Mechanism and Regulation of Protein Synthesis in *Saccharomyces cerevisiae*

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ABSTRACT In this review, we provide an overview of protein synthesis in the yeast *Saccharomyces cerevisiae*. The mechanism of protein synthesis is well conserved between yeast and other eukaryotes, and molecular genetic studies in budding yeast have provided critical insights into the fundamental process of translation as well as its regulation. The review focuses on the initiation and elongation phases of protein synthesis with descriptions of the roles of translation initiation and elongation factors that assist the ribosome in binding the messenger RNA (mRNA), selecting the start codon, and synthesizing the polypeptide. We also examine mechanisms of translational control highlighting the mRNA cap-binding proteins and the regulation of *GCN4* and *CPA1* mRNAs.

KEYWORDS GCN4; translation elongation; translation initiation

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A rapidly dividing yeast cell growing on rich medium is estimated to synthesize nearly 13,000 proteins per second (von der Haar 2008), limited by the availability of ribosomes (Shah *et al.* 2013). The average cell contains nearly 200,000 ribosomes (Warner 1999; Firczuk *et al.* 2013) and 15,000–60,000 messenger RNA (mRNA) molecules (with ~1/3 encoding ribosomal proteins) (Warner 1999; Zenklusen *et al.* 2008). With levels ranging from 10^5 to 10^6 molecules per cell, translation elongation factors are among the most abundant proteins in the cell (Firczuk *et al.* 2013). Given the vast resources the yeast cell devotes to protein synthesis, a thorough understanding of protein synthesis is critical to understanding the biology of *Saccharomyces cerevisiae*. In addition to its critical role in synthesizing all of the proteins required for cell growth, the translation apparatus is also nimble and regulates both general and mRNA-specific protein synthesis in response to environmental cues.

The basic mechanism of translating the nucleotide code of mRNA into the amino acid sequence of a protein, as performed by the ribosome, is well conserved throughout evolution. The process of protein synthesis can be subdivided into four major steps: initiation, elongation, termination, and ribosome recycling. During translation initiation, the small (40S) ribosomal subunit binds the specific initiator methionyl (Met)-transfer RNA $(tRNA)_i^{Met}$ and an mRNA. The initiation step is completed when the small subunit selects a start codon and the large (60S) ribosomal subunit joins to form a functional ribosome. The elongation phase of protein synthesis refers to the codon-dependent addition of amino acids to the growing

polypeptide chain. Finally, the termination steps involve release of the completed polypeptide chain from the ribosome, and recycling refers to dissociation of the ribosome and deacylated tRNA from the mRNA.

The initiation steps of protein synthesis have undergone the greatest changes during evolution. Whereas bacterial ribosomes locate translation start sites in part through base-pairing interactions between the ribosomal RNA (rRNA) in the ribosome and sequences immediately 5′ of the initiation codon (Kozak 2005; Laursen *et al.* 2005), eukaryotic ribosomes bind to the mRNA near the 5′ cap and scan in a 3′ direction inspecting the mRNA for start codons (Hinnebusch 2011). This change in initiation mechanisms between bacteria and eukaryotes is associated with a large increase in the number and complexity of factors required to facilitate protein synthesis. The three bacterial translation factors, IF1, IF2, and IF3 (Laursen *et al.* 2005; Schmeing and Ramakrishnan 2009), are replaced in yeast by 11 factors (Table 1). In contrast to the vastly different factor requirements for translation initiation in yeast *vs.* bacteria, the elongation and termination factors are structurally and/or functionally conserved with one exception between yeast and bacteria. The elongation factor eEF3 appears to be uniquely required in yeast as it is found neither in bacteria nor in higher eukaryotes (Belfield and Tuite 1993).

Over the last ≥15 years, molecular and biochemical studies have provided remarkable insights into the process of translation and the factors that assist the ribosome in producing proteins. Whereas the identity of most of the eukaryotic translation factors was established by biochemical studies conducted between the 1960s and 1980s, molecular investigations in yeast have provided novel insights into the functions and structure–function properties of the factors. In this review, we will focus on the initiation and elongation steps of protein synthesis, the functions of the translation factors, and the translational regulatory schemes in yeast. Due to space limitations, we will restrict our descriptions to the predominant scanning mechanism of translation initiation, and we will not provide a detailed description of the yeast ribosome nor of the tRNAs and complementary tRNA synthetases required for high-fidelity protein synthesis.

Table 1 Translation initiation factors

Factor	Subunit	Gene	Systematic name	Length (AA)
eIF1		*SUI1*	YNL244c	108
eIF1A		*TIF11*	YMR260c	153
eIF2	α	*SUI2*	YJR007w	304
	β	*SUI3*	YPL237w	285
	γ	*GCD11*	YER025w	527
eIF2B	α	*GCN3*	YKR026c	305
	β	*GCD7*	YLR291c	381
	γ	*GCD1*	YOR260w	578
	δ	*GCD2*	YGR083c	651
	ε	*GCD6*	YDR211w	712
eIF3	a	*RPG1/TIF32*	YBR079c	964
	b	*PRT1*	YOR361c	763
	c	*NIP1*	YMR309c	812
	g	*TIF35*	YDR429c	274
	i	*TIF34*	YMR146c	347
	j	*HCR1*	YLR192c	265
eIF4A		*TIF1*	YKR059w	395
		TIF2	YJL138c	395
eIF4B		*TIF3/STM1*	YPR163c	436
eIF4E		*CDC33*	YOL139c	213
eIF4G		*TIF4631*	YGR162w	952
		TIF4632	YGL049c	914
eIF5		*TIF5*	YPR041w	405
eIF5B		*FUN12*	YAL035w	1002

Mechanism of Translation Initiation

The most complex step of protein synthesis is translation initiation. In addition to the 40S and 60S ribosomal subunits, Met-tRNA$_i^{Met}$ and 11 translation initiation factors consisting of 24 independent gene products (Table 1) are required to initiate translation on an mRNA. As detailed in the scheme in Figure 1, translation initiation factors function in an ordered fashion to assemble the 80S ribosomal complex that synthesizes proteins. First, the factor eIF2 binds GTP and Met-tRNA$_i^{Met}$ forming a ternary complex (TC) that associates with the 40S ribosome along with the factors eIF1, eIF1A, eIF3, and perhaps eIF5 to form the 43S preinitiation complex (PIC). The eIF4 family of factors including the 7-methylguanosine (m^{7}G) mRNA cap-binding protein eIF4E, the RNA helicase eIF4A, and the factors eIF4G and eIF4B, are thought to prepare the mRNA for binding to the 43S PIC to form a 48S PIC. Following binding near the 5′ end of the mRNA, the ribosomal complex scans down the mRNA in search of an AUG start codon. Selection of the translation start site is accompanied by completion of GTP hydrolysis by eIF2 and release of many of the initiation factors. The factor eIF5B, a second GTPase, promotes binding of the 60S subunit to form an 80S ribosome. Subsequent GTP hydrolysis by eIF5B leads to its release from the 80S monosome, which is poised to begin translation elongation.

mRNA features in translation initiation

In addition to translation factors, mRNA features also contribute to formation of a translating 80S ribosome. While the most important feature of an mRNA is the open reading frame (ORF), other parts of the mRNA have significant impacts on protein synthesis. Nearly all yeast proteins are initiated with methionine encoded by an AUG codon. In addition, in almost all cases, protein synthesis starts at the first AUG codon from the 5′ end of the mRNA. To date, only a few exceptions to these rules have been identified, and interestingly several of the exceptional mRNAs are subject to translational regulation or encode proteins that are targeted to more than one subcellular compartment (Hinnebusch 2011).

Start codons and context nucleotides: Translation initiation in yeast has generally been thought to be restricted to AUG codons. For example, when the AUG start codon of a *CYC7* reporter gene was replaced by any of the nine single nucleotide near cognate codons (one mismatch from AUG), protein

