

Molecular Architecture of the 40S·eIF1·eIF3 Translation Initiation Complex

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SUMMARY

Eukaryotic translation initiation requires the recruitment of the large, multiprotein eIF3 complex to the 40S ribosomal subunit. We present X-ray structures of all major components of the minimal, six-subunit *Saccharomyces cerevisiae* eIF3 core. These structures, together with electron microscopy reconstructions, cross-linking coupled to mass spectrometry, and integrative structure modeling, allowed us to position and orient all eIF3 components on the 40S·eIF1 complex, revealing an extended, modular arrangement of eIF3 subunits. Yeast eIF3 engages 40S in a clamp-like manner, fully encircling 40S to position key initiation factors on opposite ends of the mRNA channel, providing a platform for the recruitment, assembly, and regulation of the translation initiation machinery. The structures of eIF3 components reported here also have implications for understanding the architecture of the mammalian 43S preinitiation complex and the complex of eIF3, 40S, and the hepatitis C internal ribosomal entry site RNA.

INTRODUCTION

Protein synthesis is catalyzed by the ribosome in a process that consists of initiation, elongation, and termination. In bacteria, three initiation factors (IF1, IF2, IF3) and the Shine-Dalgarno sequence are sufficient to accurately pair the AUG start codon in messenger RNA (mRNA) with the anticodon loop of methionyl initiator transfer RNA (Met-tRNA_i). Eukaryotes, in contrast, require at least 25 different polypeptides assembled into eight eukaryotic initiation factors (eIFs) to initiate protein synthesis and employ a complex scanning mechanism to probe the 5' leader sequences of mRNA for the correct start site (Hinnebusch, 2011, 2014; Jack-

son et al., 2010; Voigts-Hoffmann et al., 2012). Initiation is targeted by a number of regulatory pathways linked to cellular processes such as cell growth, differentiation, and environmental stress responses (Sonenberg and Hinnebusch, 2009), and the functional disruption or decoupling of these regulatory interactions has been observed in a number of cancers (Ruggero, 2013).

Eukaryotic translation initiation begins with the cooperative assembly of the 43S preinitiation complex (PIC), composed of the eIF2/GTP/Met-tRNA_i ternary complex (TC), eIF1, eIF1A, eIF5, and eIF3 on the 40S ribosomal subunit. In canonical eukaryotic translation, the 43S PIC recruits mRNAs by engaging the eIF4F cap-binding complex to form the 48S PIC. Within the 48S PIC, eIF1, eIF1A, eIF3, and eIF4G promote the accurate scanning of the mRNA leader region (Hinnebusch, 2011, 2014) and ensure the proper recognition and pairing of the start codon with Met-tRNA_i.

eIF3 is a large and structurally complex molecular assembly that, in the majority of eukaryotes, consists of 11–13 subunits (eIF3a–eIF3m) with a molecular weight of 600–800 kDa (Hinnebusch, 2006; Valásek, 2012). Six of the subunits (eIF3a, eIF3c, eIF3e, eIF3k, eIF3l, and eIF3m) contain PCI (proteasome, COP9/signalosome, eIF3) modules, and two (eIF3f and eIF3h) contain MPN (Mpr1-Pad1-N-terminal) domains. The PCI·MPN core is structurally conserved in the 26S proteasome lid, the COP9 signalosome, and eIF3, forming a distinctive, multilobed structure (Enchev et al., 2010). PCI modules are characterized by an N-terminal helical domain (HD) and a C-terminal winged helix domain (WHD) (Ellisdon and Stewart, 2012), which mediates PCI dimerization (Ellisdon et al., 2012). In the subnanometer EM structures of the proteasome lid, six WHDs oligomerize to form a horseshoe-shaped arc with their HDs radiating outward (Beck et al., 2012; Lander et al., 2012), an organization also observed in the EM reconstructions of eIF3, the 43S, and 43S·IRES complexes and the COP9 signalosome (Enchev et al., 2010, 2012; Hashem et al., 2013a, 2013b; Querol-Audi et al., 2013). *S. cerevisiae* and related yeasts lack six components of the PCI·MPN core, retaining only two PCI proteins among the six universally conserved eIF3 core subunits (eIF3a/Tif32, eIF3b/Prt1,

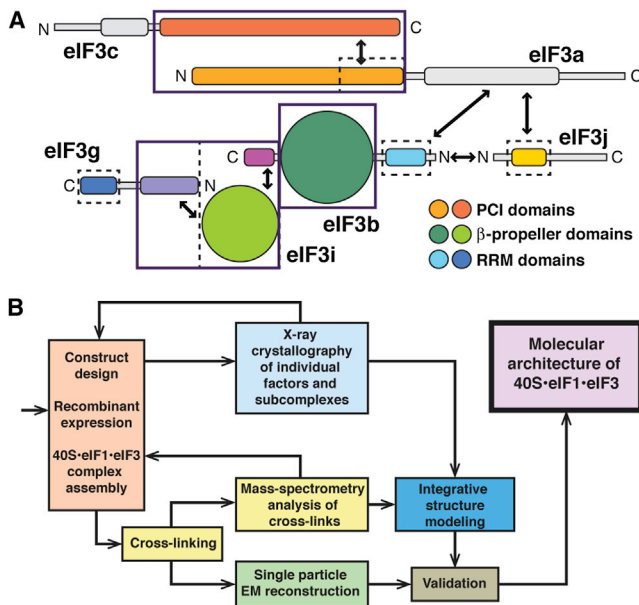


Figure 1. Domain Organization of *S. cerevisiae* eIF3 and Experimental Approach

(A) Domain map of the six subunits of *S. cerevisiae* eIF3. Structured domains are shown as rectangles or spheres and are individually colored. Domains with known structural motifs are designated in the legend. Predicted unstructured regions are shown as thin gray lines. Known eIF3 interactions are indicated by arrows. Modules whose crystal structures are described in this paper are boxed, whereas previously described structures are indicated by narrow dashed lines.

(B) Schematic outline of the hybrid experimental approach, highlighting the techniques used in this study.

eIF3c/Nip1, eIF3i/Tif34, eIF3g/Tif35, and eIF3j/Hcr1) (Figure 1A). The conserved core displays a modular architecture, with the interaction between eIF3b and the C-terminal portion of eIF3a connecting the PCI subunits to the eIF3g/eIF3i subcomplex and to eIF3j (Zhou et al., 2008).

Recent EM reconstructions as well as in vivo and in vitro experiments indicate that the N-terminal ends of the helical subdomains of the eIF3a/eIF3c PCI modules form two intermolecular bridges with the 40S subunit in the vicinity of ribosomal proteins rpS13/uS15 and rpS27/eS27 as well as rpS26/eS26, rpS1/eS1, and rpS0/uS2 (Hashem et al., 2013a; Querol-Audi et al., 2013; Valásek et al., 2003). In addition, footprinting and hydroxyl-radical probing experiments implicate helix 16 of the 40S subunit in eIF3 binding (Pisarev et al., 2008). Other studies have placed eIF3j, which interacts with the eIF3b-RRM (RNA recognition motif) and the eIF3a-CTD (C-terminal domain) (Chiu et al., 2010; ElAntak et al., 2007; Fraser et al., 2004; Nielsen et al., 2006), near the decoding center of the 40S subunit (Fraser et al., 2007). Nevertheless, information about the molecular architecture of eIF3 is incomplete due to the lack of atomic structures of eIF3 subunits and the dynamic nature of the 40S•eIF3 interaction. In this study, we present X-ray structures of all major structured domains of *S. cerevisiae* eIF3 and utilize a combination of negative-stain electron microscopy (EM), chemical cross-linking followed by mass spectrometry (CX-MS) and integrative

structural modeling to derive a detailed molecular architecture of the yeast 40S•eIF1•eIF3 complex.

