Title: Structural insights into ISRIB, a memory-enhancing inhibitor of the integrated stress response

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Abstract: The integrated stress response regulates protein synthesis under conditions of stress. Phosphorylation of translation initiation factor eIF2 by stress-sensing kinases converts eIF2 from substrate to competitive inhibitor of its dedicated nucleotide exchange factor, eIF2B, arresting translation. A drug-like molecule called ISRIB reverses the effects of eIF2 phosphorylation and restores translation by targeting eIF2B. When administered to mice, ISRIB enhances cognition and limits cognitive decline due to brain injury. To determine ISRIB's mechanism of action we solved an atomic structure of ISRIB bound to the human eIF2B decamer. We found that ISRIB acts as a molecular staple, pinning together tetrameric subcomplexes of eIF2B along the assembly path to a fully-active, decameric enzyme. In this Structural Snapshot, we discuss ISRIB's mechanism, its ability to rescue disease mutations in eIF2B and conservation of the enzyme and ISRIB binding pocket.

Conflict of Interest Statement:
P.W. [University of California, San Francisco (UCSF) employee] currently holds ISRIB-related patents. These patents are licensed by UCSF to Genentech and Calico.

Introduction

Proteins execute the majority of cellular functions and thus regulation of protein synthesis is vital for cell growth, health and survival. Translation initiation in particular offers a proteome-wide control point. The integrated stress response (ISR), a cellular signaling network, couples the detection of cellular stresses to the inhibition of translation initiation. Four kinases sense stress: PERK detects the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum, PKR senses double-stranded RNA, GCN2 responds to amino acid deprivation, and HRI senses heme deficiency. Once activated, these kinases converge on the alpha subunit of eukaryotic translation initiation factor 2 (eIF2) at serine 51 to elicit a translational and transcriptional stress response (*1*).

eIF2, a heterotrimeric GTPase composed of α, β and γ subunits, can bind GTP and methionine initiator tRNA to form a ternary complex. In conjunction with translation machinery, ternary complex scans along the 5' untranslated region of mRNAs to detect the translation start site. Once the AUG start codon is decoded, GTP is hydrolyzed and eIF2-GDP is released as a binary complex from the ribosome. Exchange of GDP for GTP enables a new round of translation initiation. This occurs with the aid of a dedicated nucleotide exchange factor, translation initiation factor 2B (eIF2B).

eIF2B is a decameric nucleotide exchange factor composed of two copies of subunits α, β, γ, δ and ε (*2–4*). Upon ISR-induced phosphorylation, eIF2 is converted from substrate to competitive inhibitor of eIF2B, arresting general protein synthesis and upregulating translation of a select few mRNAs containing upstream open reading frames (*5–7*). These mRNAs encode stress-responsive factors such as the transcription factor ATF4.

A small drug-like molecule called ISRIB (integrated stress response inhibitor) targets eIF2B (*8*, *9*) and, remarkably, enhances cognition in mice (*10*). In rodents, ISRIB is effective in a number of disease models: treatment with the molecule can reverse cognitive deficits following traumatic brain injury (*11*), protect against prion-induced neurodegeneration (*12*), and prevent metastasis of a subset of cancers . *In vitro* studies determined that ISRIB activates and stabilizes a decameric eIF2B complex (*13*). To determine ISRIB's mechanism of action we solved a structure of the small molecule-bound eIF2B complex.

Structure of ISRIB-bound human eIF2B

We established a recombinant expression and purification system for the five subunits of eIF2B that self-assemble in *E. coli* to form a stable decamer. We imaged this complex in the presence of ISRIB by cryo-electron microscopy and determined a 2.8Å average resolution structure that identified the binding site and coordination of the small molecule (Fig. 1). Our interpretation of the density afforded modeling of the majority of the ten-subunit decamer, with the exception of the flexible "ear" domains of eIF2Bγ and the leashed "HEAT" domain of eIF2Bε that is itself sufficient for basal catalytic activity (*14*, *15*). Future studies are aimed at resolving these critical regions.