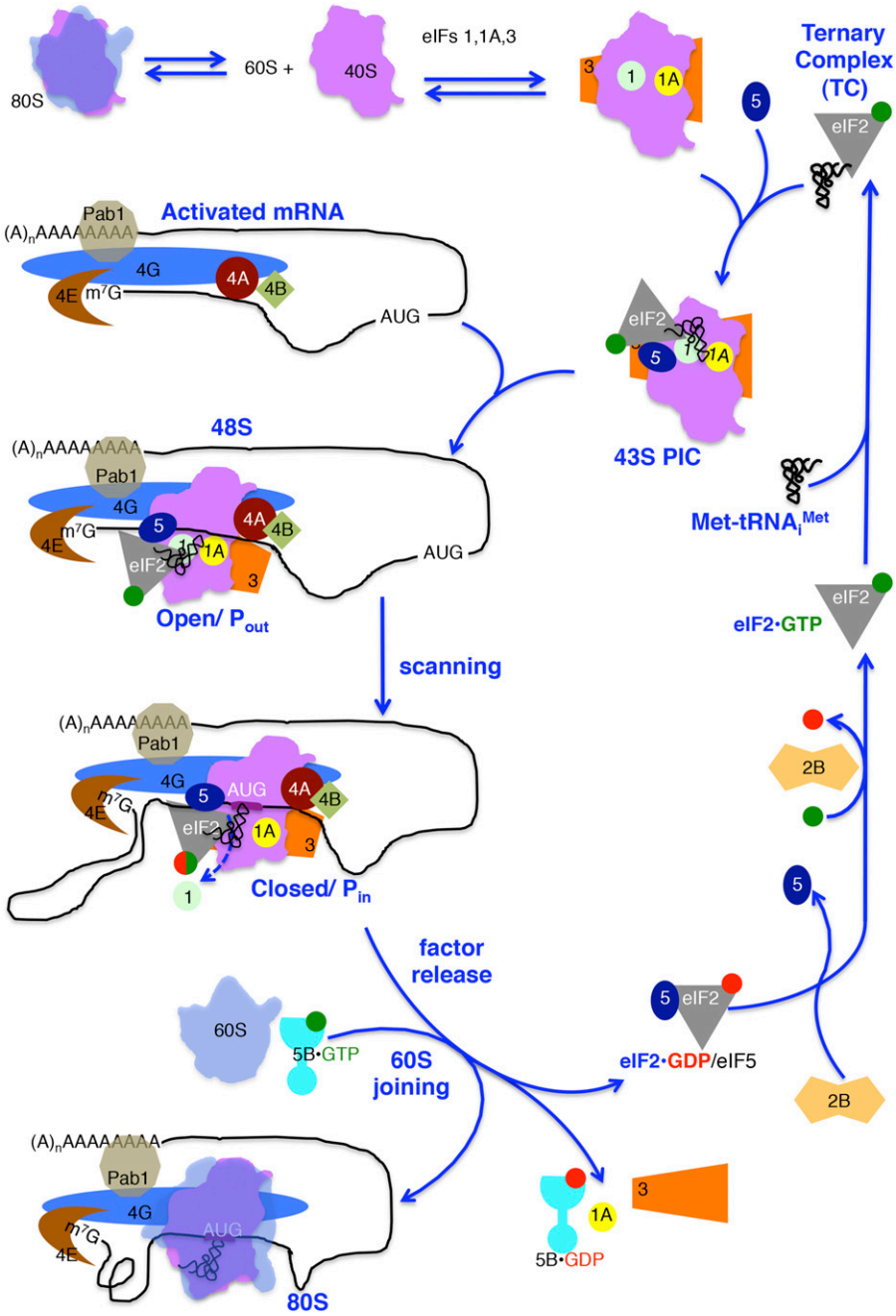

Figure 1 Pathway for yeast cytoplasmic translation initiation. Protein synthesis begins with the dissociation of ribosomal subunits and assembly of a 43S PIC. This is shown as consecutive steps in which eukaryotic initiation factors (eIFs) 1, 1A, and 3 bind to the 40S subunit first, followed by the eIF2–GTP (green circle)–Met-tRNA$_i^{Met}$ ternary complex (TC) and eIF5. The 43S PIC binds an activated mRNA near the 5′ cap, forming a 48S complex. Activated mRNAs bear eIF4E at the 5′ cap, Pab1 bound to the poly(A) tail, bridged by eIF4G to form a loop along with eIF4A and eIF4B. During scanning, the 43S PIC in an open conformation, where Met-tRNA$_i^{Met}$ is not fully base paired within the P site (P$_{out}$), moves in a 3′ direction along the 5′ UTR to the AUG codon. Either prior to or upon AUG recognition, GTP bound to TC is hydrolyzed to GDP+Pi (green and red hybrid circle), but Pi is not released until AUG recognition. Start codon selection is accompanied by release of eIF1, Pi loss from eIF2–GDP (red circle), release of eIF2 and eIF5, and reorganization of the 43S PIC to a closed state with Met-tRNA$_i^{Met}$ in the P$_{in}$ conformation and tightly bound to the complex. eIF5B–GTP promotes joining of the 60S subunit to the AUG-bound PIC. GTP hydrolysis and release of eIF5B–GDP and eIF1A forms the 80S complex with Met-tRNA$_i^{Met}$ bound in the P site and a vacant A site ready for the elongation phase of protein synthesis. Recycling of eIF2 is accomplished by eIF2B displacing eIF5 from eIF2–GDP and then facilitating nucleotide exchange on eIF2. Met-tRNA$_i^{Met}$ binds to eIF2–GTP reforming TC.

synthesis dropped to <0.5% of the AUG control (Clements *et al.* 1988). Likewise, all possible single nucleotide substitutions at the AUG start codon of a *HIS4-lacZ* reporter lowered expression to ≤2% of the AUG control (Donahue and Cigan 1988). However, the mRNAs encoding glycyl (Grs1) and alanyl (Ala1) tRNA synthetases initiate at both AUG and non-AUG codons (Chang and Wang 2004; Tang *et al.* 2004; Chen *et al.* 2008). Whereas the cytoplasmic synthetases initiate at an AUG start codon, the extended, mitochondrial enzymes initiate at upstream codons: UUG for Grs1 (Chen *et al.* 2008) and ACG for Ala1 (Tang *et al.* 2004).

Whole genome ribosomal profiling studies that mapped ribosome-protected mRNA fragments confirm the presence of ribosomes initiating translation at the non-AUG codons in the *GRS1* and *ALA1* mRNAs (Ingolia *et al.* 2009). While ribosome profiling studies have identified initiation at both AUG and non-AUG codons at short upstream open reading frames (uORFs) in the 5′ leader of yeast mRNAs, recent studies indicate that non-AUG codons rarely contribute to initiation of uORFs *in vivo* (Arribere and Gilbert 2013) and that sample processing procedures may have resulted in overrepresentation of some rarely used translation start sites (Gerashchenko and Gladyshev 2014).

The context of nucleotides around the start codon has been shown to be important in mammalian translation, but these flanking nucleotides appear to play a less significant role in

yeast. Kozak defined an optimal sequence for start codon selection in mammalian cells as CC(A/G)CCAUG(G/A) (Kozak 2002, 2005). Within this context, the nucleotides at positions −3 and +4 relative to the A of the AUG codon were shown to be most important. Substitutions of pyrimidines at these positions lead to scanning ribosomes bypassing the AUG codon and thus leaky scanning to a start codon further 3′ in the mRNA. Three studies in yeast revealed only modest impacts of flanking nucleotides on AUG start codon selection. Studying the *HIS4* gene, Cigan *et al.* (1988b) found that changing the preferred −3 A residue to C, G, or the least preferred U, reduced expression by only ∼3, 23, and 40%, respectively. Likewise, in studies of start codon context in derivatives of the *CYC1* gene, Baim and Sherman (1988) found that U or C at the −3 position resulted in roughly a twofold increase in leaky scanning as compared to when a purine was at this position. Finally, based on a high throughput screen of start codon context nucleotides, Dvir *et al.* (2013) reported a significant, but modest (<30%), impact of a −3 purine on reporter gene expression. At odds with these studies, flanking nucleotides have been shown to be important in selection of the non-AUG start codon on the *GRS1* mRNA (Chen *et al.* 2008) as well as in the selection of alternate AUG start codons on the *MOD5* and *CCA1* mRNAs (Werner *et al.* 1987; Slusher *et al.* 1991; Wolfe *et al.* 1994). In addition, the poor start codon context (C_{-3}GUAUG) of the *SUI1* gene encoding translation factor eIF1 in yeast impairs expression and enables autoregulation due to the role of eIF1 in start codon selection (Martin-Marcos *et al.* 2011).

mRNA leader length and secondary structure: In general, yeast mRNAs have short and rather unstructured 5′ UTRs (Kertesz *et al.* 2010). A genome-wide analysis of transcription start sites in yeast enabled the characterization of the 5′ UTR for ∼80% of yeast genes (Nagalakshmi *et al.* 2008). These data revealed an average 5′ UTR length of 50 nucleotides with <5% of mRNAs having an AUG codon within 10 nucleotides of the 5′ end. Interestingly, mRNAs with short leaders, <12–20 nt, are subject to nonsense-mediated mRNA decay (NMD), apparently due to ribosomes bypassing the first start codon and initiating at downstream, out-of-frame sites (Arribere and Gilbert 2013).Thus, an AUG codon too close to the 5′ end of an mRNA is not readily recognized by the translating ribosome. In contrast, expanding the length of the 5′ UTR of a luciferase reporter mRNA from 43 nt to >1700 nt had no significant effect on relative luciferase expression (Berthelot *et al.* 2004). Thus, scanning ribosomes are thought to possess a high level of processivity at least on the relatively unstructured mRNAs present in yeast.

According to the scanning model of translation, secondary structure in the 5′ UTR could block translation by at least two different mechanisms. Secondary structure near the 5′ cap of the mRNA could prevent ribosome association with the mRNA, whereas secondary structure further down the 5′ UTR could prevent ribosome scanning. Several studies in yeast have demonstrated that insertion of stem-loop structures in the 5′ UTR interferes with translation (Baim and Sherman 1988; Cigan *et al.* 1988b; Abastado *et al.* 1991; Vega Laso *et al.* 1993; Berthelot *et al.* 2004; Sen *et al.* 2015). As expected, more stable stem-loop structures are more deleterious than weaker stem loops; however, the impact of cap-proximal *vs.* more distal secondary structure varies in the different published reports.

mRNA cap and poly(A) tail: In addition to playing important roles in mRNA stability, the m^7G-(5′)ppp(5′)-N cap and poly(A) tail contribute to the translation of an mRNA. All genomically encoded mRNAs in yeast are capped at their 5′ end with m^7GTP. Capping occurs co-transcriptionally and is catalyzed by the enzymes Cet1, Ceg1, and Abd1, an RNA 5′ triphosphatase, a GTP-mRNA guanyltransferase, and an RNA guanine-7-methyltransferase, respectively (Shuman 2001). As described below, the cap structure is recognized by the translation factor eIF4E. At the 3′ end of the mRNA, the poly(A) tail is bound by the protein Pab1. Interestingly, Pab1 and eIF4E bind to separate sites on the translation factor eIF4G, and this binding has been shown to mediate mRNA circularization (Wells *et al.* 1998). The functional significance of mRNA circularization has not been resolved and it has been proposed to facilitate translation by helping shunt terminating ribosomes to the 5′ end of the same mRNA. Alternatively, mRNA circularization may serve a regulatory role to ensure translation of only intact (capped and polyadenylated) mRNAs.