RESULTS

Overall Structures of the Full-Length eIF3a and eIF3c PCI Modules and the eIF3a/eIF3c Heterodimer

As a first step in our hybrid approach (Figure 1B), we obtained a number of soluble eIF3 fragments and subcomplexes that were amenable to crystallographic analysis (Table S1 available online). The structure of the full-length PCI domain of eIF3a (*S. cer.* residues 1–496) was solved by single-wavelength anomalous dispersion (SAD) with selenomethionine (Se-Met)-labeled protein to a resolution of 3.3 Å (Table S2). As observed in a truncated structure of eIF3a and in previously characterized PCI modules (Ellisdon and Stewart, 2012; Khoshnevis et al., 2014), the WHD is structurally similar to other PCI proteins, whereas the N-terminal domain has a series of distinct, flatly arranged helical repeats (Figure 2A). The positioning of these helices is reminiscent of the concertina-like arrangement of tetratricopeptide repeats (TPR), although, as in other PCI proteins, TPR-like sequence elements are not detectable. Unlike other PCI HDs, which typically have a significant right-handed superhelical twist, eIF3a has two distinct segments to its repeat. Whereas helices 10–14 have a right-handed pitch, helices 1–9 are arranged along a flat plane that is distinct from other PCI proteins with extended helical repeats (Figure 2C).

Using the full eIF3a PCI-domain structure as a guide, N-terminal eIF3a truncations were generated to obtain crystals of a dimeric eIF3a/eIF3c assembly, encompassing the full predicted PCI domain of eIF3c (*S. cer.* residues 251–812) and residues 228–496 of eIF3a. The structure of the dimeric eIF3a/eIF3c complex was solved at 3.5 Å resolution using Se-Met SAD (Figure 2B and Table S2). The superposition of the common eIF3a WHD component in these structures provides a detailed atomic model for the full eIF3a/eIF3c PCI heterodimer. As expected, the PCI domain of eIF3c shares the basic architecture of the fold (Figure 2B) but with a more elaborate helical domain that extends over 450 residues (Figure 2C). The helical domain can be subdivided into two regions: helical element 1 (HE-1) is composed of helices 1–7, whereas helical element 2 (HE-2) contains helices 9–13. HE-1 and HE-2 form TPR-like right-handed superhelices similar to those found in other PCI proteins, with the relative orientations of HE-1 and HE-2 defined by helix 8, which disrupts the helical repeat at the HE-1/HE-2 interface, inducing an almost 90° bend in the domain (Figures 2B and 2C). An extensive, conserved protein segment formed by a 40 amino acid insertion between helices 13 and 17 meanders back into the HE-1/HE-2 interface to further stabilize the kink (Figure 2C).

The dimerization interface between eIF3a and eIF3c involves two distinct interaction regions. Helix 23 in the WHD of eIF3a engages the WHD of eIF3c (Figures 2B and 2D) in an arrangement similar to the Thp1-Sac3 PCI-dimer (Ellisdon et al., 2012). Because the WHD domains are the least well-ordered regions in our structure, the details of the interaction cannot be unambiguously established but involve a number of conserved hydrophobic side chains on eIF3a (W463, L470, I480, and I482) and eIF3c (F732, Y733, F736, L744, and F745) as well as a number of

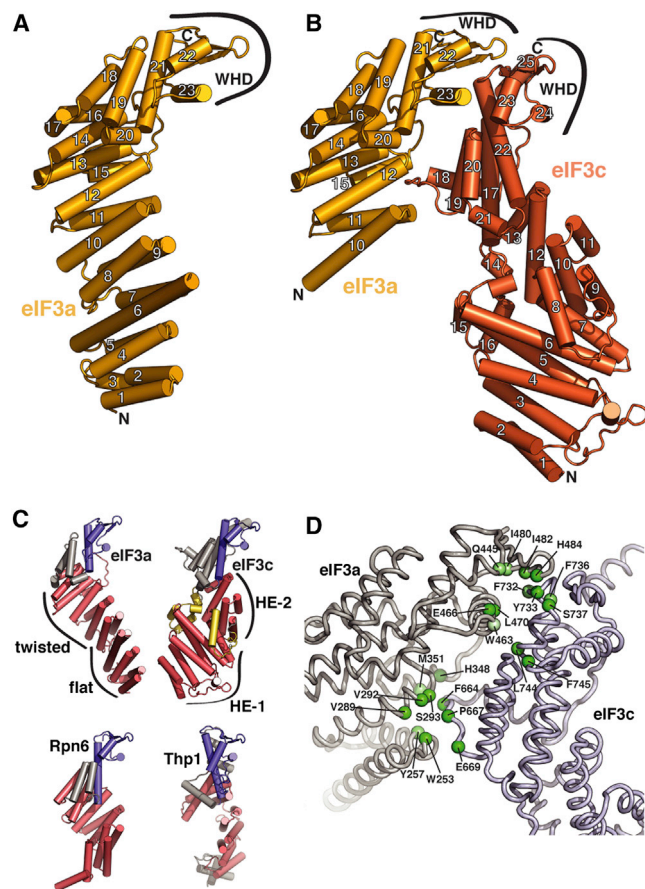


Figure 2. Structures and Interactions of *S. cerevisiae* eIF3 PCI-Domain Proteins

(A) Cartoon representation of the crystal structure of the full PCI domain of eIF3a (chain A) with numbered helices.

(B) Cartoon representation of the crystal structure of the eIF3a/eIF3c PCI dimer, colored as in Figure 1A and with numbered helices (with the exception of short 3/10 helices).

(C) Comparison of PCI helical domains. The crystal structures of eIF3a (chain A) and eIF3c were aligned by superposition of their WHDs (colored blue) to each other and to Rpn6 (PDB 3TXN, Pathare et al., 2012) and Thp1 (PDB 3T5V-chain B, Ellisdon and Stewart, 2012), previously solved PCI proteins with extended helical domains (shown in red). Features of the eIF3a and eIF3c helical regions are labeled, and elements within eIF3c that stabilize the kink in the helical domain are shown in yellow.

(D) Detail of the eIF3a/eIF3c interface. Conserved residues within the two interaction regions observed in the crystal structure are shown as spheres and labeled. Stronger green shading signifies a higher degree of conservation.

conserved polar and charged residues (Q445, E466, and H484 on eIF3a and S737 on eIF3c) (Figure 2D). A comparison between eIF3a·eIF3c and Thp1·Sac3 shows that, although the overall interaction mode is conserved between these two PCI complexes, the relative positions of the WHDs differ by up to 6 Å between these two heterodimers (Figure S1). The second interaction interface between eIF3a and eIF3c involves a loop between helices 18 and 19 of eIF3c that engages a cleft formed by helices 11, 12, and 15 of eIF3a (Figures 2B and 2D), allowing a stacking interaction between residue F664 of eIF3c and Y257

of eIF3a. This interaction induces a rearrangement of up to 3 Å at the end of eIF3a helix 15 to accommodate the eIF3c loop.

Implications for Understanding Mammalian eIF3 Architecture and eIF3a/eIF3c·HCV/CSFV IRES Interactions

Yeast eIF3 has a significantly reduced repertoire of eIF3 components, but the conservation of eIF3a and eIF3c in all eukaryotes is high. Docking of the eIF3a/eIF3c PCI heterodimer into the EM density of the mammalian 43S (Hashem et al., 2013a) reveals an excellent fit into the two structural elements that mediate 40S binding by the PCI-MPN core of eIF3 (Figure 3A). In this placement, eIF3c engages the Zn-binding knuckle of rpS27/eS27 through a conserved pocket within HE-1, whereas eIF3a primarily contacts rpS1/eS1 (Figure 3B). The two PCI modules flank ES7, the only expansion segment within the central RNA domain that defines the 40S platform (Figure 3B).

Many viral internal ribosome entry site (IRES)-containing RNA elements require the presence of eIF3. The recent structure of the 40S-bound CSFV IRES in complex with eIF3 revealed the structural basis of this interaction, with the IRES RNA occupying the eIF3 binding site at the 40S platform and creating a secondary binding site for the PCI·MPN core (Hashem et al., 2013b). Because IRES interactions are also mediated by eIF3a/eIF3c, we were able to model the interaction by fitting the eIF3a/eIF3c heterodimer into the 43S·IRES EM density map without any adjustments (Figure 3C). This fit of the eIF3a/eIF3c heterodimer places two loop elements (between helices 2 and 3 of eIF3a and helices 1 and 2 of eIF3c) important for IRES and 43S binding (Sun et al., 2013) in close contact to two extrahelical bulges conserved within domain IIIB of the CSFV and HCV IRESs (Figure 3D). Consistent with our model, these RNA elements are protected from RNase digestion when bound by eIF3 (Sizova et al., 1998; Sun et al., 2013).