A 2.7Å resolution core enabled more accurate modeling of ISRIB and identification of residues important for it activity. Our density for ISRIB is best explained by the drug binding as multiple conformers, each with a pair related by 180° rotation. Further, our interpretation of the density indicated that eIF2B βH188 contributes to ISRIB binding by forming a C-H-pi bond with the methylene bridge adjacent to the ether oxygen on ISRIB. Replacement of this histidine with either tyrosine or phenylalanine enhanced ISRIB binding, serving to validate our observation.

A number of other residues contribute to the shape specificity and hydrophobicity of the pocket, and are critical for ISRIB binding. For example, eIF2Bδ L179 blocks binding of a methylated ISRIB analog, presumably due to steric hindrance. This methylated ISRIB has greatly reduced activity that can be restored by mutation of leucine to alanine. Given this information one may surmise that ISRIB efficacy can be improved by enhancing shape complementarity towards the binding pocket. This observation may explain the high efficacy of a di-halogenated ISRIB that can occupy greater space within the binding pocket.

ISRIB mechanism of action

By analyzing eIF2B in the presence and absence of ISRIB using cryoEM and analytical ultracentrifugation techniques, we determined a functional model of ISRIB action. ISRIB staples together tetrameric eIF2B(βγδε) subcomplexes to form a more active octameric eIF2B$(\beta\gamma\delta\varepsilon)_2$, that in turn possesses an avid binding surface for the dimeric alpha subunits. Consolidation of the alpha homodimer and the ISRIB-bound octamer produces the fully-active decamer, eIF2B$(\alpha\beta\gamma\delta\varepsilon)_2$.

Given that ISRIB minimally activates a decameric complex isolated by size exclusion, we propose that complex assembly explains the majority of ISRIB action. Minute allosteric changes within the enzyme cannot be excluded at this time.

Interactions with eIF2 and phosphorylated eIF2

Structures of eIF2B bound to eIF2 (*16–19*) revealed that multiple eIF2B subunits mediate binding of the eIF2 heterotrimer. In humans, eIF2α binds across the decamer symmetry interface, bridging the eIF2Bβ and eIF2Bδ subunits (*16*, *17*). Mutational analyses based on these structures demonstrated that the composite binding surface present in the full eIF2B decamer substantially enhances recruitment of eIF2α. eIF2α binding in turn ensures close proximity between eIF2γ's GTP-binding domain and the loosely tethered eIF2Bε-HEAT to promote productive nucleotide exchange. Thus, assembly of a full eIF2B decamer leads to rapid destabilization of the eIF2-GDP binary complex.

By contrast, phosphorylated eIF2α contacts eIF2B at a distinct interface between the eIF2Bα and eIF2Bδ subunits. This new binding mode sequesters the assembled decamer in an inactive state with consequences for ISRIB's activity. Under normal conditions, ISRIB restores cellular GEF activity by replenishing the supply of uninhibited eIF2B decamers from a limiting pool of building blocks. Since the cellular concentrations of eIF2 greatly exceed that of eIF2B, high levels of eIF2 phosphorylation would sequester all decameric eIF2B complexes in an inactive state and eliminate ISRIB's effect. In accordance with these predictions, cell-based assays

indicate that high levels of stress abolish ISRIB's ability to suppress the integrated stress response (*12*, *20*).

Vanishing White Matter Disease

Numerous mutations in subunits of eIF2B lead to a neurodegenerative disease known as Vanishing White Matter Disease (VWMD). VWMD mutations have been identified in all eIF2B subunits (Fig. 2A-C), with many clustering in the eIF2Bε subunit. Since catalysis is essential, these mutations likely affect the structural stability of the protein, the entire eIF2B complex or binding to eIF2.

Previous research demonstrated that VWMD mutations can lead to a decrease in GEF activity. Furthermore, Wong et al. reported that eIF2B activation can rescue defects in GEF activity due to VWMD mutations and ameliorate disease in rodent models (*21*, *22*). A particularly deleterious variant, δR483W, was dramatically restored to near full activity by ISRIB in cells. This mutation lies directly outside the ISRIB pocket (Fig. 2D) and, when analyzed by size-exclusion chromatography, promotes dissociation of eIF2B into tetrameric subcomplexes. Given our findings on ISRIB action, it is plausible that ISRIB prevents complex dissociation by stapling eIF2B across its symmetry axis. Thus, ISRIB may have a particularly strong effect on mutations that destabilize eIF2B assembly. Two additional proximal mutations, βE213 and δY489, may similarly be rescued by ISRIB, though this remains to be shown. Finally, mutations distal to the ISRIB binding site that cause defective GEF activity may also be rescued by ISRIB through the molecule's ability to favor decameric complex production. We predict that the VWMD mutations resistant to ISRIB activity would be those that affect ISRIB binding directly or critically impair dimerization or binding to eIF2.