Experiments in animal and plant cells demonstrated that the mRNA cap and poly(A) tail act synergistically to promote translation (Gallie 1991), and experiments using *in vitro* translation systems prepared from whole yeast cell extracts revealed a similar functional coupling between the cap and poly(A) tail (Iizuka *et al.* 1994; Tarun and Sachs 1995). Whereas the cap or poly(A) tail alone stimulated translation >20-fold compared to an mRNA lacking both features, the presence of both a cap and poly(A) tail enhanced translation an additional 2- to 8-fold (Tarun and Sachs 1995). As expected, cap-dependent translation is dependent on eIF4E, and poly(A) stimulation of translation is dependent on Pab1 (Otero *et al.* 1999).

Initiator methionyl-tRNA

The $tRNA_i^{Met}$ performs a unique role in protein synthesis. Distinct initiator and elongator tRNAs are used to incorporate methionine at the start codon *vs.* internal AUG codons in ORFs, respectively. Yeast contain four to five *IMT* genes encoding $tRNA_i^{Met}$ and five *EMT* genes encoding elongator methionyl-tRNA ($tRNA_e^{Met}$) (Astrom *et al.* 1993). Whereas both sets of tRNAs contain a 5′-CAU-3′ anticodon, nucleotide and post-transcriptional modification differences restrict the function of the tRNAs to initiation *vs.* elongation. Swapping nucleotides between $tRNA_i^{Met}$ and $tRNA_e^{Met}$ has provided insights into the critical determinants for $tRNA^{Met}$ function in initiation *vs.* elongation. Functionally important features of $tRNA_i^{Met}$ include: (1) A1:U72 and C3:G70 base pairs in the

acceptor stem; (2) A54 and A60 in the T loop; and (3) three G:C base pairs in the anticodon stem (positions 29–31:39–41). In addition, the nucleotide A54 and an O-ribosyl phosphate modification of A64 restrict $tRNA_i^{Met}$ from functioning in translation elongation (Figure 2) (von Pawel-Rammingen *et al.* 1992; Astrom *et al.* 1993; Astrom and Bystrom 1994).

Substitution of the A1:U72 base pair in $tRNA_i^{Met}$ by G1:C72, as found in $tRNA_e^{Met}$, impaired yeast cell growth (von Pawel-Rammingen *et al.* 1992; Astrom *et al.* 1993), binding of Met-$tRNA_i^{Met}$ to eIF2 (Farruggio *et al.* 1996; Kapp *et al.* 2006), and TC binding to the 40S ribosome (Kapp *et al.* 2006). Thus the identity of this base pair contributes both to TC formation and to later steps in the initiation pathway. Three consecutive G:C base pairs in the anticodon stem are important for $tRNA_i^{Met}$ function in bacteria (Varshney *et al.* 1993; Mandal *et al.* 1996), and their critical role in eukaryotes has only recently been revealed (Dong *et al.* 2014). Disrupting the G31:C39 base pair in the anticodon loop altered the accuracy of translation start site selection in a manner that was sensitive to the presence of the A54 residue in the T loop (Dong *et al.* 2014). As the C3:G70 base pair in the acceptor stem likewise contributed to the accuracy of translation start site selection (Dong *et al.* 2014), and all of these mutations affected the binding of the eIF2–GTP–Met-$tRNA_i^{Met}$ ternary complex to the 40S ribosome, albeit in distinct ways, these results indicate that conserved nucleotides of the $tRNA_i^{Met}$ contribute to the accuracy of translation start site selection.

An additional important determinant in $tRNA_i^{Met}$ is a 2-O-ribosyl phosphate modification of A64. In yeast strains lacking Rit1, the enzyme that catalyzes the modification, $tRNA_i^{Met}$ can function in translation elongation (Astrom and Bystrom 1994). Interestingly, domain III of EF-Tu, and by analogy of the eukaryotic elongation factor eEF1A, contacts the T loop of the bound tRNA (Nissen *et al.* 1995). The O-ribosyl phosphate modification of position 64 in the T loop would be expected to sterically interfere with Met-$tRNA_i^{Met}$ complex formation with eEF1A. Hence, it is thought that this modification restricts $tRNA_i^{Met}$ function to initiation and thus prevents competition for methionyl-tRNA between translation initiation and elongation. Consistent with this idea, deletion of *RIT1* exacerbated the growth defect in strains with mutations in eIF2 or $tRNA_i^{Met}$; and this growth defect was partially rescued by overexpression of $tRNA_i^{Met}$ and further exacerbated by overexpression of eEF1A (Astrom *et al.* 1999).

While many nucleotides in tRNAs are post-transcriptionally modified (for example by methylation, conversion to pseudouridine, etc.), it is noteworthy that $tRNA_i^{Met}$ appears to be especially sensitive to these modifications. Most of the 11 modifications of yeast $tRNA_i^{Met}$ are nonessential; however, loss of the m^1A58 modification destabilizes $tRNA_i^{Met}$ and impairs translation initiation (Anderson *et al.* 1998, 2000). The Gcd10/Gcd14 complex catalyzes methylation of A58, and inactivation of *GCD10* or *GCD14* results in turnover of $tRNA_i^{Met}$ by the Trf4/Rrp6 pathway (Kadaba *et al.* 2004).

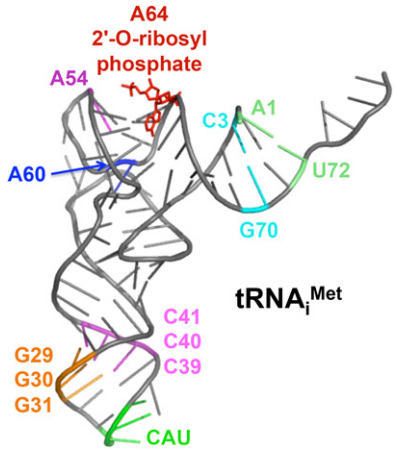

Figure 2 $tRNA_i^{Met}$ features important for translation initiation. Features that enhance $tRNA_i^{Met}$ function in initiation or restrict it from functioning in elongation are highlighted on the tertiary structure of yeast $tRNA_i^{Met}$ (pdb 1YFG). Highlighted residues include A1:U72 and C3:G70 base pairs in the acceptor stem, residues A54 and A60 in the T loop, and a 2′-O-ribosyl phosphate modification on residue A64. Three consecutive G:C base pairs in the anticodon loop are important for the accuracy of start site selection. The anticodon 5′-CAU-3′ is depicted in green. Structure was generated using the PyMol Molecular Graphics System (version 1.7.6.6, Schrödinger).

Ternary complex formation

The translation factor eIF2 is responsible for binding Met-$tRNA_i^{Met}$ to the 40S ribosome. A TC is formed between Met-$tRNA_i^{Met}$ and the GTP-bound form of eIF2. The eIF2 is a heterotrimeric complex consisting of α (Sui2), β (Sui3), and γ (Gcd11) subunits. The yeast eIF2α (*SUI2*) and eIF2β (*SUI3*) genes were first discovered by Donahue *et al.* (1988) in a screen for mutations that suppress the histidine auxotrophy of *his4-303* strains in which an AUU codon is substituted for the initiating AUG codon of the *HIS4* gene. Spontaneous mutations in unlinked *sui* (suppressors of initiator codon) genes, including *SUI2* (Cigan *et al.* 1989) and *SUI3* (Donahue *et al.* 1988), enable translation to initiate at an in-frame UUG codon that normally encodes Leu as the third residue in *HIS4* (Donahue *et al.* 1988). As discussed below, analysis of Sui^- mutations in eIF2 and other translation factors has provided insights into the mechanism of start codon selection during translation initiation.

Structures of yeast (Dhaliwal and Hoffman 2003; Hussain *et al.* 2014; Llacer *et al.* 2015), archaeal (Schmitt *et al.* 2012), and mammalian eIF2α (Ito *et al.* 2004) revealed that the protein consists of three domains: an N-terminal OB-fold domain and a central α-helical domain that are connected through a flexible linker to a C-terminal α/β domain that binds to eIF2γ (Figure 3A) (Schmitt *et al.* 2012; Hussain *et al.* 2014; Llacer *et al.* 2015). A key mode of translational control in yeast and other eukaryotes involves phosphorylation of eIF2α. The yeast kinase Gcn2, which is conserved in all eukaryotes, phosphorylates the conserved Ser51 residue in a mobile loop of the OB-fold domain (Dever *et al.* 1992).

