Molecular Landscape of the Ribosome Pre-initiation Complex during mRNA Scanning: Structural Role for eIF3c and Its Control by eIF5

Highlights
- eIF3c N-terminal domain is divided into three regions, 3c0, 3c1, and 3c2
- 3c1 and eIF5 anchor eIF1 to the 40S ribosome during mRNA scanning
- On AUG, 3c0 binds eIF1 ribosome-binding site, facilitating eIF1 release
- eIF5 prevents 3c0 from binding eIF1 before AUG selection

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In Brief
During translation initiation, eIF3 binds the solvent-accessible side of the 40S ribosome. Obayashi et al. propose that the N-terminal domain of eIF3c reaches into the decoding center to not only anchor the gate-keeper eIF1 but also facilitate eIF1 release on AUG selection. eIF5 appears to play a role in this regulation.

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Molecular Landscape of the Ribosome Pre-initiation Complex during mRNA Scanning: Structural Role for eIF3c and Its Control by eIF5

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SUMMARY

During eukaryotic translation initiation, eIF3 binds the solvent-accessible side of the 40S ribosome and recruits the gate-keeper protein eIF1 and eIF5 to the decoding center. This is largely mediated by the N-terminal domain (NTD) of eIF3c, which can be divided into three parts: 3c0, 3c1, and 3c2. The N-terminal part, 3c0, binds eIF5 strongly but only weakly to the ribosome-binding surface of eIF1, whereas 3c1 and 3c2 form a stoichiometric complex with eIF1. 3c1 contacts eIF1 through Arg-53 and Leu-96, while 3c2 faces 40S protein uS15/S13, to anchor eIF1 to the scanning pre-initiation complex (PIC). We propose that the 3c0:eIF1 interaction diminishes eIF1 binding to the 40S, whereas 3c0:eIF5 interaction stabilizes the scanning PIC by precluding this inhibitory interaction. Upon start codon recognition, interactions involving eIF5, and ultimately 3c0:eIF1 association, facilitate eIF1 release. Our results reveal intricate molecular interactions within the PIC, programmed for rapid scanning-arrest at the start codon.

INTRODUCTION

Ribosomes initiate translation with levels of stringency varying between bacteria (low) and eukaryotes (high) (Asano, 2014). The high accuracy of initiation in eukaryotes results from suppressing initiation from non-AUG codons like GUG and UUG. This stringency is imposed partly by eukaryotic initiation factors (eIFs) that bind the small (40S) ribosomal subunit in the 43S preinitiation complex (PIC), i.e., eIF1A, eIF1, eIF2, eIF3, and eIF5 (Asano, 2014; Hinnebusch, 2014). Like its bacterial counterpart IF1, eIF1A binds the 40S A-site. The other four factors engage in numerous mutual interactions to form the multifactor complex (MFC) with Met-tRNAiMet bound to eIF2-GTP in the ternary complex, whereby MFC can be isolated free of ribosomes from various eukaryotes (Asano et al., 2000; Dennis et al., 2009; Meleppattu et al., 2015; Sokabe et al., 2012). eIF4F, comprising m7G-cap binding subunit eIF4E, RNA helicase eIF4A, and scaffold eIF4G, mediates attachment of the mRNA 5’ end to the PIC in its open, scanning-competent conformation (Kumar et al., 2016). A key event in start codon selection is dissociation from the 40S of eIF1, a gatekeeper molecule that maintains the open conformation of the PIC (Pestova and Kolupaeva, 2002; Saini et al., 2010). During scanning, the eIF1 physically opposes full accommodation of tRNAi in the P-site, keeping it in the P_OUT conformation (Lomakin and Steitz, 2013; Rabl et al., 2011; Weisser et al., 2013). Once tRNAi, base pairs to the AUG start codon, eIF1 is released, Met-tRNAi is fully accommodated in the P-site (P_IN state), and the PIC adopts the closed conformation incompatible with scanning. The resulting 40S initiation complex is ready for subsequent 60S subunit joining.