Conservation of eIF2B and ISRIB pocket

Structures of eIF2B show a conserved organization of subunits between *S. pombe* and *H. sapiens*. Furthermore, a tetrameric structure of *C. thermophilum* eIF2Bβ and eIF2Bδ indicates the likelihood of a similar arrangement (*23*). Additionally the subunits of the regulatory subcomplex contain homology to archaeal sugar-phosphate isomerases, and subunits of the catalytic subcomplex are structurally similar to sugar transferases (*4*). These latter data raise the possibility that eIF2B may bind nucleotides or sugars that regulate its function. Our structure of human eIF2B does not contain density that is immediately identifiable as nucleotide, but these possibilities remain open.

Intriguingly, the yeast eIF2B forms polymers under conditions of stress *in vivo* (*24–27*). While this has not yet been shown for the mammalian counterpart, we ponder whether the flexible, extended "ear" domains of eIF2Bγ may be involved in templating eIF2B filaments, and if this domain becomes stabilized in an elongated, multi-enzyme complex.

Strikingly, the cavity within which ISRIB binds is structurally conserved between *S. pombe* (Fig. 3A), *C. thermophilum* (Fig. 3B) and *H. sapiens* eIF2B. This raises the question of whether an

endogenous ligand may bind within eIF2B and regulate its activity in a manner similar to ISRIB. Sequence alignments of eIF2B across yeast, zebrafish, fruit fly, round worm, mouse and human indicate that certain residues within the ISRIB pocket are more conserved than others (Fig. 3E-F). The critical βH188 is not conserved in worms, flies or yeast, suggesting that ISRIB may not function in these organisms and that modifications of the compound may be required for use in these species. Empirical data is essential to validate these predictions.

We analyzed the structure using UCSF Chimera to visually render residues by conservation across eleven species (Fig. 3C) as a saturation gradient of either blue or gold for eIF2Bβ and eIF2Bδ. Since eIF2B is a highly conserved enzyme, we amplified the differences in conservation by defining white as 50% conserved and the most saturated blue and gold as 100% conserved. As expected based on the sequence alignments, βH188 is a light blue in a close-up view of the ISRIB pocket (Fig. 3D), as are other pocket residues. This suggests that the bonds between the enzyme and molecules within the pocket can change by species.

Interestingly the residues adjacent to βH188, βF187 and βV189, are more highly conserved and point away from the pocket. These presumably stabilize the fold of the pocket. Taken together, these data suggest that the shape of the pocket is more conserved than the sequence lining the pocket. Thus, with the correct pharmacological tools, we may be able to exploit this regulatory site for species-specific control of eIF2B activity and the integrated stress response. Endogenous ligands, if they exist, may be specific to certain species. In humans, such a molecule would be of great interest, especially if it functions similarly to ISRIB in the nervous system.

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Figure 1: *Human eIF2B bound to ISRIB*. Four distinct views (A-D) of ISRIB-stabilized eIF2B with subunits colored in different shades (α in red, β in blue, γ in green, δ in gold, ε in gray, and ISRIB in CPK coloring). Dotted lines (C) indicate connection to the "ear" domains of gamma and HEAT domain of epsilon for which density is not clearly defined. (E) Model for ISRIB's mechanism of action: eIF2B(βγδε) tetramers are dimerized upon ISRIB addition to form a stable octamer that can in turn bind the dimeric eIF2B(α_2) to assemble the fully-active decamer.

Figure 2: *Vanishing white matter disease mutations*. (A-C) Structure of human eIF2B depicted in light gray with disease mutations mapped in colored spheres (α in red, β in blue, γ in green, δ in gold, ε in gray). (D) Close-up view of ISRIB pocket labeled with the three most proximal identified mutations.

