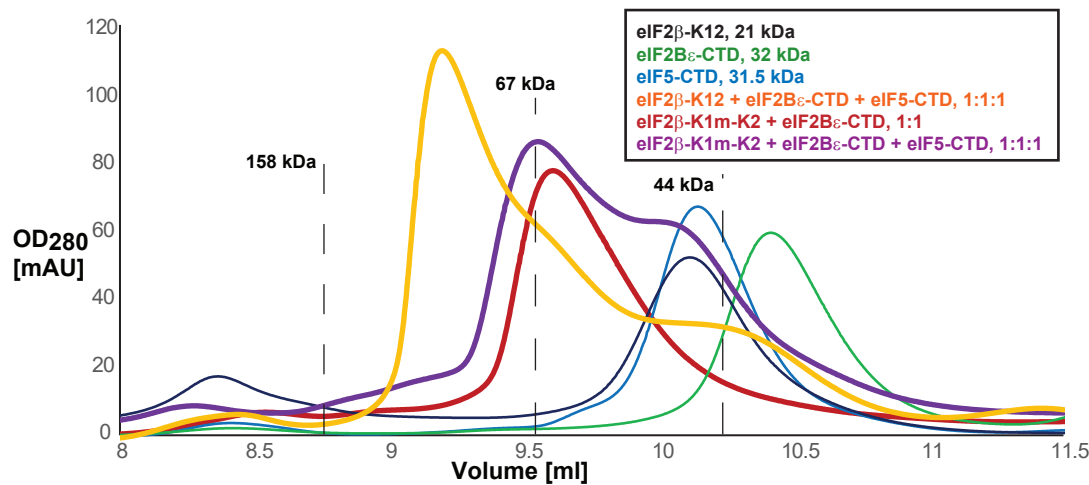
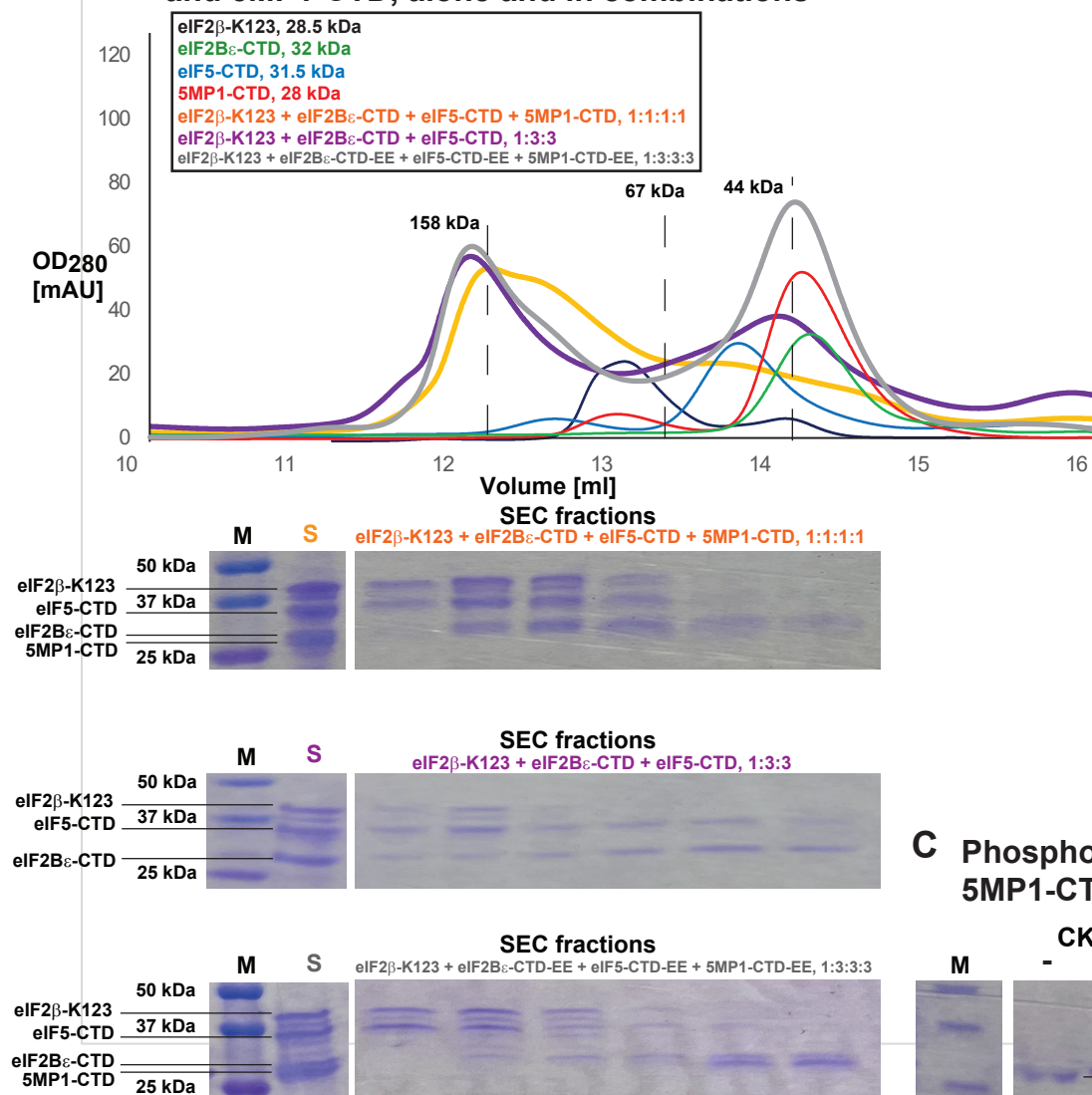
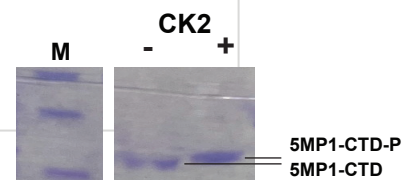
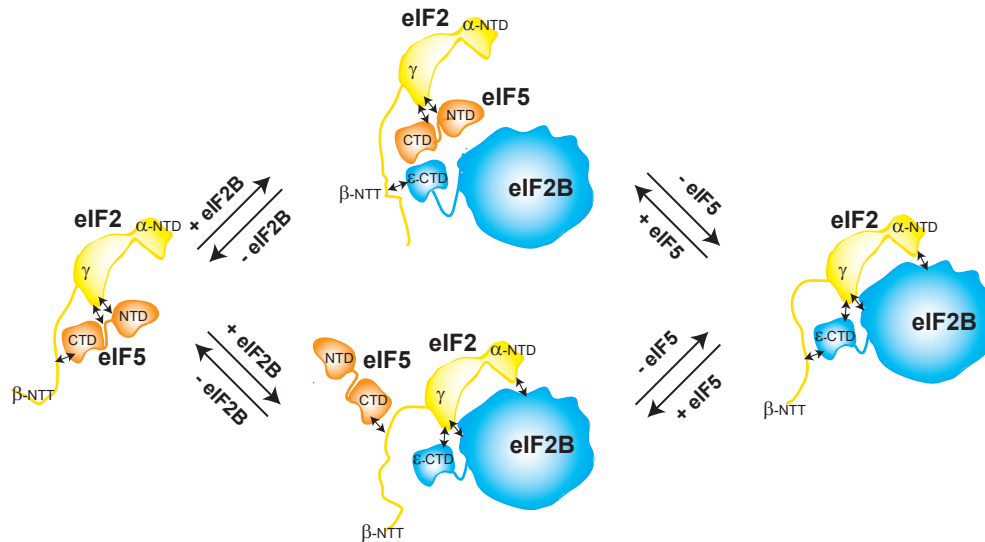
Figure 4**A** Overlay of SEC traces of Hs eIF2 β -K12, eIF2 β -K1m-K2, eIF5-CTD, and eIF2B ϵ -CTD, alone and in combinations**B** Overlay of SEC traces of Hs eIF2 β -K123, eIF5-CTD, eIF2B ϵ -CTD, and 5MP1-CTD, alone and in combinations**C** Phosphorylation of 5MP1-CTD by CK2