In this work, we examine the structural role of the N-terminal domain (NTD) of the eIF3c-subunit of eIF3, a crucial binding partner of eIF1 and eIF5 in the MFC, and key regulator of start codon
solved cryo-EM PIC structure (Erzberger et al., 2014). Based on start codon selection (Asano et al., 2000, 2001a; Karásková et al., 2012; Phan et al., 1998; Valášek et al., 2004). elf3 is a multisubunit complex (Asano et al., 1997) that binds the solvent-accessible side of the 40S (Srivastava et al., 1992). Cross-linking and integrated modeling studies suggest that elf3c-NTD extends into the 40S decoding center proximal to elf1 (Erzberger et al., 2014). elf5 is the GTPase activating protein for elf2 (Asano et al., 2001b; Huang et al., 1997). Independently of the catalytic NTD, the elf5 C-terminal domain (CTD) interacts with elf1A, elf2B, elf3c, and elf4G at various stages of initiation (Luna et al., 2012, 2013; Reibarkh et al., 2008; Singh et al., 2012; Yamamoto et al., 2005). While an initial cryoelectron microscopy (cryo-EM) study revealed density potentially corresponding to elf5-CTD facing elf1 and elf2 in the PIC (Hussain et al., 2014), this was not observed in more recent PIC structures (Lácer et al., 2015). Thus, the location and structural role of elf5-CTD in the PIC also remains unclear.

Genetic studies have revealed that elf3c-NTD contains two distinct elements with opposing roles in initiation accuracy. Box12 is required for accurate initiation, and substitution mutations in this element increase non-AUG initiation (for the Sui− or suppressor of initiation codon mutation phenotype). The Box6 element is required for initiation at non-AUG codons, and substitutions in Box6 suppress effects conferred by a Sui+ mutation (for the Ssu+ or suppressor of Sui phenotype) (Karásková et al., 2012; Valášek et al., 2004). Henceforth, Box6 and Box12 are designated as an Ssu+ (Box6Ssu+) and a Sui+ element (Box12Sui+), respectively. Certain Box6 or Box12 mutations decrease elf1 binding to the elf3c-NTD, suggesting that the elf3c-NTD helps to stabilize elf1 in the PIC not only during mRNA scanning, but also during the switch to the closed state. Based on these results, we identify 3c1 as the core elf1-binding site in elf3c-NTD. Low-affinity elf1 binding by flanking region 3c0 containing Box6Ssu+ contributes to the high-affinity elf1 binding (~1 μM) by fragments containing 3c1 and 3c0, likely through interaction with more than one site on elf1. Because we failed to generate an elf3c segment containing only 3c1, the contribution of C-terminal flanking 3c2 remained unclear. However, based on the low-affinity elf1 binding to elf3c-E87–163, 3c2 containing Box12Sui+ likely contributes to the relatively high-affinity binding (~8 μM) observed for elf3c-D58–163.

RESULTS

Functional Dissection of elf1-Binding Elements in elf3c-NTD

To map elf1 binding sites in the elf3c-NTD, we divided the latter into three regions: 3c0 encompassing amino acids (aa) 1–58, including the conserved N terminus required for elf5 binding (Karásková et al., 2012) and most of Box6Ssu+; 3c1 encompassing aa 59–87, which contains a conserved hydrophobic segment; and 3c2 comprising aa 88–163, including predicted α helices (http://bioinf.cs.ucl.ac.uk/psipred/) and Box12Sui+ (Figures 1A and 1A). GST fusions to elf3c-NTD fragments with different combinations of these regions (elf3c-A to -G, Figure 1A) were tested for elf1 binding using GST pull-down assays. Fragment elf3c-D58–163 essentially covers the previously determined minimal elf1-binding site (aa 60–137) (Karásková et al., 2012).