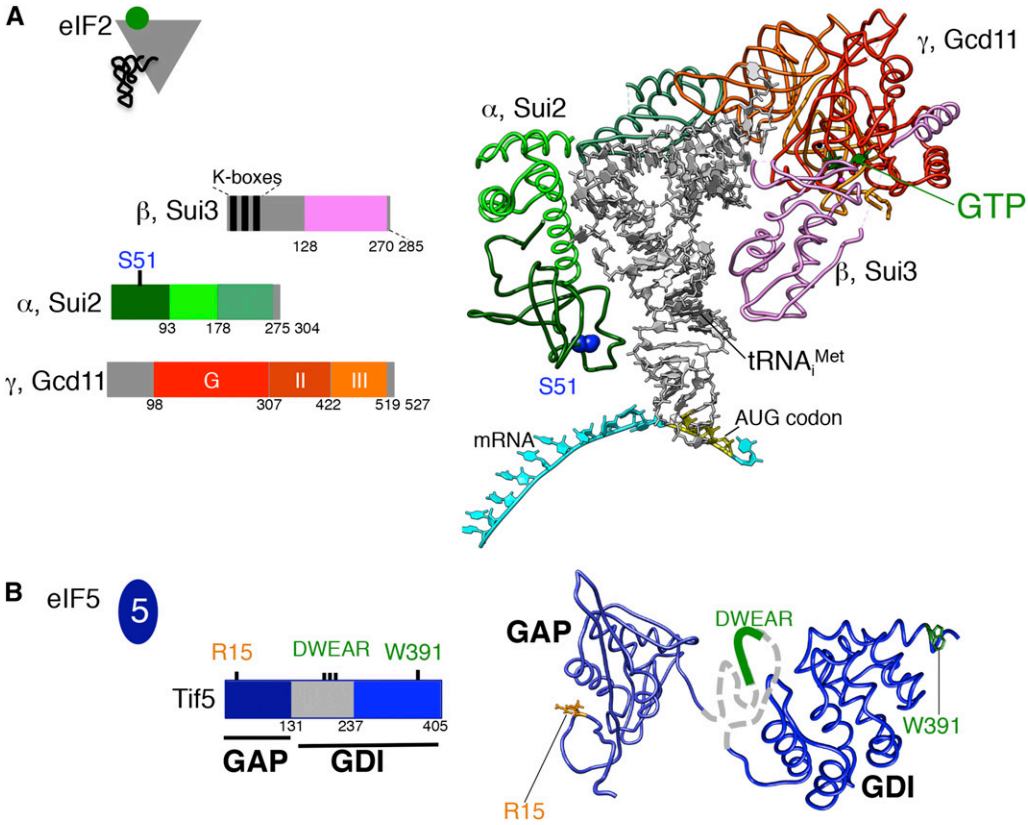

Figure 3 Schematic and structural models of eIF2 and eIF5. (A) Structural model of the eIF2–GTP–Met-tRNA$_i^{Met}$ ternary complex bound to an mRNA AUG codon (right) and cartoons depicting the eIF2 α, β, and γ subunit structural domains (left) using the same color schemes. The structural model is adapted from the structure of the yeast 48S complex (pdb 3JAP) with the 40S ribosome and other initiation factors omitted for clarity (Llacer *et al.* 2015). The eIF2α residue Ser51 (blue), GTP analog (green), Met-tRNA$_i^{Met}$ (gray), and mRNA (cyan) with AUG codon (yellow) are indicated. (B) eIF5 domains and activities (left) and structural models (right) for the human GAP domain bearing R15 (pdb 2E9H) and the yeast CTD bearing W391 (pdb 2FUL) (Wei *et al.* 2006). Structures were drawn using Chimera software (University of California, San Francisco, UCSF).

Whereas translation of the *GCN4* mRNA in yeast is typically repressed by the presence of uORFs in the mRNA leader, phosphorylation of eIF2α enables ribosomes to bypass the inhibitory uORFs and initiate translation at the *GCN4* ORF (reviewed in Hinnebusch 2005). Yeast lacking *GCN2* are unable to grow under amino acid starvation conditions due to the failure to derepress *GCN4* expression (Wek *et al.* 1989, 1990). Gcd$^-$ mutations, including mutations that impair eIF2 function (Williams *et al.* 1989), derepress *GCN4* expression in the absence of *GCN2*, mimicking the effect of eIF2 phosphorylation in reducing TC assembly (Hinnebusch 2005).

The C-terminal half of yeast eIF2β shows significant sequence homology to archaeal aIF2β and consists of three elements: an N-terminal α-helix, a central helix–turn–helix domain, and a C-terminal zinc-binding domain (Figure 3A) (reviewed in Schmitt *et al.* 2010). The N-terminal α-helix, which is unstructured in the free form of aIF2β, binds to the backside of the aIF2γ GTP-binding (G) domain in the aIF2 complex (Sokabe *et al.* 2006; Yatime *et al.* 2007). Point mutations in this helix of yeast eIF2β, as well as in the docking site on yeast eIF2γ, disrupt eIF2 complex formation and confer Gcd$^-$ and Sui$^-$ phenotypes (Hashimoto *et al.* 2002; Borck *et al.* 2012). In the archaeal aIF2 complex, the C-terminal zinc-binding domain of aIF2β packs against the central α–β domain (Sokabe *et al.* 2006; Yatime *et al.* 2007). While the function of the zinc-binding domain has not been resolved, removal of this domain impairs RNA binding to isolated yeast eIF2β (Laurino *et al.* 1999) and confers a dominant Gcd$^-$ and recessive lethal phenotype (Castilho-Valavicius *et al.* 1992), while point mutations confer a dominant Sui$^-$ phenotype (Donahue *et al.* 1988; Castilho-Valavicius *et al.* 1992).

The N-terminal half of eIF2β is not present in the archaeal protein. Key features of this portion of eIF2β are three elements referred to as K-boxes K1, K2, and K3, each containing seven Lys residues and one Ser or Thr residue. Whereas deletion of any single or two K-boxes does not affect cell viability, removal of all three K-boxes is lethal (Asano *et al.* 1999; Laurino *et al.* 1999). Consistent with these findings, substituting Ala residues in place of the K3 Lys residues in a *SUI3* allele lacking K1 and K2 was also lethal. In contrast, substituting Arg residues in place of the K3 Lys residues in the same allele was viable and had no impact on cell growth (Laurino *et al.* 1999). Thus, the positively charged character of at least one K-box is required for cell viability. Biochemical analyses revealed that removal of the K-boxes impairs mRNA, but not Met-tRNA$_i^{Met}$, binding to isolated eIF2 complexes (Laurino *et al.* 1999). Moreover, mutating the K-boxes in eIF2β impairs the binding of isolated eIF2β, as well as the eIF2 complex, with both the eIF2 GTPase stimulatory factor eIF5 and the catalytic ε-subunit of the eIF2 guanine-nucleotide exchange factor (GEF) eIF2B (Asano *et al.* 1999). A bipartite element consisting of acidic and aromatic amino acids is conserved at the C termini of eIF5 and eIF2Bε and mediates the K-box-dependent interaction with the N terminus of eIF2β (Asano *et al.* 1999).

The γ-subunit of eIF2, encoded by *GCD11*, was first identified based on the Gcd$^-$ phenotype of several mutants (Hannig *et al.* 1993). Interestingly, mutations in eIF2γ were independently isolated in a screen for Sui$^-$ mutants (Huang *et al.* 1997). Consistent with these findings, the *GCD11-R510H* mutant, originally isolated based on its ability to derepress *GCN4* expression, also confers a Sui$^-$ phenotype (Dorris *et al.* 1995). The eIF2γ protein consists of three domains: an N-terminal GTP binding domain and β-barrel domains II and III (Figure 3A). Based on structural studies of the archaeal and yeast complexes, eIF2γ is the keystone of the eIF2 complex with separate docking sites for the eIF2α and eIF2β subunits (Schmitt *et al.* 2012; Hussain *et al.* 2014; Llacer *et al.* 2015). The incorporation of eIF2γ in the eIF2 complex is dependent on the apparently eIF2-specific chaperone Cdc123 (Perzlmaier *et al.* 2013). The amino acid sequence and structure of eIF2γ and aIF2γ show striking similarity to elongation factor EF-Tu from bacteria (Hannig *et al.* 1993; Schmitt *et al.* 2002; Roll-Mecak *et al.* 2004). Whereas EF-Tu binds diverse aminoacyl-tRNAs (aa-tRNAs) to the ribosomal A site, eIF2 specifically binds Met-tRNA$_i^{Met}$ to the ribosomal P site. The structure of Phe-tRNA bound to EF-Tu revealed that the amino acid and acceptor stem of the tRNA bind in a pocket formed between the G domain and domain II. Supporting the notion that eIF2γ uses a similar pocket for Met-tRNA$_i^{Met}$ binding, the slow-growth phenotype of the *gcd11-Y142H* mutant, which alters a residue in the proposed Met-tRNA$_i^{Met}$ binding pocket, was partially suppressed by overexpression of tRNA$_i^{Met}$, and purified eIF2 complexes containing the mutant eIF2γ subunit showed defects in Met-tRNA$_i^{Met}$ binding (Dorris *et al.* 1995; Erickson and Hannig 1996; Shin *et al.* 2011). Thus, at least the acceptor stem and amino acid binding site appears to be shared between eIF2 and EF-Tu. In contrast, a contact between the body of the tRNA, especially the T stem, and domain III of EF-Tu is apparently not conserved in eIF2γ (Nissen *et al.* 1995; Sanderson and Uhlenbeck 2007a,b; Shin *et al.* 2011; Schmitt *et al.* 2012; Hussain *et al.* 2014; Llacer *et al.* 2015). Instead, hydroxyl radical probing experiments and cryo-EM structures of 48S PICs indicated that domain III of eIF2γ projects toward, but does not contact, helix h44 on the subunit interface surface of the 40S ribosomal subunit (Shin *et al.* 2011).