Based on the convincing fit of the eIF3a/eIF3c structure into the EM density, we attempted to dock the recently solved paralogs of eIF3e (CSN1) (Lee et al., 2013) and the eIF3h-eIF3f heterodimer (Rpn8/Rpn11) (Pathare et al., 2014; Worden et al., 2014) into the eIF3·IRES EM density along with older models for eIF3k, eIF3m, and eIF3l (Figure S2A). Using the previously identified positions of the various eIF3 subunits as a guide (Querol-Audi et al., 2013), we fitted the models to agree with features of the EM density (Figure 3E). The proposed arrangement defines the molecular interactions within the core subunits, with the HCV·IRES, and with the 40S subunit. Remarkably, even the helical bundle structure recently proposed for the 26S proteasome lid (Estrin et al., 2013) fits the remaining density after placement of the PCI and MPN domains (Figure 3E), suggesting that it is a conserved feature of the PCI·MPN core of these functionally diverse complexes. The final model also fits the lower-resolution mammalian 43S EM maps (Figure S2B).

Structure of the eIF3b β -Propeller Domain

The eIF3b subunit plays a critical anchoring role within the 5 subunit core of eIF3. Though structural information is available for the N-terminal RRM domain (ElAntak et al., 2007; Khoshnevis et al., 2010) and a segment of the C-terminal tail (Herrmannová et al., 2012), great uncertainty remained about the central region of the protein, predicted to form either a single or a double

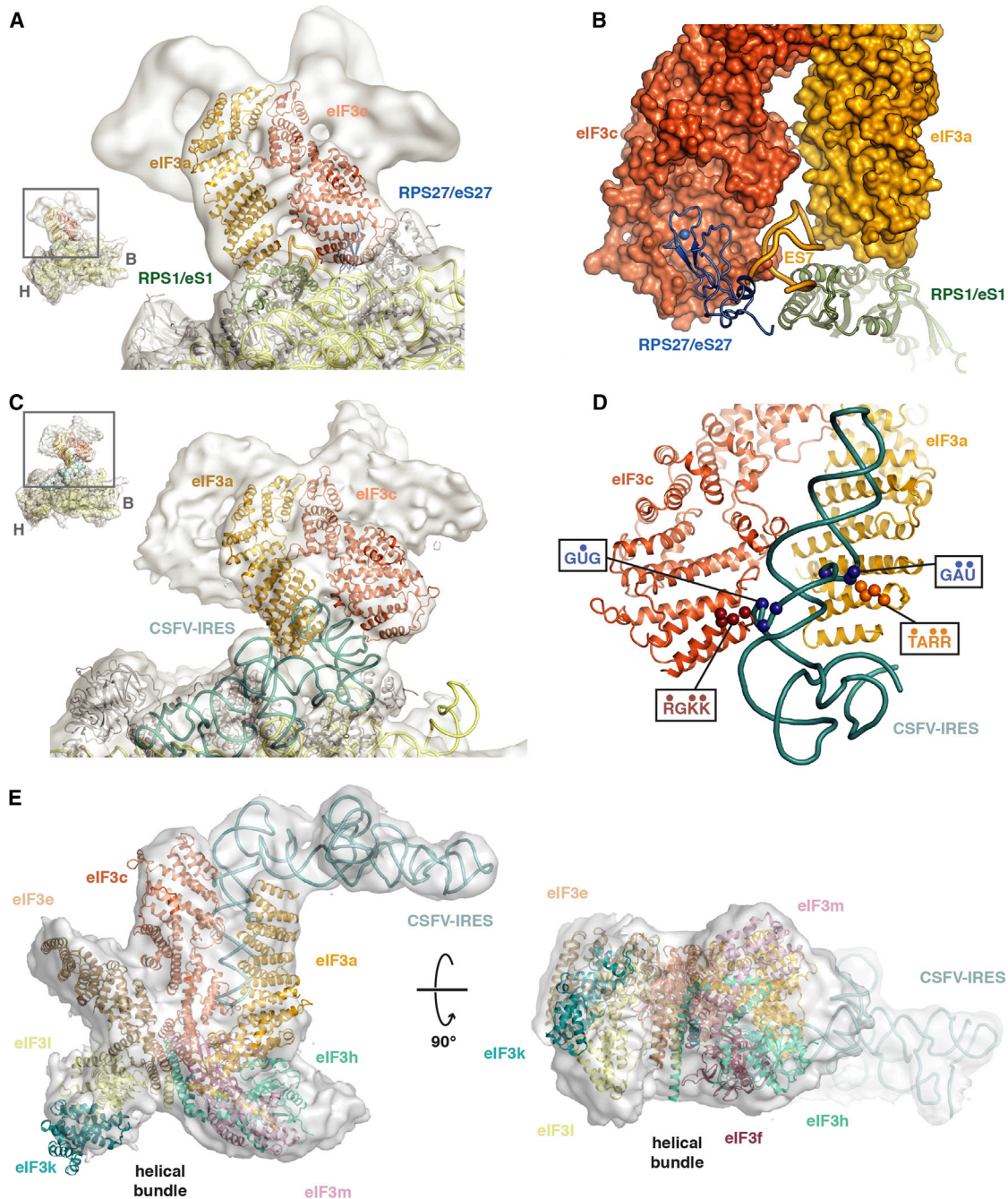


Figure 3. Docking of eIF3a/eIF3c in the PCI-MPN Core Density of Mammalian 43S and 43S-IRES EM Maps

(A) Rigid-body fitting of the eIF3a/eIF3c PCI heterodimer model into the EM density of the 43S EM map. EIF3a and eIF3c are labeled and colored as in Figure 1A. Ribosomal proteins rpS1/eS1 (green) and rpS27/eS27 (blue) from the docked yeast 40S structure (Ben-Shem et al., 2011) are highlighted. Other ribosomal proteins are shown in gray and the ribosomal RNA in yellow.

(B) Detail of the position of the eIF3a/eIF3c helical domains after rigid-body fitting and their proposed interaction with ribosomal proteins rpS1/eS1 (green), rpS27/eS27 (blue), and RNA expansion element ES7 (orange).

(C) Rigid-body fitting of the eIF3a/eIF3c PCI heterodimer model into the 43S-IRES EM map.

(D) Detail of the interaction between the CSFV-IRES and the eIF3a/eIF3c dimer in the docked structure. Residues of eIF3a and eIF3c important for IRES binding are shown as spheres and identified by their sequences. The dots indicate positions mutated in the equivalent human complex (Sun et al., 2013). RNA elements protected by eIF3 in IRES domain IIIIB are shown as blue spheres and are identified by their sequences. The blue dots indicate extrahelical bases.

(E) Model of the complete PCI-MPN core of eIF3 (based on currently available crystal structures and models) (Figure S2A) docked into the 43S-IRES EM map (Hashem et al., 2013b).

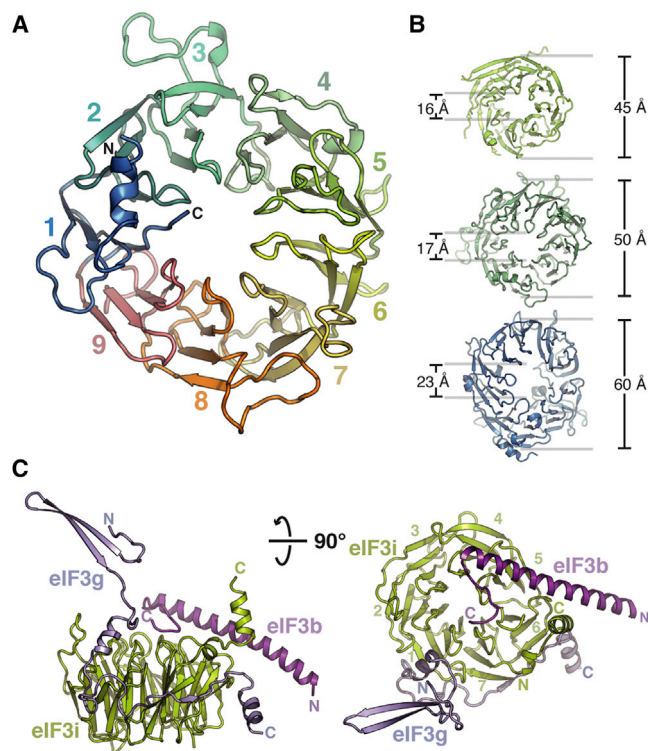


Figure 4. Structures of the eIF3b β Propeller and of the eIF3b-CTD/eIF3i/eIF3g-NTD Complex

(A) Cartoon representation of the crystal structure of the middle domain of eIF3b (monomer A, unswapped). Individual blades are colored and labeled, and the position of the N and C termini are indicated.