Figure 5

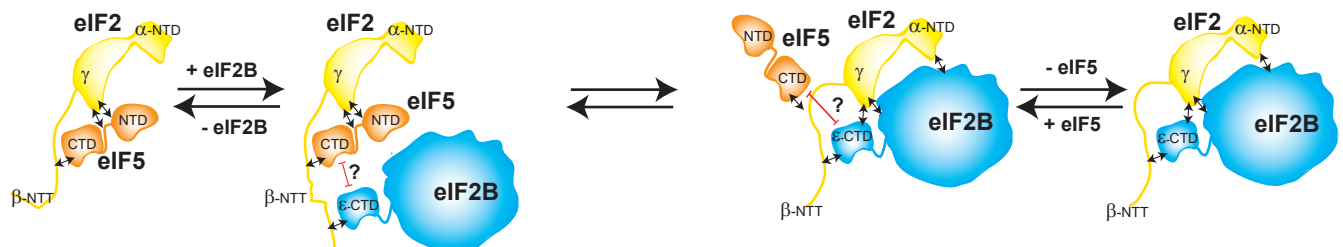
A One eIF2 β K-box.

eIF5 and eIF2B can only bind simultaneously to eIF2 if one is bound to eIF2 β -NTT and the other - to eIF2 γ



B Two or more eIF2 β K-boxes

eIF5-CTD and eIF2B ϵ -CTD can bind simultaneously to eIF2 β -NTT, allowing faster binding of one protein to eIF2 in the presence of the other





RNA

A PUBLICATION OF THE RNA SOCIETY

Molecular basis for the interactions of eIF2 β with eIF5, eIF2B, and 5MP1 and their regulation by CK2

Paul A Wagner, Meimei Song, Paul Doran, et al.

RNA published online July 16, 2025

Supplemental Material

<http://rnajournal.cshlp.org/content/suppl/2025/07/16/rna.080652.125.DC1>

P<P

Published online July 16, 2025 in advance of the print journal.

Accepted Manuscript

Peer-reviewed and accepted for publication but not copyedited or typeset; accepted manuscript is likely to differ from the final, published version.

Creative Commons License

This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see <http://rnajournal.cshlp.org/site/misc/terms.xhtml>). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at <http://creativecommons.org/licenses/by-nc/4.0/>.

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).



The NEW Vortex Mixer

US
SCIENTIFIC
BY DESIGN

To subscribe to RNA go to:

<http://rnajournal.cshlp.org/subscriptions>