Purified yeast eIF2 binds either GTP (K_d ~1.7 μM) or GDP (K_d ~0.02 μM) (Kapp and Lorsch 2004a). As for a number of G proteins, this 100-fold higher affinity for GDP relative to GTP introduces the requirement for eIF2B to recycle eIF2–GDP complexes to the functional eIF2–GTP form. Whereas eIF2–GTP complexes bind Met-tRNA$_i^{Met}$ (K_d ~9 nM) to form a ternary complex, eIF2–GDP binary complexes are defective for Met-tRNA$_i^{Met}$ binding (K_d ~150 nM) (Kapp and Lorsch 2004a). Thermodynamic coupling between GTP and Met-tRNA$_i^{Met}$ binding to eIF2 results in a 10-fold increase in GTP binding affinity in the presence of Met-tRNA$_i^{Met}$ (GTP K_d ~0.2 nM) (Kapp and Lorsch 2004a). Consistent with this biochemical result, the slow-growth phenotype of an eIF2γ-K250R mutation, which impairs GDP and GTP binding to eIF2, is suppressed by overexpression of tRNA$_i^{Met}$ (Erickson and Hannig 1996).

As noted above, conserved features of tRNA$_i^{Met}$ contribute to ternary complex formation. However, the Met on Met-tRNA$_i^{Met}$ appears to be the most important determinant for TC formation. Deacylation of Met-tRNA$_i^{Met}$ decreases its affinity for binding to eIF2 by >10-fold (K_d ~130 nM), comparable to the binding of Met-tRNA$_i^{Met}$ to eIF2–GDP (Kapp and Lorsch 2004a). It is postulated that the thermodynamic coupling between eIF2 and the methionine residue on Met-tRNA$_i^{Met}$ serves to ensure that translation initiates exclusively with Met.

Interestingly, the eIF2γ-K250R mutation in addition to weakening GDP binding enables cell survival in the absence of eIF2α (Erickson *et al.* 2001). The growth of the *gcd11-K250R sui2Δ* strain is further enhanced by overexpression of tRNA$_i^{Met}$ (*IMT4*) and by overexpression of *gcd11-K250R* and *SUI3* (Erickson *et al.* 2001). As weakening GDP binding to eIF2 enables elimination of eIF2α, these findings suggest that eIF2α plays a role in stimulating the eIF2B-catalyzed guanine nucleotide exchange on eIF2.

43S PIC formation

Binding of the eIF2 TC to the 40S subunit is facilitated by the factors eIF1 and eIF1A that bind directly to the 40S ribosome (Figure 1). The factor eIF1, encoded by *SUI1*, is a small (108 amino acid) protein that, based on structures of the yeast or the analogous *Tetrahymena* factor, binds to the platform of the 40S subunit near the P site (Figure 4, A and B) (Rabl *et al.* 2011; Hussain *et al.* 2014). The factor eIF1A, encoded by *TIF11*, is homologous to the bacterial factor IF1 (Battiste *et al.* 2000; Choi *et al.* 2000; Olsen *et al.* 2003; Hussain *et al.* 2014). Like IF1, eIF1A binds to the ribosomal A site and likely functions, in part, to prevent Met-tRNA$_i^{Met}$ binding in the A site (Figure 4, A and B) (Carter *et al.* 2001; Hussain *et al.* 2014). Cryoelectron microscopy of the yeast 40S ribosome has revealed conformational changes accompanying the binding of eIF1 and eIF1A (Passmore *et al.* 2007; Hussain *et al.* 2014). In the absence of factors, the "latch" of the mRNA entry channel, composed of 18S rRNA helices h34 in the head and h18 in the body of the 40S subunit, is closed. Binding of eIF1 and eIF1A to the 40S subunit is accompanied by rotation of the head of the subunit (Hussain *et al.* 2014), perhaps providing access for the Met-tRNA$_i^{Met}$ and TC and by weakening of the latch interactions to enable binding of mRNA (Passmore *et al.* 2007; Hussain *et al.* 2014). Interestingly, when only eIF1A is bound to the 40S subunit, the density corresponding to the latch is stronger than that observed in the apo-40S structure. As described below, this so-called "closed" complex in the absence of eIF1 is thought to be associated with selection of the translation start codon.

Yeast eIF1 is composed of an ~20-residue unstructured N-terminal tail (NTT) followed by an ~88-residue folded α/β core (Figure 4A) (Reibarkh *et al.* 2008). The α/β core of eIF1 resembles similar domains in eIF2β, the N terminus of eIF5 (Figure 3C), and several ribosomal proteins (Reibarkh

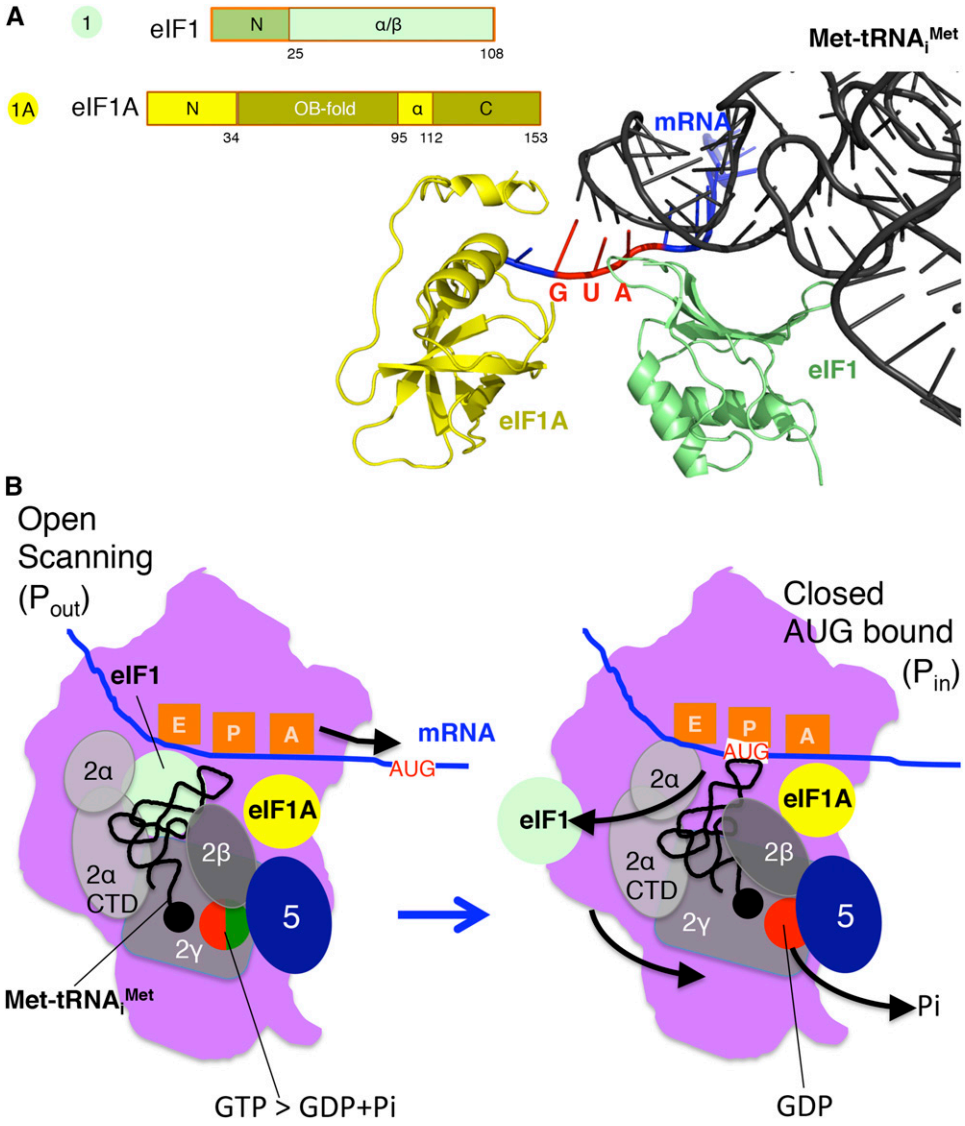

Figure 4 Schematic and structural models of eIF1, eIF1A, and AUG codon selection. (A) Structural model (right) and schematics (left) of eIF1 (green) and eIF1A (yellow) bound to the 48S PIC (pdb 3JAP) along with Met-tRNA$_i^{Met}$ (black) and mRNA (blue, AUG codon in red), but other factors and the ribosome are removed for clarity. Structure was generated using the PyMol Molecular Graphics System (version 1.7.6.6, Schrödinger). (B) Cartoon showing approximate positions of eIFs 1 and 1A with TC and eIF5 in the open scanning conformation (left) with Met-tRNA$_i^{Met}$ not fully engaged in the P site (P$_{out}$), and factor movements (black arrows) induced by AUG codon recognition (right) and the transition to the closed complex (P$_{in}$) signaled by movement of eIF1 that triggers Pi release prior to eIF2–GDP–eIF5 release from the PIC.

et al. 2008). In addition to binding the ribosome and regulating TC binding, eIF1 directly contacts eIF2β, the C-terminal domain of eIF5, and eIF3c. As these contacts have been mapped to distinct regions of eIF1, it is thought that eIF1 can simultaneously bind all three factors, and consistent with this idea, eIF1 can be found in a multifactor complex (MFC) with the eIF2 TC, eIF3, and eIF5 (Asano *et al.* 2000).