(B) Comparison of the β propeller diameters and pore dimensions among eight- (PDB 1R5M, Cerna and Wilson, 2005), nine-, and ten-bladed (PDB 3F6K, Quistgaard et al., 2009) β propellers.

(C) Cartoon representation of the crystal structure of the eIF3b-CTD/eIF3i/eIF3g-NTD trimer, colored as in Figure 1A.

β -propeller structure. We purified and crystallized the 57 kDa central domain of eIF3b (*S. cer.* residues 132–626) and solved its structure using Se-Met SAD to a resolution of 2.2 Å (Table S3). The middle domain of eIF3b forms a β propeller with nine blades, a configuration not previously observed in a native polypeptide (Figure 4A). In our crystals, two β propellers are present in the asymmetric unit, with a domain swap encompassing segments of blades 1 and 9 (Figures S3A and S3B). For clarity, our other figures depict a monomeric, unswapped eIF3b model.

The middle domain of eIF3b begins with a short helical element, followed by the first blade of the β propeller. The A strand of this blade is provided by the most C-terminal strand in a 3+1-type velcro closure. The blades of eIF3b are fairly symmetric, with extensive loops decorating blades 3, 4, 5, 7, and 8 (Figure 4A). The central channel of eIF3b is marked by a conserved structural loop motif that constricts the “bottom” entrance of the central cavity, creating a funnel-like shape (Figure S3C). The structure and underlying sequences of the loops are conserved among the various blades of *S. cerevisiae* eIF3b and in the blades of eIF3b β propellers from other organisms (Figures S3C and S3E). This particular loop orientation is also

seen in the six-bladed ToIB β propeller, which has a similar consensus sequence (the AxSPD motif) linking strands A and B (Figure S3D) (Abergel et al., 1999; Chen et al., 2011). The nine-bladed eIF3b β propeller fills the gap between previously characterized eight- and ten-bladed β propellers (Figure 4B). A search for other nine-bladed β propellers in the *S. cerevisiae* genome revealed that the conserved sequence element and its associated nine blades are also present in eIF2A (Figure S3E), a repressor of IRES-mediated translation initiation that is down-regulated during stress events in *S. cerevisiae* (Kim et al., 2011). It remains to be investigated whether these shared structural features reflect functional commonalities with eIF3b.

Structure of the Trimeric eIF3b-CTD/eIF3i/eIF3g-NTD Complex

The C-terminal helical domain of eIF3b mediates the association of eIF3b with the dimeric eIF3i/eIF3g complex (Herrmannová et al., 2012). Crystals were obtained for a complex containing the eIF3b-CTD (*S. cer.* residues 655–698), full-length eIF3i, and the eIF3g-NTD (N-terminal domain) (*S. cer.* residues 1–135). The heterotrimeric structure was solved to a resolution of 2 Å using molecular replacement with the eIF3b-CTD/eIF3i complex as the search model (Table S3) (Herrmannová et al., 2012). The arrangement of eIF3i and the eIF3b fragment was nearly identical to the previously solved dimeric complex, with a root-mean-square deviation (rmsd) of 0.4 Å. Of the 135 residues in the crystallized eIF3g fragment, clear density was observed for the first 96 residues. The first 46 residues of eIF3g form a β hairpin that makes an important crystal contact but does not directly interact with eIF3i or eIF3b. Indeed, no residue of the eIF3g-NTD makes a direct contact with the eIF3b-CTD (Figure 4C). Residues 47–90 of eIF3g are responsible for the tight association with eIF3i, meandering along one-third of the outside surface of the eIF3i β propeller and making extensive contacts with blades 1, 6, and 7 of eIF3i (Figures 4C, S4A, and S4B). The molecular interaction between the eIF3g-NTD and eIF3i can be divided into three areas. The first is defined by the insertion of three eIF3g residues (R52, W55, and Y58) between blades 1 and 2 of eIF3i and involves both hydrophobic and polar interactions (Figure S4A). The second is characterized by extensive polar contacts along the outside of blades 1 and 7, including a short β strand (residues E77–V79) extending the sheet of blade 7 of eIF3i (Figure S4B). Finally, another set of exclusively hydrophobic interactions (residues L81, L83, and W87 of eIF3g) occurs between blades 6 and 7 of eIF3i (Figure S4B). The most prominent elements in the interaction surface are highly conserved among all organisms (Figures S4C and S4D), suggesting that the interaction mode between eIF3i and eIF3g is universally conserved.

Single-Particle Reconstructions of 40S·eIF1·eIF3 Particles from *Lachancea kluyveri*

Electron microscopy studies of initiation events have been hampered by the labile nature of 40S·eIF1 interactions during cryoelectron microscopy (cryo-EM) sample preparation as described for both yeast and mammalian 40S·eIF1 complexes (Hashem et al., 2013a; Passmore et al., 2007). Because eIF1 is critical for eIF3 binding to 40S, we similarly observe no consistent eIF3 occupancy in 40S samples prepared for cryo-EM,