The strongest elf1 binding was observed with elf3c-A1,163-B36–163, -C36–87, and -F1–87, which all include the C-terminal half of 3c0 and the entire 3c1 (Figures 1B, lanes 2, 3, 6, and 8, and 1C). Isothermal titration calorimetry (ITC) assays demonstrated apparent Kd values of ~1 μM for these constructs (Figures 1A and S2A) with SDs of <15% (n = 3, Figure S2B). elf3c-D58–163 containing regions 3c1 and 3c2, exhibited weaker association with elf1 (Figure 1B, lane 4) with an apparent Kd of ~8 μM (Figures 1A, S2A, and S2B). elf3c-D58–163 regions therefore bind elf1 with a significantly lower affinity than the constructs with regions 3c0-3c1 (p < 0.006, n = 3). In contrast, the two NTD segments lacking 3c1, elf3c-E87–163 (3c2), and elf3c-G1–58 (3c0) did not appear to interact with elf1 in GST pull-down assays (Figure 1B, lanes 5 and 7), but did display appreciable binding when the elf1 concentration was increased ~50-fold to ~30 μM (Figure 1B, lanes 5 and 9, elf1 detected by anti-elf1; Figure 1C, lanes 3 and 7, and elf1 indicated by arrowheads in Coomassie staining). Note that in Figure 1B, amounts of elf1 bound to GST-elf3c-E87–163 (lane 9) and elf3c-G1–58 (lane 5) are <10% of that bound to GST-elf3c-F1–87 with 3c0 and 3c1 (lanes 7 and 10; where 10% and 90% of the pull-down fraction were loaded. Anti-elf1 signal in lane 10 is saturated due to overloading). Consistent with the pull-down results, the Kd for elf3c-E87–163 binding to elf1 is >100 μM (Figure 1A).

The ITC assay revealed that elf3c-D58–163 forms a stoichiometric complex with elf1 (N = 1.0, Figures 1A and S2B), while other segments containing 3c1 and 3c0 (A1–163, B36–163, C36–87, and F1–87) display N values (number of elf3c molecules bound per elf1 molecule) significantly less than 1.0 (p < 0.03, n = 3). These results suggest that elf1 has more than one binding site for elf3c regions 3c0 and 3c1.

Based on these results, we identify 3c1 as the core elf1-binding site in elf3c-NTD. Low-affinity elf1 binding by flanking region 3c0 containing Box6Ssu+ contributes to the high-affinity elf1 binding (~1 μM) by fragments containing 3c1 and 3c0, likely through interaction with more than one site on elf1. Because we failed to generate an elf3c segment containing only 3c1, the contribution of C-terminal flanking 3c2 remained unclear. However, based on the low-affinity elf1 binding to elf3c-E87–163, 3c2 containing Box12Sui+ likely contributes to the relatively high-affinity binding (~8 μM) observed for elf3c-D58–163.

CSP Mapping with 15N-elf3c-NTD Identifies aa Involved in elf1 Binding

Next, we used NMR chemical shift perturbation (CSP) mapping to delineate elf3c residues directly involved in elf1 binding. We first determined the structure of elf3c-NTD by NMR spectroscopy using [13C, 15N] elf3c-B36–163 segment (see Supplemental Information and Table S3 for details), which demonstrated that the region covering most of 3c2 (residues 105–159) folds into α-helical globule (Figure 1C). The elf3c backbone resonance assignments were then used for CSP studies. As shown in Figure S3, CSPs induced by elf1 binding are nearly identical between 15N-elf3c-A1,163 and -B36–163, which is consistent with our GST-pull down and ITC studies (Figures 1A, S1B, S1C, and S2) (Karásková et al., 2012). We further observed large CSPs for A67 (circled in blue in Figure 1D; indicated by arrow in Figure S3, lower panels), and E51 residues (circled in blue in Figure 1D).
accompanied by the strong resonance line broadening in the stretch: K68(P)YG(P)DWFKK77 (K68, Y70, and F75 highlighted in Figure 1D; others highlighted in Figures S3A and S3B; prolines are in parentheses). In contrast, all CSPs in 3c2 were minor (<0.04 ppm) (Figure 1E) except for F90, which was considered spurious inasmuch as it was not eliminated by an eIF1 mutation that abolishes interaction with eIF3c (shown below in Figure S4, panel 1). Collectively, these results indicate that the eIF1-binding site on eIF3c NTD resides in the area covering Box6Ssu+ of 3c0 (containing E51) and core region 3c1 (aa 58–87, contains A67K(P)YG(P)DWFKK77) (Figures 1A and S1A).