The 153-residue yeast eIF1A consists of a central OB-fold domain that resembles the bacterial factor IF1 (see Fekete *et al.* 2005). The core of eIF1A is buttressed on its C-terminal side by a helical region consisting of a long α2 helix and a short 3_{10} helix. In addition, the factor has long unstructured N(~25-residues)- and C(~34 residue)-terminal tails (Figure 4A). In the 43S complex, the C-terminal tail (CTT) of eIF1A crosses through the P site (Hussain *et al.* 2014; Zhang *et al.* 2015). The Met-tRNA$_i^{Met}$ is thus prevented from fully engaging the P site (P$_{in}$ state) and instead is thought to be in a P$_{out}$ state that is more conducive to scanning (Hinnebusch 2011, 2014). As discussed below, the CTT of eIF1A also interacts with the N-terminal domain (NTD) of eIF5 and with domain IV of eIF5B in subsequent steps of the initiation pathway.

Despite interacting with distinct sites, binding of eIF1 and eIF1A to the 40S subunit is thermodynamically coupled (Maag and Lorsch 2003). Moreover, both factors are required to achieve stable binding of the eIF2 TC *in vitro* (Algire *et al.* 2002). Consistent with these findings, mutations in the eIF1 core domain or in eIF1A that weaken their binding to the 40S ribosome likewise decrease the rate of TC binding *in vitro* and confer Gcd$^-$ phenotypes *in vivo* (Fekete *et al.* 2005; Cheung *et al.* 2007).

Whereas eIF1 and eIF1A are critical for TC binding to the 40S in the reconstituted yeast *in vitro* translation system, the factor eIF3 has been reported to stabilize TC binding by only approximately twofold (Kapp and Lorsch 2004b). Yeast eIF3 is composed of five essential subunits (a/Tif32, b/Prt1, c/Nip1, i/Tif34, and g/Tif35) and one nonessential subunit (j/Hcr1) (Figure 5) (note that the unusual nomenclature of the yeast eIF3 subunits is due to the presence of additional

subunits in the mammalian factor that are not present in yeast eIF3). In addition, eIF5 (Tif5) purifies stoichiometrically with tagged forms of eIF3 from yeast (Phan *et al.* 1998). Extensive mapping studies of protein–protein interactions have provided insights into the structure of eIF3 and its interaction with other factors (reviewed in Valasek 2012). The eIF3b/Prt1 is thought to form the primary scaffold of the multisubunit complex. The N terminus of eIF3b/Prt1 contains an RNA recognition motif (RRM) that serves as a protein–protein interaction site for eIF3j/Hcr1 as well as for the C-terminal part of eIF3a/Tif32, which resembles eIF3j/Hcr1. The central part of eIF3b/Prt1 binds to eIF3c/Nip1, and the eIF3i/Tif34 and eIF3g/Tif35 subunits bind cooperatively to the C-terminal portion of eIF3b/Prt1. Finally, the N-terminal portion of the eIF3c/Nip1 subunit binds directly to eIF1 and to eIF5, which in turn binds the eIF2 TC (Figure 5). Thus, eIF3 and in particular the eIF3b subunit plays a central role in assembly of the 43S PIC. In contrast to eIF1, eIF1A, and the TC, which bind to the intersubunit face of the 40S subunit, cryo-EM studies revealed that the core of yeast eIF3 binds to the solvent-exposed face of the 40S with arm-like projections, including the PCI domains of eIF3a and eIF3c, that bind near the mRNA entry channel reaching around to the intersubunit face of the 40S (Erzberger *et al.* 2014; Aylett *et al.* 2015; Llacer *et al.* 2015). Consistent with this model of the eIF3–40S complex, yeast eIF3 subunits have been found to interact with 18S rRNA and ribosomal proteins on the solvent-exposed side of the 40S subunit (Valasek *et al.* 2003; Kouba *et al.* 2012a,b). The C terminus of eIF3a/Tif32 was shown to bind to a region of 18S rRNA encompassing helices h16–h18, and in two-hybrid assays this same portion of eIF3a/Tif32 bound to ribosomal proteins Rps2 and Rps3. These interactions place eIF3a near the mRNA entry channel of the 40S subunit.

Inactivation of a temperature-sensitive eIF3b/Prt1 mutant (Phan *et al.* 1998; Nielsen *et al.* 2004) or depletion of eIF3c/Nip1 (Phan *et al.* 1998; Valasek *et al.* 2004) impairs general translation *in vivo* and *in vitro*. Moreover, extracts from these strains exhibit a defect in binding Met-tRNA$_i^{Met}$ to 40S subunits that was rescued by adding back the eIF3 complex (Phan *et al.* 1998). The Met-tRNA$_i^{Met}$ and mRNA binding defects in extracts from the *prt1-1* strain were also rescued by addition of an eIF3abc, but not an eIF3big, partial complex (Phan *et al.* 2001). It is noteworthy that the factors eIF5, eIF1, and eIF3j/Hcr1 co-purified with the eIF3abc partial complex, raising the possibility that these latter factors contributed to the complementing activity. These results uncover a functional specialization within the eIF3 complex and they also support previous studies in mammalian systems, indicating that Met-tRNA$_i^{Met}$ binding to the 40S subunit is a prerequisite for the ribosome to bind to an mRNA (see Hinnebusch 2000).

In addition to the sequential assembly of the 43S complex with eIF1 and eIF1A binding to the 40S subunit prior to association of the TC, an *en masse* assembly of the 43S complex has also been proposed. A MFC consisting of eIF1, eIF2, eIF3, and eIF5 plus Met-tRNA$_i^{Met}$ has been isolated from cells, and in crude cell extracts the MFC can be separated from the 40S ribosome (Asano *et al.* 2000). It has been proposed that preassembly of the MFC facilitates proper binding of Met-tRNA$_i^{Met}$ to the 40S subunit. Consistent with this hypothesis, the protein–protein interactions required for MFC integrity, including the binding of eIF1 to eIF3 (Singh *et al.* 2004; Valasek *et al.* 2004), eIF5 to eIF1, eIF2β, and eIF3c (Singh *et al.* 2004, 2005; Valasek *et al.* 2004; Yamamoto *et al.* 2005) and eIF2 to eIF3a (Valasek *et al.* 2002; Nielsen *et al.* 2004), are also important for protein synthesis *in vivo*, and mutations that disrupt eIF3c interaction with eIF1 or eIF5 confer Sui$^-$ phenotypes (Valasek *et al.* 2004). While the *in vivo* data support the idea that MFC integrity is important for translation initiation, additional experiments are needed to define the function of the MFC. In particular, it is important to determine whether the MFC binds to the 40S *en masse* and serves as a more efficient means to bind Met-tRNA$_i^{Met}$ to the 40S subunit. Alternatively, it has been proposed that the MFC might serve as a depot for the initiation factors that are critical for stable binding of Met-tRNA$_i^{Met}$ to the 40S (Aitken and Lorsch 2012).

mRNA recruitment of the 43S PIC

The 5′ cap and 3′ poly(A) tail of mRNAs serve as binding sites for eIF4E and the poly(A) binding protein Pab1, respectively, that act synergistically to assist in recruiting additional translation initiation factors including eIF4G and the 43S PIC to near the 5′ end (Figure 1 and Figure 6A) (Tarun and Sachs 1995; Preiss and Hentze 1998). Yeast mRNA 5′ leader sequences are of variable length and can contain secondary structures that impede 43S binding and scanning to AUG initiation codons. As a consequence ATP-dependent RNA helicases such as eIF4A and Ded1 are recruited. Our understanding of the roles of factors in these key steps is outlined below.

eIF4E and mRNA 5′ cap recognition: eIF4E has a compact single structural domain containing a cleft for mRNA 5′ cap binding. A pair of tryptophan residues (W58 and W104) form a 5′ cap-trapping sandwich (Altmann *et al.* 1988; Gross *et al.* 2003). A central region of eIF4G (eIF4G1$^{393–460}$) binds to eIF4E on the opposite face to the 5′ cap interaction (Figure 6B). The eIF4E-4G binding interface overlaps with the surface important for binding 4E-binding proteins (4E-BPs) Caf20 and Eap1 that inhibit eIF4F assembly by competing with eIF4G to bind eIF4E (Altmann *et al.* 1997; Ptushkina *et al.* 1998; Cosentino *et al.* 2000).