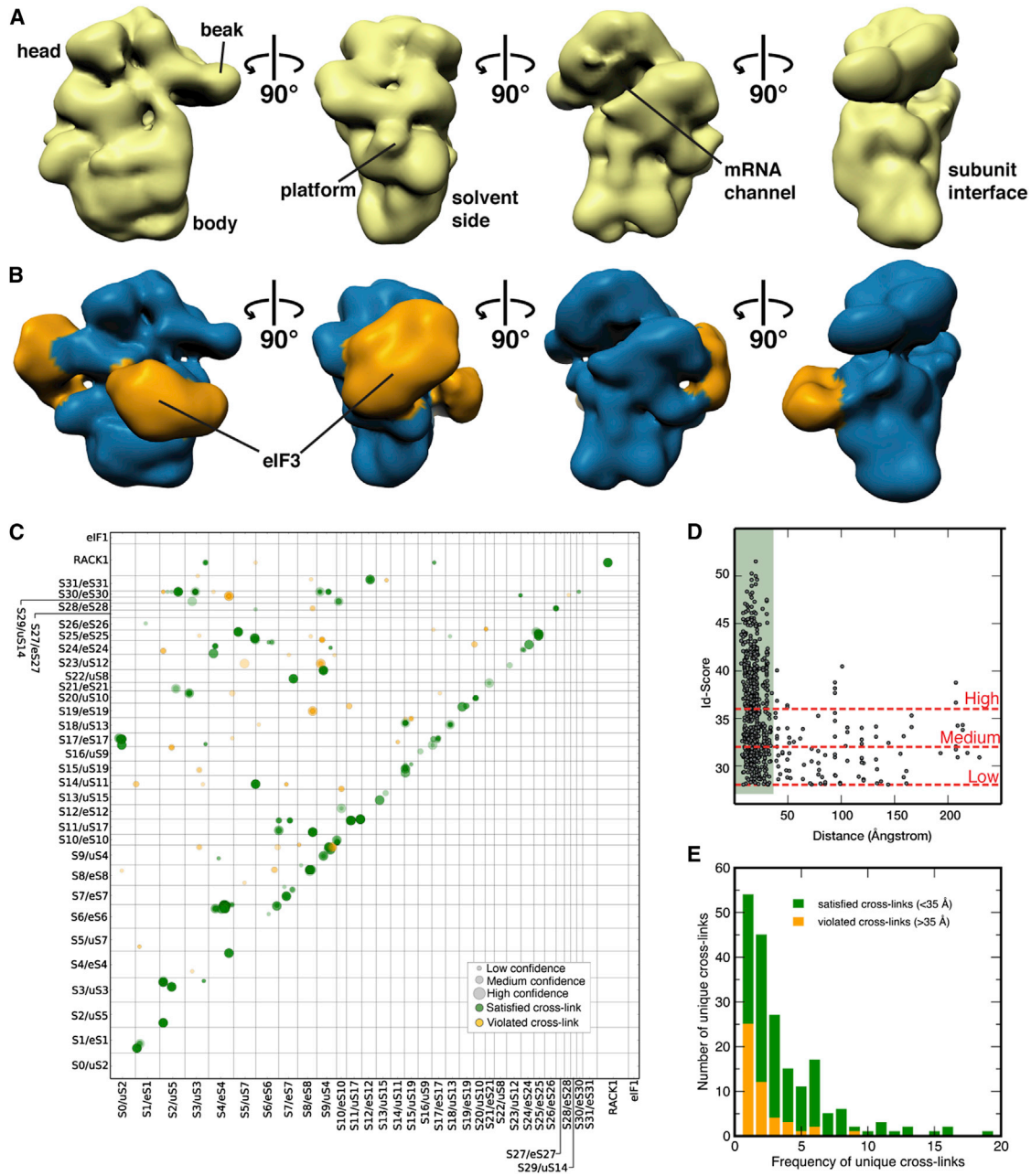


Figure 5. Single-Particle Reconstruction and CX-MS Analysis of the Yeast 40S·eIF1·eIF3 Complex

(A and B) Views of EM reconstructions of the (A) unoccupied and (B) occupied fractions of the *L. kluveri* 40S·eIF1·eIF3 complex sample with labeled ribosomal landmarks.

(C) Matrix of all unique crosslinks between and within subunits of 40S from CX-MS analyses of multiple 40S·eIF1·eIF3 samples. Crosslinks were mapped onto the X-ray structure of the yeast 40S particle and were colored in green (crosslink distance <35 Å) or orange (crosslink distance >35 Å). The size of the circle for each mapped crosslink is proportional to its Id score. Multiple identifications of a particular crosslink are indicated by a stronger color intensity due to overlaid circles.

(D) Scatter plot of mapped crosslink distances against Id score of the 40S data set. Crosslinks are grouped into three classes of similar size (high = Id score >36; medium = 32 < Id score < 36; and low = 28 < Id score < 32). Satisfied crosslinks (distance <35 Å) are within the green background.

(E) Frequency distribution (as defined in Table S4) of detected unique crosslinked residues within the 40S matrix. Satisfied and violated crosslinks are colored as in (C).

even with samples crosslinked by GraFix (Kastner et al., 2008). In contrast, identical samples prepared by negative stain show clear density for eIF3, suggesting that this preparation method

is more compatible with a functional 40S·eIF1·eIF3 assembly. The 28 Å single-particle reconstruction of the *L. kluveri* 40S·eIF1·eIF3 complex (Figure 5B) reveals two large areas

of extra density on the solvent-exposed side of 40S compared to complexes without eIF3 (Figure 5A). Similar to the eIF3 density in the mammalian 43S structure, one extra density is localized below the platform, matching the eIF3a/eIF3c PCI heterodimer in size and shape, whereas the other region of extra density is on the solvent-exposed side of the 40S subunit, halfway between the platform and the beak. In our hybrid structural approach, we use the EM envelope as a boundary to validate the results of our integrative structure modeling outlined below.

Crosslink Mass Spectrometry Analysis of the 40S•eIF1•eIF3 Complex

We carried out CX-MS experiments to identify contact points between eIF3 subunits and 40S•eIF1 and to guide our placement of eIF3 subunits on 40S. Because of the propensity of *S. cerevisiae* eIF3 samples to form dimers due to the blade swapping within the eIF3b subunit (Khoshnevis et al., 2012) (Figure S3A), we carried out the CX-MS experiments on eIF3 samples from the budding yeasts *Lachancea kluyveri* and *Debaryomyces hansenii* (see the Extended Experimental Procedures for details). These organisms, like *S. cerevisiae*, only possess the universally conserved, six-component eIF3.

To define the position of eIF3 on 40S, we prepared and crosslinked samples of the yeast eIF3 core bound to 40S•eIF1. Samples were either crosslinked in high salt to prevent nonspecific ribosome binding and eIF3 aggregation or, alternatively, in low salt but with an additional sucrose gradient ultracentrifugation step to remove dimeric 40S/40S•eIF3 species and unbound eIF3. Samples were subjected to LC-MS/MS analysis, yielding 965 interlinks and intralinks (i.e., crosslinks between different proteins or within the same protein, respectively) (Table S4). Specifically, we were able to detect 155 interlinks between eIF3 subunits and 40S and 461 interlinks between 40S ribosomal proteins.

The extensive number of crosslinks connecting 40S ribosomal proteins permitted a detailed analysis of the characteristics of our data set. When mapped onto the X-ray structure of the *S. cerevisiae* 40S subunit (Ben-Shem et al., 2011), 86% of these crosslinks fall within 35 Å (Figure 5C and Table S5), the maximal lysine C α -C α distance that our crosslinker can bridge (Leitner et al., 2012). Within this crosslink test set, the distribution of all mapped distances versus the Id score (Figure 5D) (Rinner et al., 2008) or versus the FDR rate (Figure S5A) (Walzthoeni et al., 2012) shows the expected correlation, as 96% of crosslinks with an Id score of >36 and 94% crosslinks with an FDR of <0.05 are satisfied (Table S5). However, this analysis also showed that a majority of crosslinks in the lower confidence score ranges are also satisfied, suggesting that useful information is lost if standard data cutoffs are applied. Another characteristic observed in our test data set is that certain unique crosslinks are detected more frequently across independent samples than others (Figure 5C and Table S4). These highly redundant crosslinks were more likely to be satisfied than those detected once or only a few times (Figure 5E), suggesting that the number of occurrences of a specific crosslink should be an important consideration when analyzing our data set.

Integrative Structural Modeling of the 40S•eIF1•eIF3 Complex Architecture

The observed characteristics of the 40S crosslink test set influenced our strategy for the integrative structural modeling of the 40S•eIF1•eIF3 complex (Figure S6). In addition to promoting crosslinks with high redundancy, we decided to include all crosslinks with an Id score greater than 28 (FDR <0.3) and used the information gained from the test set to split the crosslink data set into three confidence classes (high, medium, and low; Figures 5D, S5, and Table S5). In order to fully explore the contributions of high-, medium-, and low-confidence crosslinks, we opted for a Bayesian framework to allow us to qualify every piece of information based on the test set. The goal was to lessen the impact of inconsistent crosslinks while benefitting from accurate crosslinks exclusively present within the low and medium confidence ranges (Figure 6B and Experimental Procedures). The 965 crosslinks from the 40S•eIF1•eIF3 data set were used to model the positions and orientations of the six eIF3 subunits on the 40S•eIF1 complex, using crystallographic structures and comparative models of the 40S subunit, eIF1 protein, eIF3 domains, and eIF3 interfaces (Figures 6A, S6, and S7, and Extended Experimental Procedures). 40S, eIF1, and eIF3 domains were represented by sets of beads arranged into either rigid bodies or flexible strings (Figures 6A and S7). None of the available EM maps were integrated in the calculation. Candidate models were ranked by a scoring function (described in the Extended Experimental Procedures) that reflected how well the models satisfied crosslinking data as well as excluded volume and sequence connectivity restraints (Ruszel et al., 2012).

We computed 90,000 structural models by sampling the positions and orientations of the rigid bodies as well as the positions of the remaining beads, guided by the scoring function. The 500 best-scoring models were grouped into two clusters, using the rmsd as a structural similarity criterion (Figure S8 and Extended Experimental Procedures). The cluster of models with the higher population and lower average score was chosen as the final solution set.

These solutions satisfied the excluded volume of the beads and their sequence connectivity restraints. As expected from our experimental design, most of the satisfied crosslinks within our data set of 126 unique eIF3-eIF3 and 40S•eIF1-eIF3 crosslinking restraints were redundant and of high confidence (Figures 6B and S5 and Table S5). Importantly, the eIF3-eIF3 and 40S•eIF1-eIF3 data set had crosslink confidence and frequency distributions comparable to the 40S•eIF1 crosslink data set (Table S5 and Figure S5).