Structure of eIF3c-NTD105–159 and Integrated Modeling of eIF3c:eIF1:40S Complex Structure Define Two Globular Units within eIF3c-NTD

In a recent cryo-EM study of the eIF1/eIF3/40S complex, which integrated extensive crosslinking information, it was proposed that the eIF3c-NTD projects from the solvent side along the 40S subunit into the decoding center, where eIF1 is bound (Erzberger et al., 2014). However, structural information for the eIF3c-NTD was lacking. We therefore incorporated NMR structure of eIF3c segment 105–159 (Figure 1C) into the integrated modeling platform and calculated a new localization for the whole eIF3 complex (Figure S5). The resulting localization densities for eIF3c-NTD had a resolution of 18 Å (Figure 2A, left), guided by four high-confidence crosslinks (Figure 2A, right), which is a clear improvement from the 38 Å precision in our previous model (Erzberger et al., 2014). The eIF3c-NTD is resolved into two globular units that span the /C24 60 Å distance between eIF1 and rpS13/uS15 (Figures 2A and S5). The one is located near rpS13/uS15 and was assigned as a /-helical globular structure in 3c2 (aa 105–159) shown in Figure 1C. The other is adjacent to eIF1 and, thus, was assigned as the core eIF1-binding region 3c1 (aa 59–87) (Figure 1A). Indeed, recent medium-resolution
cryo-EM reconstructions (Aylett et al., 2015; Liácer et al., 2015) reveal densities consistent with the positions of 3c1 and 3c2 (Figure 2B). The N-terminal region 3c0 (Figure 1A) was not localized in the eIF3:eIF1:40S structure, presumably because it cannot bind eIF1 when eIF1 is bound to the 40S subunit (as discussed below). Therefore, integrative modeling that incorporates the NMR structure of eIF3c D58–163 pinpointed the locations of the 3c1 and 3c2 elements within the PIC, with 3c1 directly contacting eIF1.

**NMR Evidence that eIF3c-NTD Segments 3c1-3c2 Interact with a Limited Surface of eIF1 Compatible with 40S Binding**

eIF1 comprises an unstructured N-terminal tail (NTT) and a globular domain with a β1-β2-α1-β3-β4-α2-β5 fold (Fletcher et al., 1999; Reibarkh et al., 2008) (Figure 3A; Table S4). To determine the eIF1 residues contacted by the 3c1-3c2 units in the complex formed with eIF3c D58–163, we performed CSP experiments using 15N-eIF1. As shown in Figure 3B and summarized in Figure 3A, strong CSPs were observed for R53, K56, I93, and L96 residues on eIF1 thereby indicating that these residues on eIF1 direct its interaction with eIF3c D58–163. In contrast, resonances corresponding to residues within or nearby the two eIF1 ribosome-binding sites (Martin-Marcos et al., 2013; Rabl et al., 2011), including K60 at the α1 C terminus and T40/T41 near the β1-β2 loop (loop 1), were only marginally affected (Figures 3A and 3B). As summarized in Figure 4A, the eIF3c-D58–163-binding site on eIF1 comprises the N-terminal and central portions of α1 and the adjacent hydrophobic area containing I93 (residues...
Figure 3. NMR CSP Mapping of eIF3c Binding Site on eIF1
(A) Primary structure of yeast eIF1 (brown horizontal line) with boxes indicating secondary structure elements. aa whose resonance was shifted due to addition of distinct eIF3c fragments are shown in colors based on the code on the bottom.
(B–E) Top, CSP of $^{15}$N-eIF1 resonances caused by eIF3c-D58–163 (B), eIF3c-E78–163 (C), eIF3c-C36–87 (D), and eIF3c-B36–163 (E) were highlighted with arrows in the specified areas of $^1$H–$^{15}$N HSQC spectra. The spectra taken in the presence and absence of eIF3c fragments (1:1.2) are shown in color and gray, respectively. The
(legend continued on next page)
NMR Evidence that Segment 3c0-Box6$_{Sea}$ Interacts with the Ribosome-Binding Surface of eIF1

Relative to 3c1-3c2 segment D$_{58}$-163, fragment C$_{36}$-87, containing 3c1 and part of 3c0, displayed CSPs of greater intensity for a larger number of $^{15}$N-eIF1 resonance peaks (Figure 3D). Herein, in addition to R53 and L96 eIF1 residues, extensive CSPs were also observed for D$_{61}$, A$_{63}$, and N$_{65}$, which are localized in the z1-$\beta$3 loop, T$_{41}$ in P$_{2}$ near loop 1, and L$_{80}$ (Figure 3D, summarized in Figure 3A). This suggests that C$_{36}$-87 fragment binds an entire side of $\beta$ sheets 1–4 of eIF1 that is adjacent to K60 at the z1 C terminus, the 40S contact site, and is likely to overlap with the second 40S contact site in loop 1, R36 (cyan lettering in Figure 4B). Interestingly, the resonance corresponding to I$_{93}$ was slightly shifted in the presence of C$_{36}$-87 without attenuation of its signal (Figure 3D, yellow for weak/moderate interaction) but did not disappear (line broadening) as observed for D$_{58}$-163 (red in Figure 3B). As summarized in Figure 4B, this pattern suggests that C$_{36}$-87 still retains interaction with R53 and L96 of eIF1 through the core element, 3c1, while its interaction with eIF1-I$_{93}$ is diminished due to lack of 3c2. This supports an indirect stimulatory role for 3c2 in eIF1 binding to eIF3c-NTD (dotted line in Figure 4A).