While all mRNAs are capped and can bind eIF4E, they likely have differential affinity for eIF4E. Single-molecule FRET measurements with short model mRNAs calculated eIF4E affinity of 90 nM for an unstructured capped mRNA that was enhanced approximately fourfold by the presence of a modest secondary structure element 12 nt from the cap (O'Leary *et al.* 2013). Similarly, binding eIF4E to eIF4G enhances affinity for capped mRNA to ∼15–20 nM (Mitchell *et al.* 2010; O'Leary *et al.* 2013). Analysis of mRNAs bound to eIF4E in cells generally mirrors their levels in total RNA;

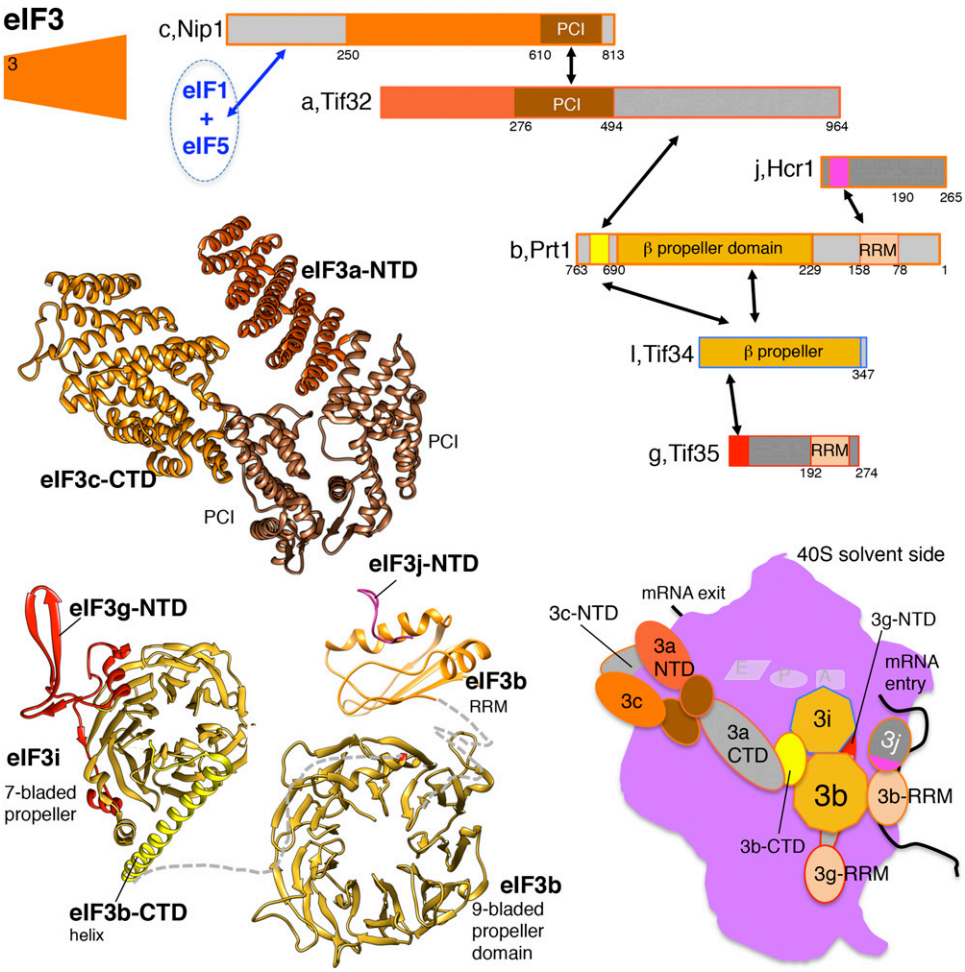

Figure 5 Schematic and structural models of eIF3. Schematics depict the eIF3 subunit organization and indicate major structural domains and protein–protein interactions (black arrows) within the eIF3 core complex. Structural models depicting these interactions are shown using Chimera software (UCSF) using pdb coordinates 4U1C (eIF3a/c), 4U1E (eIF3b-CTD/eIF3i/eIF3g-NTD), 4U1F (eIF3b β-propeller domain) (Erzberger *et al.* 2014), and 2KRB (eIF3b RRM/eIF3j peptide) (Elantak *et al.* 2010). The cartoon depicting eIF3 binding to the 40S solvent-exposed surface is based on cryo-EM reconstructions (Erzberger *et al.* 2014; Aylett *et al.* 2015; Llacer *et al.* 2015). The same color scheme is used for consistency between images.

however, >1000 mRNAs were enriched and a similar number were relatively depleted in eIF4E binding experiments (Costello *et al.* 2015). A simple conclusion is that eIF4E does not bind equally to all mRNAs *in vivo*.

The eIF4E-G113D (*cdc33-1*) temperature-sensitive (*ts*) mutant causes cell cycle arrest in G1 that was attributed to reduced translation efficiency of the *CLN3* mRNA (Danaie *et al.* 1999). When Cln3 levels are elevated, *cdc33-1* cells arrest randomly in the cell cycle rather than at G1, suggesting that translation of *CLN3* mRNA, which bears an upstream ORF that contributes to its translational control (Polymenis and Schmidt 1997), becomes rate limiting for passage through G1 upon eIF4E inactivation. As *cdc33* cells retain some protein synthesis activity at nonpermissive temperatures, the mutated factor may retain partial function (Altmann and Trachsel 1989). However, cell fluorescence studies suggest that when eIF4E is inactivated, the nuclear cap-binding protein Sto1 remains bound to mRNAs following their exit from the nucleus (Garre *et al.* 2012) and there is evidence that the nuclear cap complex, composed of Sto1 and Cbc2, may promote continued translation initiation, but with lower efficiency (Fortes *et al.* 2000).

eIF4G and Pab1 bring mRNA ends together: eIF4G binds several translational components including eIF4E, eIF4A, Pab1, eIF5, 40S ribosomes, and mRNA (Figure 6, A–C). Although the two yeast eIF4G proteins (Table 1) are smaller than their mammalian counterparts, they share many of the same domains and interactions. Yeast eIF4G1 (Tif4631) and eIF4G2 (Tif4632) are 51% identical and appear to be functionally redundant. eIF4G1 expression levels are higher than eIF4G2 and poor growth phenotypes associated with *tif4631*-Δ can be overcome by expressing eIF4G2 at higher levels from the stronger *TIF4631* promoter (Clarkson *et al.* 2010).

eIF4G1 and 4G2 share an eIF4E interaction domain (eIF4G1$^{393–460}$). This fragment adopts an α-helical structure on binding eIF4E (Figure 6B) (Gross *et al.* 2003) and bears a core conserved "YxxxFLL" motif that is critical for binding eIF4E. The *tif4631-459* allele with both Leu residues (positions 457 and 458) mutated to alanine has greatly weakened eIF4E interaction and confers a *ts* phenotype in strains lacking eIF4G2, demonstrating that the eIF4E–eIF4G interaction is critical *in vivo* (Tarun and Sachs 1997). *In vitro* studies indicate eIF4G–eIF4E interactions both promote translation of mRNAs bearing a 5′ cap and suppress translation of uncapped mRNAs (Tarun and Sachs 1997; Mitchell *et al.* 2010).

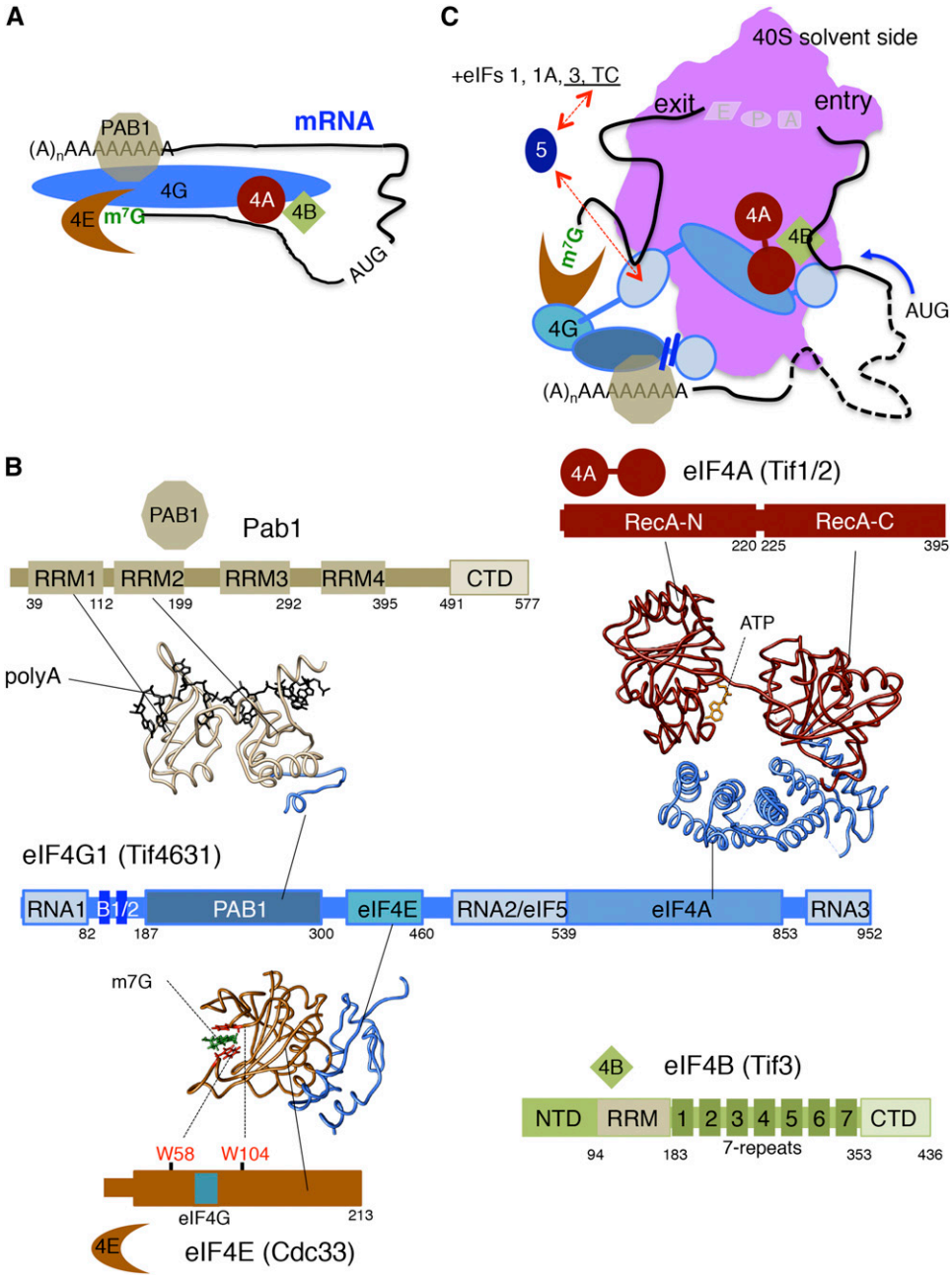

Figure 6 Interactions among the m^{7}G cap- and mRNA-binding factors. (A) Cartoon of mRNA recruitment step as in Figure 1. (B) Schematics of eIF4G (middle), Pab1 and eIF4A (top), and eIF4E and eIF4B (bottom). Factor binding domains on eIF4G are labeled, and structural models of the interacting factors are depicted. Structural models of human Pabp-poly(A)–eIF4G (Safaee *et al.* 2012), yeast eIF4A–eIF4G (Schutz *et al.* 2008), and yeast eIF4E–eIF4G (Gross *et al.* 2003) were drawn using Chimera software (UCSF). (C) Model for interactions of eIF4G domains with initiation factors and with the mRNA 5′ UTR on both the mRNA entrance and exit sides of the 40S ribosome to enhance mRNA binding to the ribosome.