The average pairwise rmsd of the solutions in the favored cluster is 36 Å, or 30 Å if the poorly determined eIF3g-RRM domain is omitted from the rmsd calculation. This precision allowed us to determine the positions and orientations of the eIF3 domains (Table S6). We represented the cluster of solutions by individual localization densities (Figures 6C and S8), defined as the probability of observing a specific eIF3 domain at a given point in space. Our modeling results, as described by these localization densities, place eIF3 at the back of 40S, arranged into a continuous structure that encircles the 40S•eIF1 complex, comprising two large modules and three linker regions

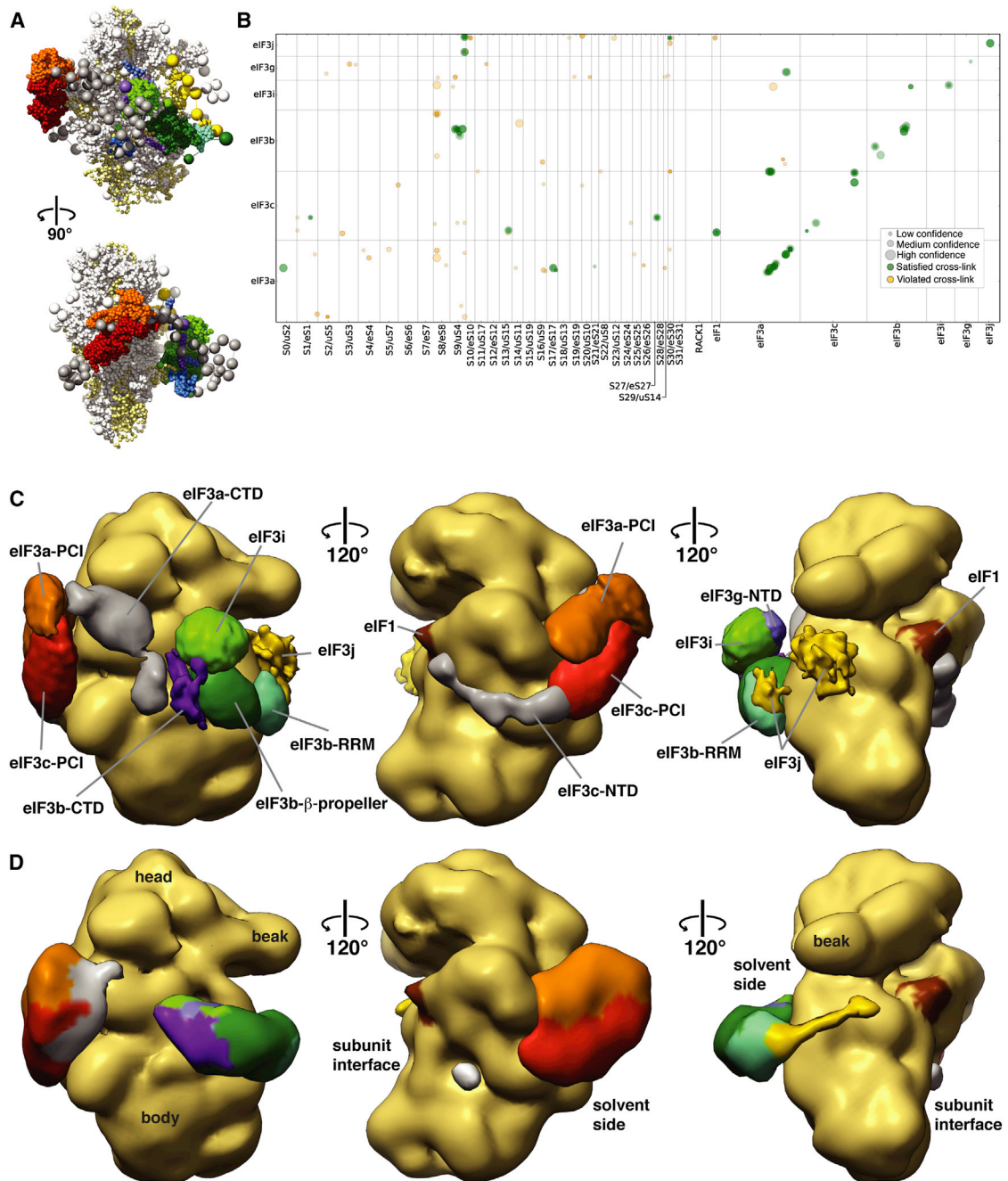


Figure 6. Integrative Modeling of the 40S-eIF1-eIF3 Complex

(A) Representative example of one of our modeling solutions, showing the bead models used in our calculations. Domains of eIF3 are colored as in Figure 1A. (B) Matrix of unique crosslinks between eIF3 and the 40S-eIF1 particle and within subunits of eIF3. Intensity, size, and color code of crosslinked residues are as in Figure 5C.

(C) Localization densities for eIF3 domains superposed on the unoccupied 40S EM reconstruction. The localization densities for each domain are contoured at 1.6 times their estimated molecular volumes. eIF3 domains are colored as in Figure 1A, and linker regions are labeled and colored gray. The position of eIF1 is indicated in brown.

(D) Difference density of the occupied and unoccupied 40S-eIF1-eIF3 EM structures. The density is colored according to the positions of eIF3 domains given by the localization densities shown in (C).

(Figure 6C). These results are fully validated by the EM reconstructions, as our localization densities are in remarkable agreement with our difference density EM map (Figure 6D). In fact, the extensive overlap between the localization densities and the EM map allowed us to assign specific eIF3 domains to regions of the EM difference density (Figure 6D).

EIF3 Subunit Placement on 40S and Comparison between Mammalian and Yeast 40S·eIF3 Complexes

The localizations of the eIF3b β propeller, the eIF3b-RRM, and the PCI modules of eIF3a and eIF3c have a precision of <30 Å and are the best-defined components within our modeling solutions (Table S6 and Figure 6C). The high precision of the eIF3a/eIF3c PCI heterodimer localization stems, in part, from three unique crosslinks between our structural model of the eIF3a/eIF3c PCI heterodimer and 40S (Figures 6B, 7A, and 7B). However, an extensive network of crosslinks involving the peripheral, non-PCI regions of eIF3a (*S. cer.* residues 496–964) and eIF3c (*S. cer.* residues 1–251) provide critical additional restraints (Figure 6B). These linking elements connect the PCI modules with the eIF3b/eIF3i/eIF3g module and with eIF1, eIF2, and eIF5, respectively. Because no structural information is available for these regions, they are represented by beads comprising 20 residue segments in our modeling (Figure S7). Despite this coarse representation, eIF3c-NTD and eIF3a-CTD form tightly clustered interaction networks containing both inter- and intralinks that provide additional restraints on either side of the eIF3a/eIF3c PCI heterodimer-binding site (Figures 7A and 7B). The localization densities for the linker regions match regions of weak difference density in our EM reconstructions (Figures 6D, 7A, and 7B), further suggesting that portions of the eIF3c-NTD and eIF3a-CTD form small ordered regions. In particular, the extensive crosslink network involving the first part of the eIF3a-CTD and the C-terminal helix of eIF3c hints at the formation of a structure analogous to the helical bundle present in the larger PCI·MPN core (Figures 7B, 7E, and 7F). Biochemical evidence suggests that the second half of the eIF3a-CTD extends near the entrance of the mRNA channel, interacting with h16/h18, rpS2/uS5, rpS3/uS3, and the eIF3b-RRM (Valásek et al., 2001, 2003). Although we do not observe any specific crosslinks that support this orientation, an extended orientation for this region of eIF3a is consistent with our overall model.