Importantly, these data also suggest that the presence of the C-terminal half of 3c0 in C$_{36}$-87 confers more extensive interactions with the ribosome-binding surfaces of eIF1 (Figure 4B).

eIF1 residues assigned to the resonances are shown with their aa numbers. aa of high relevance (R53, K56, K60, I$_{93}$, L$_{96}$) are highlighted in red. Bottom, the eIF1 residues affected by each eIF3c segment are painted orange or yellow for strong or moderate CSP of >0.1 ppm or 0.05–0.1 ppm, respectively, in the ribbon diagram of yeast eIF1 structure. The eIF1 residues whose resonances caused line broadening were painted red. Locations of aa of high relevance are indicated. Prolines (11, 46, and 72) and unassigned residues (23, 34–36, 66, and 107) are painted green and gray, respectively. In (B), note that, upon eIF3c-D$_{58}$-163 addition, the cross peak for K60 was shifted only slightly (green arrowhead in the spectrum), overlapping with that for K56, which shifted a greater distance (long black arrow).

See also Tables S1 and S4.
This is in agreement with its $N$ value in ITC experiments of 0.7, indicating more than one binding site on eIF1 (Figures 1A and S2B). Hence, we propose that 3c0 does not engage eIF1 in the scanning PIC because its binding site on eIF1 overlaps with the 40S-binding surface.

The conclusion that the C-terminal half of 3c0 containing Box6R engages ribosome-binding surface of eIF1 is further supported by ITC analysis indicating that the K37E substitution in eIF1 loop 1 reduces eIF1 binding to eIF3c-NTD by 4-fold (Figures 5B and S6A). Importantly, 3c0:eIF1 interaction may explain the Ssu− phenotype of the Box6R mutation (Valašek et al., 2004). Notwithstanding that due to the relatively low affinity of eIF1 for eIF3c-NTD ($K_d = 1 \mu M$) 3c0 is unlikely to displace eIF1 from PIC (eIF1:40S subunit $K_d = 1–10 \text{nM}$) (Martin-Marcos et al., 2013), by competing with the eIF1:40S subunit interaction 3c0 may increase the chance that eIF1 is inappropriately released from the 40S subunit at a non-AUG codon. By disrupting this competition, the Box6R mutation of 3c0 is expected to stabilize the scanning PIC and diminish non-AUG initiation (Ssu− phenotype). Thus, combined with the genetic findings (Valašek et al., 2004), the CSP study in Figure 3D suggests that 3c0:eIF1 interaction impedes eIF1 binding to the ribosome.

The largest fragment examined, eIF3c-B36–163, containing the C-terminal half of 3c0, and full 3c1 and 3c2, induced a combination of CSPs observed for both eIF3c-D58–163 (3c1+3c2) and eIF3c-C36–87 (3c0+3c1) (Figure 3E). These included major perturbations in the following eIF1 residues: R53 and L96 (due to 3c1), I93 (due to 3c2) and residues in the proximity of both eIF1 ribosome-binding surfaces (attributed to 3c0). Altogether, these results suggest that 3c0 and 3c2, flanking the core eIF1 binding element 3c1, may differentially modulate interaction of eIF3c-NTD with eIF1.