Figure 3: *Conservation of the ISRIB pocket*. eIF2Bβ and eIF2Bδ from (A) *S. pombe* (*4*) and (B) *C. thermophilum* (*28*) depicted in pale blue and pale gold respectively, and overlaid on the human structure (dark blue and gold, with ISRIB). (C) eIF2B tetrameric core rendered by residue conservation across 11 species (*S. cerevisiae*, *S. pombe*, *C. elegans*, *D. melanogaster*, *D. rerio*, *X. laevis*, *M. musculus*, *R. norvegicus*, *B. taurus*, *H. sapiens*). Extent of conservation is illustrated by saturation of blue for eIF2Bβ or gold eIF2Bδ. (D) Close-up view of ISRIB-binding pocket in C. (E-F) Sequence conservation for residues lining the ISRIB pocket (*13*) highlighted in blue for eIF2Bβ and gold for eIF2Bδ.

Figure 2

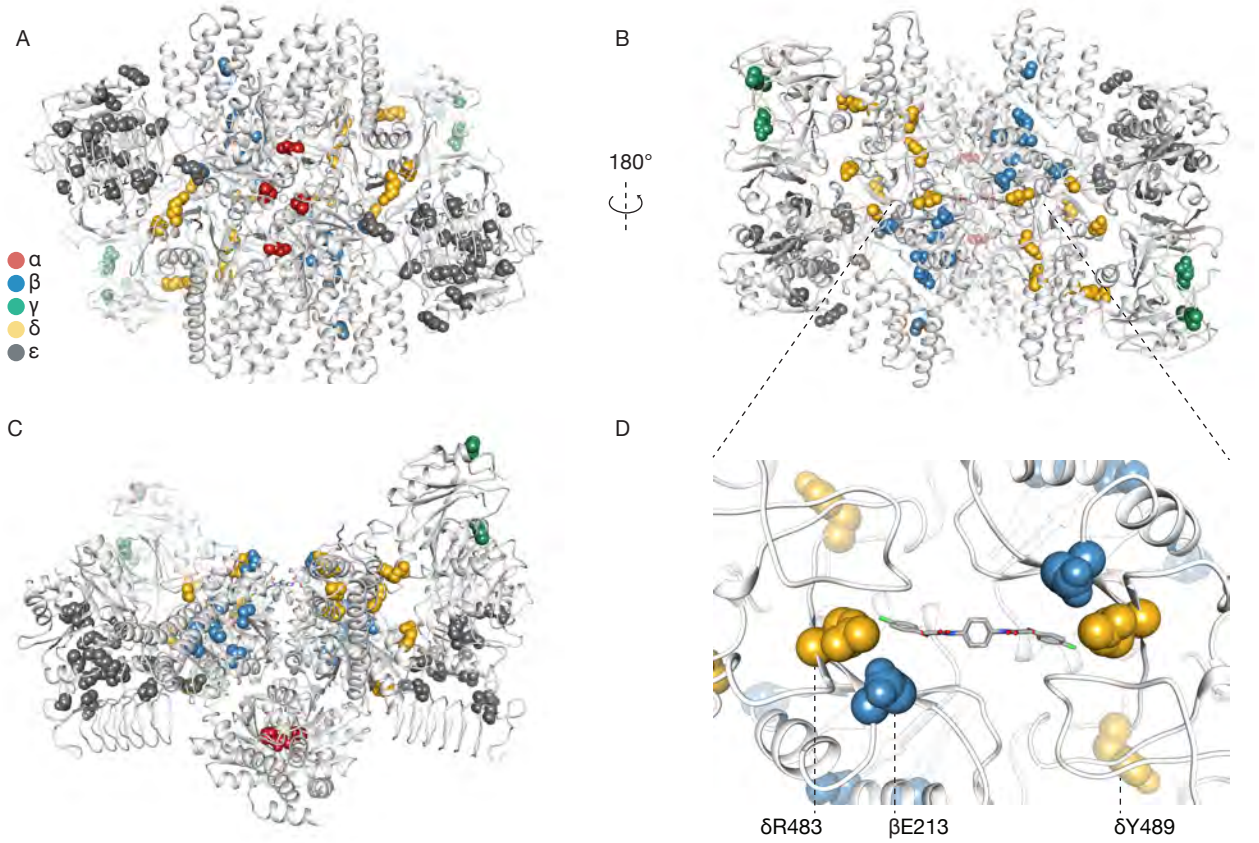


Figure 1

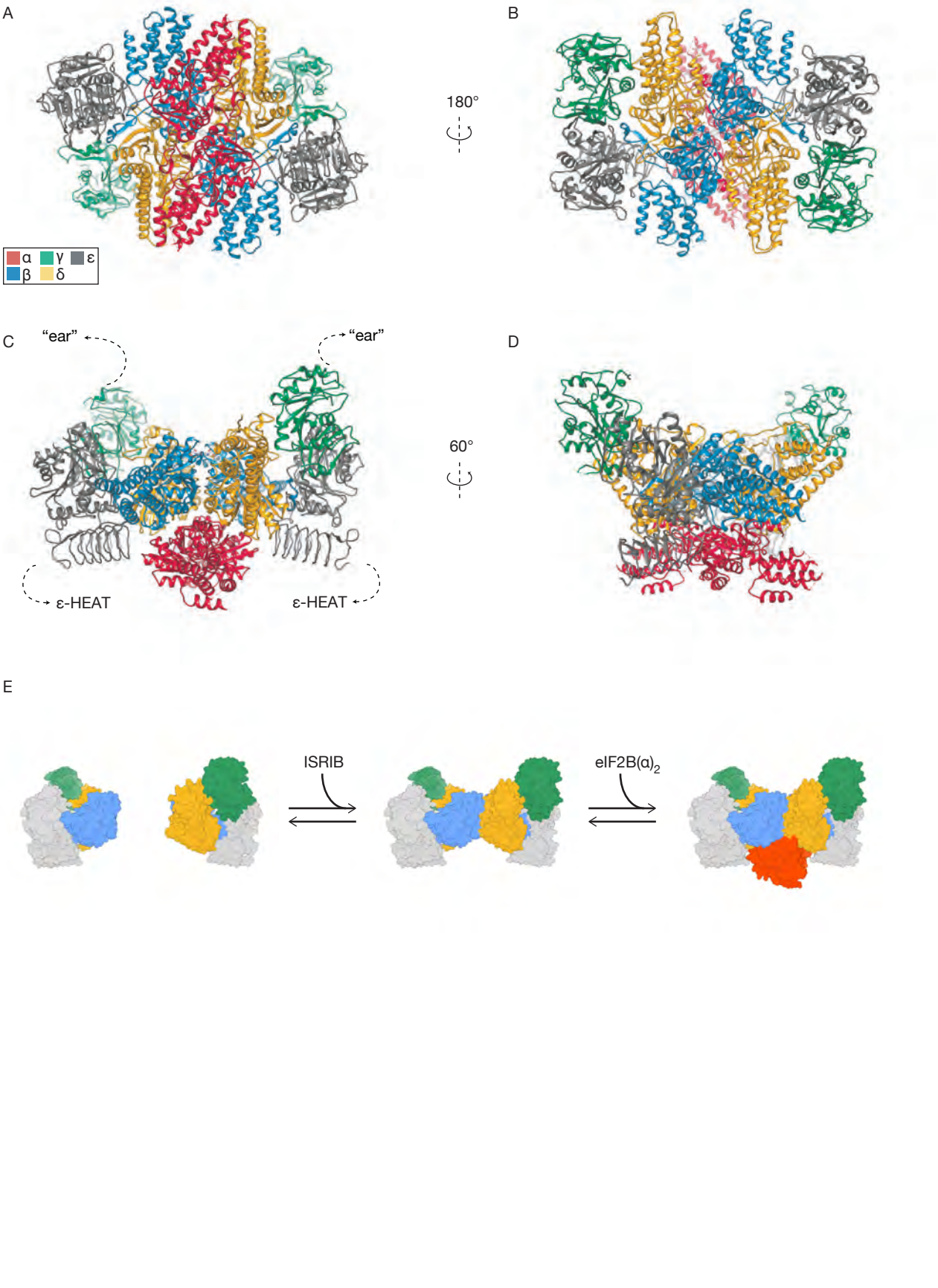
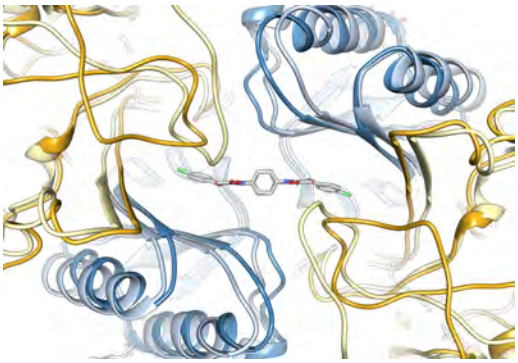