In our solutions, the eIF3b β propeller localizes between RNA helices ES6A and h16 and above rpS9/uS4, rpS4/eS4, and rpS24/eS24 with a high precision of 15 Å (Table S6). The placement is guided by ten unique crosslinks between four eIF3b residues and seven rpS9 residues (Figures 6B and 7C) and is consistent with previous RNase protection experiments that mapped an interaction between mammalian eIF3 and h16 (Pisarev et al., 2008). To obtain a representative set of structures from our solutions, we further clustered the eIF3b β propeller conformations on the 40S subunit using an rmsd cutoff of 3.5 Å (Extended Experimental Procedures). This analysis allowed us to select ten structures from our solutions that represent the most frequent positions of the eIF3b β propeller. The overlay of the selected eIF3b β -propeller structures reveals a tight cluster of structures that share a common interaction surface with 40S (Figure 7C). The placements of the eIF3b-CTD/eIF3i/eIF3g-

NTD and of the eIF3b-RRM are less precise, relying exclusively on crosslinks and sequence connectivity constraints to the eIF3b β -propeller domain, as there are no satisfied crosslinks with 40S (Figure 6B). Indeed, the position of the eIF3b-CTD/eIF3i/eIF3g-NTD complex is the feature that defines the two clusters within our 500 best-scoring solutions. The position of our best-solution set relies heavily on a redundant, medium-confidence crosslink between eIF3i and the eIF3b β propeller, whereas the position in the less-populated cluster is dominated by connectivity restraints that are less constrained in this orientation, leading to the lower precision observed for this cluster (Figure S8 and Table S6). In our final solution set, the position of the eIF3i β propeller within the eIF3b-CTD/eIF3i/eIF3g-NTD subcomplex is consistently oriented orthogonally to the eIF3b β propeller (Figure 6C), with the “bottom” face of the eIF3i β propeller facing the “top” surface of the eIF3b β propeller (Figures 7E and 7F). This arrangement places the C-terminal helices of eIF3b in an elongated conformation along the “bottom” and “top” surfaces of the eIF3b and eIF3i β propellers, respectively, and the eIF3g-NTD is localized between eIF3i and 40S near the interface of the β propellers, consistent with evidence that eIF3g stabilizes the eIF3b/eIF3i interaction (Herrmannová et al., 2012; Khoshnevis et al., 2012) and interacts with ribosomal proteins rpS3/uS3 and rpS20/uS10 (Cuchalová et al., 2010). At the N-terminal end of eIF3b, the localization density of the eIF3b-RRM is adjacent to the eIF3b β propeller near the mRNA entrance channel, linking eIF3b to eIF3j, which our solutions place in its expected position near the mRNA channel entrance (Fraser et al., 2007).

Consistent with our localization densities, the EM envelope of the mammalian 43S complex, despite the presence of the DHX29 helicase (Hashem et al., 2013a), shows features that agree with our model. Of the density elements assigned to eIF3, a toroid-shaped element between the platform and the beak of 40S closely matches our position for the eIF3b β propeller (Figure 7D). Previously, this density was tentatively attributed to eIF3i (Hashem et al., 2013a). Furthermore, the localization densities of the eIF3b-CTD/eIF3i/eIF3g-NTD complex and the eIF3b-RRM match regions of weak additional density in the mammalian 43S structure (Figure 7D), supporting the localization and orientation of the eIF3b/eIF3i/eIF3g module in our solution set. This extensive agreement between the EM densities and our modeling solutions gives us confidence that our models for the yeast and mammalian 40S·eIF1·eIF3 complexes reveal the regions of the 40S subunit involved in eIF3 interactions as well as the positions and relative orientations of nearly all eIF3 domains and the likely paths of all major linker domains (Figures 7E and 7F).

DISCUSSION

The eIF3 complex plays a prominent role in regulating the intricate molecular events of translation initiation. Its large size and modular structure have made molecular studies of this assembly challenging, and a comprehensive molecular understanding of the 40S·eIF3 interaction has remained elusive.

Our hybrid approach allowed for a large number of low-resolution restraints to be effectively integrated and optimized,

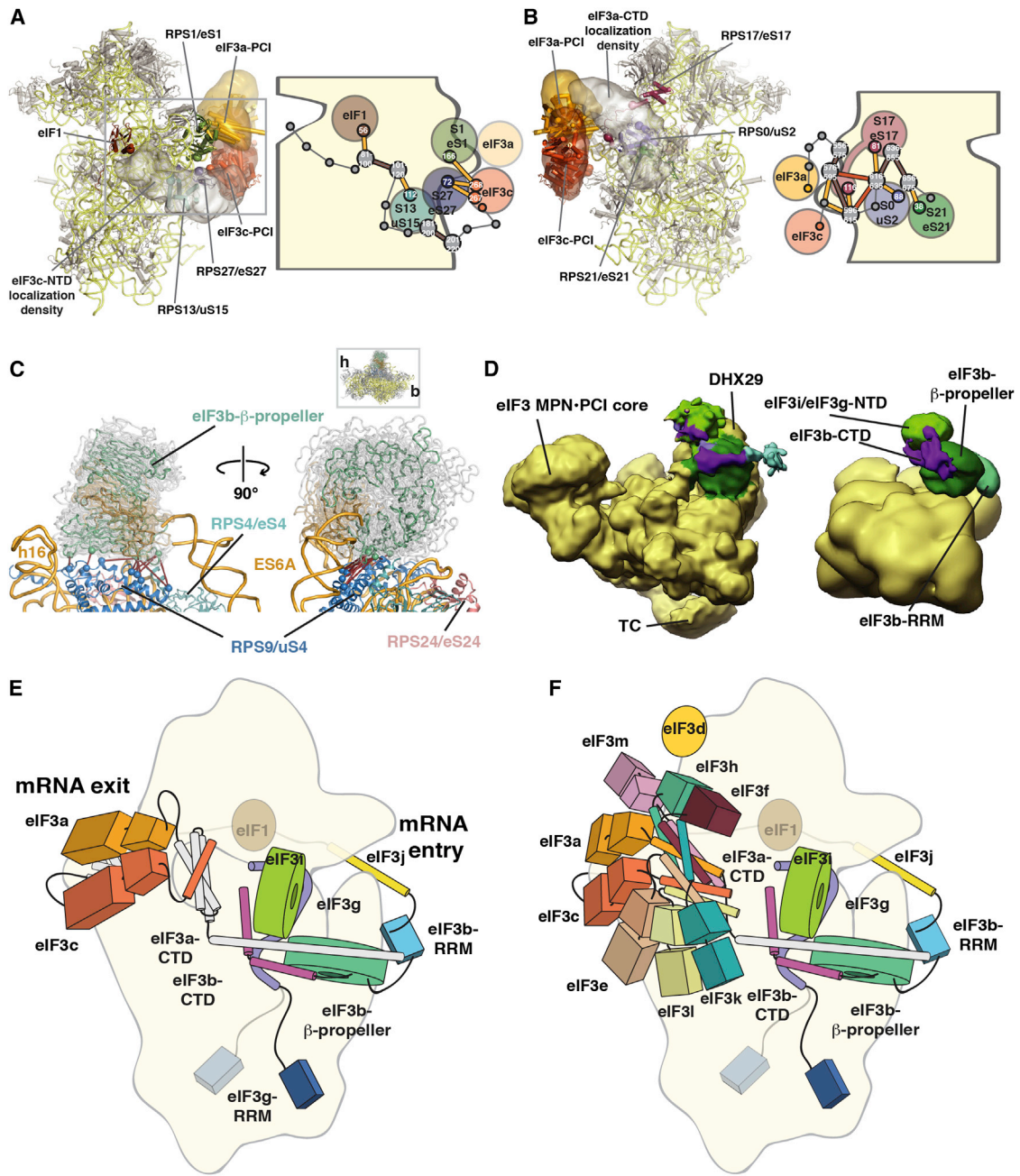


Figure 7. Placement and Interactions of eIF3 Components on 40S

(A) (Left) Cartoon depiction of the 40S•eIF1 structure model, showing ribosomal proteins in gray and RNA in yellow. Proteins that are crosslinked to eIF3c are highlighted and labeled. The localization densities for the eIF3a/eIF3c PCI heterodimer and the eIF3c-NTD are shown as transparent densities and are colored as in Figure 1A. (Right) Schematic depiction of the crosslinking pattern of the eIF3c-NTD. The bead model of the eIF3c-NTD is shown as gray spheres linked by thin gray lines and is labeled with the residue range it represents. Interlinks are shown in gold and intralinks in red.

(B) Same as (A) but showing the localization density for the eIF3a-CTD and highlighting the inter- and intralink network that helps define its orientation.