Arg-53 and Leu-96 of eIF1 Make Critical Connections to the eIF3c-NTD within the Scanning PIC

CSP analysis implicated eIF1 residues R53 and L96, in the N-terminal end of $z1$ and nearby hydrophobic patch, in interaction...
with all three eIF3c-NTD constructs that bind eIF1 with strong affinity (Figures 3B, 3D, and 3E). Accordingly, we tested the effect of substituting these residues on eIF1 binding to eIF3c-B36–163 in vitro. As controls, we examined eIF1 substitutions K56A and K60E, which are involved in 40S binding (see Figures 3A and 5A for eIF1 residues altered). In the ITC assay, R53S substitution reduced eIF3c binding below the detection limit, whereas L96P substitution reduced the affinity by 10-fold (Figures 5B and 5D, blue and green curves). Moreover, GST pull-down assays revealed that R53S has only a slight effect on 40S binding (Figures 5B and S4), which is consistent with NMR data. These results were verified by CSP experiments (Figures 5F and S4). These results also agree with our previous CSP and spin-labeling studies identifying the hydrophobic patch harboring L96 as the eIF5-CTD binding site (Luna et al., 2012; Reibarkh et al., 2008) (Figure 4A). However, L96P only slightly reduced eIF1 binding to the eIF2β-NTT (Figure 5E; see Figure 5A for summary of interaction involving eIF1-L96). Thus, the strong Sui– phenotype of L96P (Figure 6A) likely arises from combined defects of reduced eIF1 binding to the eIF3c-NTD, eIF5-CTD (Figure 5E), and perhaps the 40S subunit (Figure 5B).

Despite the fact that eIF1 substitution R53S essentially abolishes binding to the eIF3c-NTD (Figure 5B), it has no effect on initiation accuracy (Figure 6A), implying that eIF1-R53S retains other interactions with the PIC that compensate for impaired interaction with eIF3c. Employing a variation of the FA assay in which excess unlabeled eIF1 competes with wild-type (WT)-labeled eIF1 for ribosome binding (Figure 5D, left), we found that R53S has only a slight effect on 40S binding (Figures 5B and 5D, green curve). Moreover, GST pull-down assays revealed only modest effects of R53S on binding to the eIF2β-NTT and eIF5-CTD (Figure 5E, right). The CSP assay with 15N-eIF1-R53S also demonstrates robust eIF5-CTD interaction with this mutant, as observed with WT 15N-eIF1 (Figure S7) (Reibarkh et al., 2008). Thus, R53S specifically abolishes eIF1 interaction with the eIF3c-NTD (Figure 5B), which is not sufficient to impair accuracy of start site selection in vivo.

To demonstrate a role for eIF1-R53 in stabilizing the scanning PIC in vivo, we generated double mutants. Combining R53S and K56A in eIF1 did not alter the defect in eIF3c-NTD:eIF1 binding seen for R53S alone (Figure 5B) and conferred only a moderate decrease in 40S:eIF1 binding affinity beyond the 11-fold reduction in Kd observed for single mutants (Figure 6A, row 6). Since K56A has no effect on eIF1 binding to eIF2β-NTT and eIF5-CTD when
Figure 7. GST Pull-Down and AUC Experiments Characterizing Interaction between eIF3c-NTD, eIF1, and eIF5

(A) GST pull-down assay demonstrating inhibition of eIF5 binding to eIF3c by excess eIF1. 100% of indicated GST-eIF3c fusion proteins (~0.2 nmol) were allowed to bind 5 mg of eIF5 (~0.1 nmol) in the presence of 70 mg of recombinant eIF1 (~5 nmol) present in induced (I) lysates and the complex analyzed by SDS-PAGE and Coomassie staining. U and –, uninduced lysate or buffer, respectively, was added in place of induced lysates.

(B) Summary of AUC interaction studies. Left, eIF3c-NTD fragments used are shown with bars indicating their relative locations in eIF3c primary structure. Second, third, forth, and fifth columns list sizes of eIF3c species or complexes formed. –, no complex formation.

(C) AUC analysis. The sedimentation coefficient (c(s)) distributions of reactions containing eIF3c-F (red), B (cyan) and A (blue), either alone (panel 1) or in the presence of eIF1 (panel 2), eIF5 (panel 3) or both (panel 4). Panel 5, eIF1 (pink), eIF5 (orange) and the mixture thereof (green). Proposed peak assignments are presented for each experiment.