Figure 3

A



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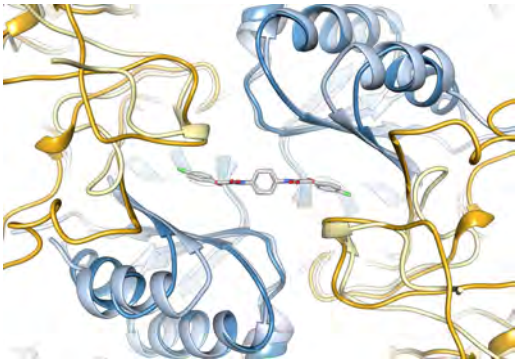
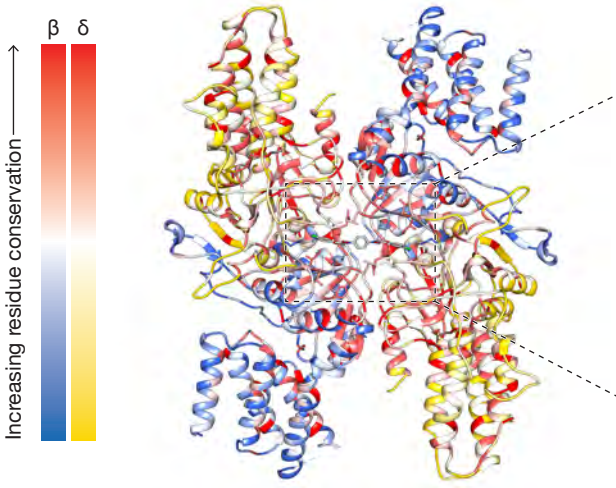


C



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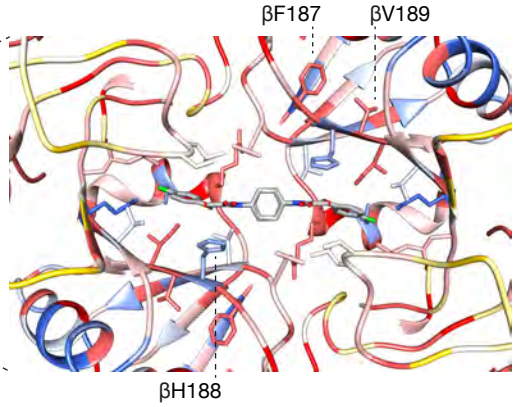

E

eIF2Bβ	162	164	188	190	215	217	225	228
H. sapiens	…HS	N	E	V	IM…F	H	-V	I	V…T	T	V	M	TDAAIFA	V	MSR	V	NKV…
M. musculus	…HS	N	E	V	IM…F	H	-V	I	V…T	T	V	M	TDAAIFA	V	MSR	V	NKV…
C. elegans	…FN	N	D	V	VL…H	R	-V	I	D…--	A	I	E	LYEVAS	K	MPE	A	SKV…
D. melanogaster	…HS	S	E	I	IL…F	L	TI	I	V…I	V	V	I	PDAAIFA	M	MSR	V	NKV…
D. rerio	…HS	N	E	V	IM…F	H	-V	I	V…T	T	V	I	PDAAIFA	V	MSR	V	NKV…
S. cerevisiae	…HD	H	E	I	LL…F	T	-V	L	V…T	L	V	V	PDSAVFA	L	MSR	V	GKV…

F

eIF2Bδ	177	178	179	485
H. sapiens	…K	V	S	L	FS…L	L	N
M. musculus	…K	V	S	L	FS…L	L	N
C. elegans	…A	S	E	I	ES…--
D. melanogaster	…R	V	K	L	FN…P	L	N
D. rerio	…K	V	S	L	FS…L	L	N
S. cerevisiae	…S	S	V	I	PT…I	V	N