(C) Molecular model of the 40S-bound eIF3b β -propeller domain. 40S RNA and protein elements in the vicinity of the binding site are individually colored and labeled. Ten representative eIF3b structures are shown as gray ribbons, with an average structure colored as in Figure 1A. Residues involved in crosslinks are shown as spheres, and crosslinks are in red.

(D–F) (D) Comparison of the mammalian 43S EM map (left) and the localization densities (superposed on the unoccupied yeast EM map) for the eIF3b/eIF3i/eIF3g complex (right). Domains are colored as in Figure 1A and are labeled. Model of the consensus positions of various eIF3 elements in the yeast (E) and mammalian (F) 40S•eIF1•eIF3 complexes, integrating the information from our hybrid approach with known interactions of eIF3 elements to derive a comprehensive model of the molecular architecture of eIF3 on the 40S subunit. Individual eIF3 components are labeled and colored as in Figures 1A and 3E. The position of the eIF3d subunit is based on extra density observed in the mammalian 43S EM structure.

resulting in a sophisticated and surprisingly detailed interaction map of eIF3 on the ribosome (Figures 7E and 7F). This arrangement enables eIF3 components to completely encircle the 40S subunit and to engage and coordinate the binding of other components of the translation initiation machinery at both ends of the mRNA channel. EIF3 can therefore act as a scaffold, ensuring that factors required at both the mRNA channel entry and exit are assembled simultaneously, allowing eIF3 to act as the “orchestra conductor” (Valásek, 2012) during initiation, coordinating the input from a number of “sections” to ensure that mRNA recruiting and scanning at one end occurs only when elements critical for AUG recognition and scanning modulation are assembled at the opposite end. Interestingly, because eIF1 and eIF3j have high 40S-binding affinities, interactions at the ends of the two eIF3 “arms” may enable the cooperative assembly of the full 43S PIC.

This model is consistent with the vast majority of biochemical and genetic data that has accumulated over the years (Hinnebusch, 2006, 2014; Marintchev and Wagner, 2004; Valásek, 2012). Mutations and disruptions in factors mapped to the mRNA channel entrance result in a leaky scanning phenotype and defects in rescanning downstream of short uORF sequences (Chiu et al., 2010; Elantak et al., 2010; Nielsen et al., 2006; Cuchalová et al., 2010), suggesting that eIF3b, eIF3g, eIF3i, and eIF3j prevent AUG codon bypass and modulate rescanning downstream of uORFs by engaging the mRNA in the vicinity of the channel entrance. At the other end of the eIF3 “clamp,” the eIF3c-NTD extends across the subunit interface, connecting the PCI-MPN core of eIF3 with the mRNA channel and making critical contacts with eIF1, eIF5, and TC and, in mammals, with eIF4 to create an assembly that is highly proficient at codon differentiation during 5' UTR scanning (Karásková et al., 2012). In contrast to mutations in the eIF3b/eIF3i/eIF3g/eIF3j module, mutations in the N-terminal tail of eIF3c result in defects in TC recruitment and scanning fidelity, suggesting distinct roles for the two “arms” of eIF3 that extend into the mRNA channel (Phan et al., 2001; Valásek et al., 2004).

The interactions mediated by the six universally conserved eIF3 subunits probably represent the core functions of eIF3 during translation initiation. The remaining six PCI-MPN eIF3 components not found in budding yeasts are likely to act as “gatekeepers,” engaging elements within mRNPs (including the cap-binding complex) to regulate their access to the ribosome. In *S. cerevisiae*, the loss of eIF3 PCI-MPN core components correlates with the simplified 5' UTR structures of most transcripts and the low intron density found in these organisms (Hinnebusch, 2014). Our detailed molecular description of the interaction between eIF3 and the 40S ribosomal subunit maps individual functionalities of eIF3 to distinct regions of the 43S PIC and provides an intricate structural framework to integrate biochemical, biophysical, and genetic studies of eukaryotic translation initiation.

EXPERIMENTAL PROCEDURES

Cloning, Purification, and Complex Assembly

A full list of constructs used in this study is given in Table S1. The purification protocols for eIF1, various eIF3 subunits and complexes, and the 40S subunit are described in Extended Experimental Procedures.

Crystallization, Data Collection, and Structure Determination

All proteins (15–20 mg/ml) were crystallized by sitting-drop vapor diffusion. Native and Se-Met SAD data were collected at beamline PX of the Swiss Light Source and processed in XDS (Kabsch, 2010). Structures were solved using AutoSol or Phaser MR in Phenix (Adams et al., 2010) and models built using Phenix AutoBuild (Adams et al., 2010) and COOT (Emsley et al., 2010). Model refinement was performed with Phenix (Adams et al., 2010). More details are provided in the Extended Experimental Procedures and Tables S2 and S3.

Chemical Crosslinking Coupled to Mass Spectrometry (CX-MS)

eIF3 samples were assembled from purified, recombinant subunits and were subsequently mixed with 40S subunits as described in the Extended Experimental Procedures. Final 40S-eIF1-eIF3 samples were assembled under various buffer conditions and crosslinked. Some samples were purified on sucrose gradients before RNase treatment, enzymatic digestion, and enrichment of crosslinked peptides was carried out. LC-MS/MS analysis was performed on an Orbitrap Elite mass spectrometer, and the data were searched using the xQuest/xProphet software pipeline. Further details are provided in the Extended Experimental Procedures.

Electron Microscopy

The *Lachancea kluyveri* 40S-eIF1-eIF3 complex was prepared and crosslinked as described for CX-MS. The sample was then subjected to gradient fixation using GraFix (Kastner et al., 2008), grids were prepared by negative staining using uranyl acetate, and images were acquired in a Tecnai F20 electron microscope under low-dose conditions. Single-particle images were split into initiation factor occupied and unoccupied ribosomal complexes based on an initial reconstruction of the whole data set using supervised classification (Imagic Spider RRR). The resolution of the final structure of the initiation factor occupied and unoccupied ribosomal complex was estimated as 28.1 and 24.6 Å, respectively (Fourier shell = 0.5 criterion, semi-independent data half sets).

Integrative Modeling

The integrative modeling approach proceeded through four steps, as described in Extended Experimental Procedures. To benefit from the entire crosslink data set, the Bayesian scoring function separately modeled the uncertainty of high-, medium-, and low-confidence crosslinks (as defined by Id score and FDR), thereby modulating their relative weights (Rieping et al., 2005). The 275 eIF3-eIF3 and 40S-eIF1-eIF3 crosslinks restrained the positions and orientations of eIF3 domains relative to 40S-eIF1, whereas the remaining 690 40S-eIF1 crosslinks were included as a training set to increase the accuracy of the uncertainty estimation. Because the frequency of crosslink observation across eight independent experiments correlates with their accuracy in our test set (Figure 5E), the unique crosslinks were weighted proportionally to their frequency. An extensive description of the methodology is provided in the Extended Experimental Procedures, and the modeling scripts and models are available at <http://sailab.org/40S-eIF1-eIF3>.

ACCESSION NUMBERS

The Protein Data Bank accession numbers for the structures reported in this paper are 4U1D (eIF3a), 4U1C (eIF3a/eIF3c complex), 4U1F (eIF3b β propeller) and 4U1E (eIF3b-CTD/eIF3i/eIF3g-NTD complex), 3J7J (PCI-MPN core model docked into 43S-IRES EM map) and 3J7K (PCI-MPN core model docked into 43S EM map). The EMDB accession numbers for the EM density maps reported in this paper are 2670 (eIF3-occupied 40S) and 2671 (unoccupied 40S).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, eight figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.07.044>.

AUTHOR CONTRIBUTIONS

J.P.E., F.S., R.P., and N.B. conceived the study and experimental approach; J.P.E., S.Z., and T.S. cloned, purified, and crystallized eIF3 samples, collected and processed X-ray diffraction data, solved the structures, and built the atomic models; J.P.E., T.S., F.S., and C.H.S.A. prepared samples for EM and CX-MS experiments. C.H.S.A. and D.B. collected EM data and calculated the EM reconstructions; F.S. performed CX-MS experiments; R.P. carried out the integrative modeling with the help of P.C.; J.P.E., F.S., and R.P. analyzed the integrative modeling results, and J.P.E., F.S., R.P., A.S., R.A., and N.B. wrote the paper with input from all authors.

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