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combined with four other elf1 substitutions in α1 (M5 mutation) (Reibarkh et al., 2009), we conclude that the synthetic Sui phenotype of the R53S,K56A substitution (Figure 6A) results from the combined loss of elf1 interaction with elf3c-NTD conferred by R53S and weakened 40S binding conferred by K56A (Figure 5B). As shown in Figure 6B (panel 2, row 2 versus 4), the elf1-R53S substitution also exacerbates the elevated UUG initiation caused by the elf2-β-S254Y variant (encoded by SUI3-2), previously attributed to increased GTP hydrolysis (Huang et al., 1997) and stabilizing the P_N conformation of Met-tRNA, at UUG codons (Martin-Marcos et al., 2014). Our findings imply that the defective stabilization of the closed/P_IN (l-1) of eIF1 are open for 40S binding (arrows). E51, showing CSP with eIF1; Y–WF; Y70, W74, F75, showing line broadening with eIF1 (Figures 1E and S3).

(Figure 3). eIF5 (dark green circle) is depicted as contacting both ends of eIF3c-NTD. eIF1 (brown circle) is bound to 3c1 via R53 and L96 (labeled). K60 and loop 1 (E) Locations of eIF2 ternary complex (blue drawing) (Lla´ cer et al., 2015), eIF5-CTD (dark green circle, this study) and 3c0 (aa 1–58) (orange line, this study) are superimposed onto the re-calculated cryo-EM structure, as shown in Figure 2B, right.

DISCUSSION

The results of NMR and complementary quantitative binding assays presented in this work revealed two distinct elf1 complexes formed with overlapping elf3c-NTD segments that appear to function at different stages of the initiation pathway. The C-terminal segment of the elf3c-NTD (fragment D59–163)
contains the core eIF1-binding unit 3c1 (aa 59–87) and the adja-
cent globular domain 3c2 (aa 105–159), which bind to a limited
surface on eIF1, including R53 and L96, in a manner compatible
with eIF1 binding to the 40S subunit (Figures 4A and 4C). We
have assigned two densities projecting from the main body of
eIF3 in the eIF1:eIF3:40S cryo-EM structure (Erzberger et al.,
2014) as 3c1, which contacts eIF1, and 3c2 interacting with
uS15 (Figure 2). In contrast, eIF3c fragment C36–87, containing
3c1 and the C-terminal half of 3c0, interacts with a broader sur-
face of eIF1 that includes R53 and L96 but additionally contains
residues surrounding the two 40S binding sites at the C terminus
of a1 and loop 1 (Figures 4C and 4B). Based on the Ssu−phenotype
of a mutation in Box6 (aa 51–60) within 3c0, we propose that
interaction of eIF1 with 3c0 occludes the 40S-binding surface in
eIF1 and thus facilitates eIF1 dissociation at the start codon—the
event diminished at UUG codons by the Ssu−mutation. This destabiliz-
ing effect is likely to be opposed in the scanning
PIC through eIF5-CTD binding to 3c0, which shifts eIF1 interac-
tion from eIF3c-NTD elements 3c0/3c1 to 3c1/3c2 and thereby
eliminates occlusion of the 40S binding surface on eIF1 by
PIC, eIF1 substitution L96P, which perturbs the interface with
3c1, reduces eIF1 binding to the eIF3c-NTD. By also impairing
eIF1 binding to the eIF5-CTD, L96P dramatically elevates UUG
initiation in the manner expected for destabilization of the scan-
ing complex (Martin-Marcos et al., 2013). eIF1 substitution
K56A, which weakens eIF1:40S interaction, we conclude that a
network of eIF1 interactions with the eIF3c-NTD, eIF5-CTD,
and 40S subunit cooperate to anchor eIF1 to the scanning
PIC and block initiation at non-AUG codons (Figure 5A). Based on
the cryo-EM model in Figure 2A, the role of 3c2 in anchoring
eIF1 to the PIC appears to be indirect. Consistently, mutations
altering Box12Sui+ (aa 111–120) within 3c2 can elevate UUG initi-
ation by either increasing or decreasing eIF1 retention in native
PICs (Karašková et al., 2012). This complexity may reflect dual
role of 3c2 in promoting eIF1 binding to segment 3c1 and
eIF5-CTD binding to 3c0 in the scanning PIC, while preventing

the more stable eIF1 complex formed with 3c0/3c1 on AUG recognition. In addition, by directly contacting 40S protein uS15/S13, 3c2 is likely to stabilize eIF1 binding to the scanning PIC (Figure 2A).

Recent studies reveal structural rearrangements within 43S/48S PICs between different steps of initiation (Hussain et al., 2014; Läcker et al., 2015; Simonetti et al., 2016). However, it is unclear exactly how start codon selection induces transition from the open to closed conformations of the PIC. Based on our findings and other work done using yeast S. cerevisiae as a model system, we propose that the eIF1A-NTT plays such a signaling role (Saini et al., 2010) (Figure 8B). During mRNA scanning, eIF1A-NTT interaction with the eIF5-CTD helps to retain eIF1 in the PIC (Luna et al., 2013) (Figure 8A). Thus, in addition to binding the 3c0 element and eIF1, the eIF5-CTD binds the basic eIF1A-NTT through a distinct acidic surface. This interaction is also important as it antagonizes eIF5-CTD interaction with the positively charged eIF2β-NTT, which would otherwise promote eIF1 release (Luna et al., 2012; Nandala et al., 2013). On Met-tRNAMet anticodon pairing to AUG, eIF1A-NTT binds to the codon:anticodon duplex in the P-site (Hussain et al., 2014) (Figure 8B). This releases the eIF5-CTD for interaction with eIF2β-NTT, which, in turn, disrupts eIF5-CTD interaction with both eIF1 (Luna et al., 2012) and 3c0 (Figures 8B and 8C). The 3c0 segment is now free to engage eIF1 and occlude its ribosome-binding surface, interfering with eIF1 re-association with the 40S subunit and thus allowing Met-tRNAMet to remain stably anchored in the Pn state (Figure 8D). These effects are expected to amplify the subtle distortion of eIF1 structure and perturbation of its 40S binding site that accompanies Met-tRNAMet isomerization to the Pn state (Hussain et al., 2014). In this way, 3c0 ensures irreversible eIF1 release from the decoding center in response to AUG recognition and subsequent closure of the ribosome structure and formation of the 40S initiation complex.

It is noteworthy that human eIF1 also binds eIF3c-NTD (Fletcher et al., 1999) and eIF5-CTD (Luna et al., 2012). While the eIF3c-NTD segments corresponding to 3c0 are shorter in animals and plants, they contain an acidic element similar to Box6, lying next to the conserved core element 3c1 (Boxed in Figure S1B). Moreover, eIF3c-NTD in animals and plants is predicted to form an α-helical structure, as found in yeast 3c2 (Figure 1C). Further work on the human and yeast systems is expected to reveal Eukarya-wide conservation of the MFC’s role in promoting scanning and AUG selection through the coordinated interactions of the eIF3c-NTD with eIF1, eIF5 and potentially other parts of eIF3 (Hussain et al., 2014; Simonetti et al., 2016; Valášek et al., 2003).

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Yeast Methods**

Isotopically labeled or unlabeled proteins were expressed in E. coli transformants carrying appropriate plasmids (Table S1) and purified as described in Supplemental Information. Yeast Saccharomyces cerevisiae strains used in this study are constructed as described in Supplemental Information and listed in Table S2. Standard yeast molecular biology methods including growth and β-galactosidase assays were used throughout (Lee et al., 2007) (see Supplemental Experimental Procedures for details).

**Biophysical Methods**

ITC, NMR spectroscopy, FA, and AUC are all performed as described in Supplemental Experimental Procedures. Detailed NMR data and structural statistics for eIF3c-B36-163 and eIF1 are summarized in Tables S3 and S4, respectively. We re-run integrative modeling prediction including the new information from the eIF3c-B NMR structure, with parameters and methods identical to those previously described (Erzberger et al., 2014).

**ACCESSION NUMBERS**

The accession numbers for the yeast eIF1 and eIF3c (aa. 36-163) data reported in this paper are PDB: 2rvh and 5H7U, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Results, eight figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.052.

**AUTHOR CONTRIBUTIONS**

E.O., R.E.L., T.N., P.M.-M., H.H., C.R.S., J.P.E., and K.A. designed and performed experiments and analyzed data. F.Z., H.A., and J.M. performed experiments and analyzed data. R.P., S.U., F.S., and T.U. analyzed data. C.M., I.H., E.P., H.Y., M.L.N., B.T., E.A., S.H., E.D., A.N., and P.G. performed experiments. R.E.L., A.G.H., G.W., and K.A. wrote the paper.

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