eIF4G1 has three separate RNA-binding regions [termed RNA1$^{(1–82)}$, RNA2$^{(492–539)}$, and RNA3$^{(883–952)}$] (Figure 6B) that likely contribute to the enhanced affinity for binding eIF4E to 5′-capped mRNAs (O'Leary *et al.* 2013), analogous to RNA-binding activities shown to promote mammalian eIF4G–eIF4E interactions (Yanagiya *et al.* 2009). In yeast, deletion of RNA1 or RNA3 is *ts*, when removed from the sole isoform of eIF4G, and further RNA motif removal is lethal (Berset *et al.* 2003). However, singly deleting RNA2 or RNA3 is lethal when combined with the *tif4631-459* mutations that impair eIF4E binding (Park *et al.* 2011). This suggests that there is redundancy in the requirements for eIF4G–eIF4E and eIF4G–mRNA interactions for promoting translation initiation. In addition to the Pab1 binding region (eIF4G$^{188–299}$), the adjacent RNA1, box 1 and box 2 conserved elements of eIF4G (Figure 6B) also promote binding to Pab1 and mRNA (Park *et al.* 2011).

The interaction between eIF4G1$^{188–299}$ and Pab1 is primarily via the second of four RRMs in Pab1 (Figure 6B) (Kessler and Sachs 1998). eIF4G binding to Pab1 provides a further route for RNA recruitment. eIF4G can therefore bridge both the 5′ and 3′ ends of an mRNA as visualized as a "closed loop" (Wells *et al.* 1998) and supported by *in vivo* evidence (Preiss and Hentze 1998; Archer *et al.* 2015).

Closed loop promotes 43S ribosome recruitment: The 5′ cap and poly(A) tail act synergistically to promote translation. eIF4G bridges these mRNA ends (Figure 1 and Figure

6A) and is implicated in recycling both 40S and 60S ribosome subunits to the start codon following termination. Using *in vitro* translation experiments and toe-printing techniques, 48S PIC association with the AUG start codon on a short mRNA was resistant to 5′ cap analog (m^7GDP) in a manner that was dependent on both the 5′ cap and poly(A) tail as well as intact eIF4G, Pab1, and the termination factors eRF1 and eRF3 (Amrani *et al.* 2008). Further support for the eIF4E–4G–Pab1–mRNA closed loop comes from the capture of mRNAs bound to each factor from live cells and quantified by RT-PCR or high-throughput sequencing (Archer *et al.* 2015; Costello *et al.* 2015). However, it should be noted that the Pab1–eIF4G interaction is dispensable for cell growth unless the eIF4E–eIF4G interaction is also impaired (Tarun *et al.* 1997; Park *et al.* 2011). Thus, closed-loop assembly is not essential, but is rate enhancing for translation.

eIF4G–eIF5 interactions promote PIC recruitment: eIF4E–eIF4G complexes play a role in ensuring that the AUG codon closest to the 5′ end is selected for translation. The 43S PIC is directed to bind an mRNA at the 5′ end. In mammalian cells, eIF4G binds the 43S complex via contacts with the c, d, and e subunits of eIF3 (Korneeva *et al.* 2000; LeFebvre *et al.* 2006; Villa *et al.* 2013). Neither eIF3d, eIF3e, nor the eIF4G domain used by mammals is conserved in yeast. Instead, a central region of eIF4G (eIF4GII residues 439–577) (He *et al.* 2003) encompassing RNA2 (Figure 6B) can bind to the eIF5 carboxyl terminal domain (CTD) (Asano *et al.* 2001) with high affinity (<15 nM) (Mitchell *et al.* 2010). Because the eIF5 CTD binds to both eIF3c/Nip1 and eIF2β (Yamamoto *et al.* 2005) it can recruit the 43S PIC to eIF4G-bound mRNAs near their 5′ end. It is noteworthy that eIF3, as well as eIF4 factors, are critical for recruitment of natural mRNAs to the PIC in the reconstituted *in vitro* system (Mitchell *et al.* 2010) and that depletion of eIF3 subunits *in vivo* causes a more dramatic impairment of mRNA binding to 40S subunits than does depletion of eIF4G (Jivotovskaya *et al.* 2006). Thus, in addition to stabilizing TC binding to the 43S PIC, eIF3 plays an important role in mRNA binding. As eIF3 binds directly to eIF5 (Asano *et al.* 1998; Phan *et al.* 1998), perhaps these factors cooperate with eIF4G to promote mRNA binding to the PIC.

eIF4A: A C-terminal segment of eIF4G ($eIF4G1^{542–883}$) interacts with the DEAD-box RNA helicase eIF4A (encoded by *TIF1* and *TIF2*) (Figure 6B) (Dominguez *et al.* 1999; Neff and Sachs 1999). The α-helical HEAT repeats in this domain of eIF4G interact with both folded domains of eIF4A to form a stable complex where the ATPase and RNA-binding features of eIF4A are poised for action (Figure 6B) (Schutz *et al.* 2008). FRET measurements suggest that eIF4G HEAT domain binding to eIF4A shifts eIF4A from an "open" conformation with its two RecA homology domains separated, to one that is partially closed or "half open" as observed in the eIF4G/eIF4A co-crystal structure (Schutz *et al.* 2008; Andreou and Klostermeier 2014). This structural rearrangement is thought to stimulate eIF4A ATPase activity in the presence of RNA and facilitate PIC recruitment and movement along the mRNA in a 5′ to 3′ direction. Using model RNA templates and purified factors, it was shown that eIF4E/eIF4G both enhances ATPase activity and provides directional bias to eIF4A to unwind 5′ RNA duplexes (Rajagopal *et al.* 2012). The RNA1, RNA2, and RNA3 domains of eIF4G enhanced both of these activities, consistent with the ability of eIF4G to link different factors together (Figure 6), enhancing successive steps in translation initiation (Rajagopal *et al.* 2012).

The RNA helicase activity of eIF4A may be more important for creating a single-stranded stretch of mRNA for efficient 43S PIC recruitment to the mRNA 5′ end than for unwinding strong secondary structure elements within many 5′ leader sequences. Support for this idea comes from several genome-wide experiments. First, depletion of eIF4G reduced overall protein synthesis by only 75% and narrowed the range of translational efficiencies genome-wide (Park *et al.* 2011). The mRNAs most affected were not ones with longer 5′ UTRs, suggesting that eIF4G's role in 43S PIC recruitment to mRNAs is more critical than its role in promoting scanning on long or structured 5′ UTRs (Park *et al.* 2011). Second, ribosome profiling of an eIF4A *ts* mutant found that the translational efficiency of most mRNAs were similarly affected by loss of eIF4A (Sen *et al.* 2015). As reporter mRNAs with 5′ UTRs of differing lengths and secondary structures were all affected by 30–50% in the eIF4A mutant, it was concluded that eIF4A is globally important for optimal initiation on all mRNAs (Sen *et al.* 2015).

eIF4B: eIF4B, encoded by *TIF3*, enhances eIF4G–eIF4A complex formation and eIF4A helicase activity, and it also stimulates 43S recruitment to mRNA. Studies by Altmann and Trachsel (1989) demonstrated that yeast eIF4B is structurally divergent from its mammalian counterpart, but performs similar functions. *TIF3* deletion causes both slow and cold-sensitive growth (Altmann *et al.* 1993). eIF4G interacts independently with both eIF4B and eIF4A and excess eIF4B suppresses *ts* mutations in the eIF4G HEAT domain and stabilizes eIF4G–eIF4A interactions, perhaps by altering the conformation of eIF4G in this complex (Park *et al.* 2013). *In vitro* measurements show that eIF4B stimulates RNA helicase activity of eIF4A, when eIF4G is also present. The eIF4A–eIF4G–eIF4B complex stimulated ATP hydrolysis and RNA unwinding by >12-fold over eIF4A alone, with eIF4B enhancing eIF4A RNA unwinding activity and eIF4G stimulating ATP hydrolysis (Andreou and Klostermeier 2014). FRET studies adding eIF4B to complexes including ATP, mRNA, and eIF4A/eIF4G promoted a switch from a half-open to a closed eIF4A conformation with juxtaposed RecA domains (Andreou and Klostermeier 2014). These structural transitions in eIF4A promoted by eIF4B and eIF4G are proposed to be important for efficient helicase activity during mRNA scanning (Andreou and Klostermeier 2014; Harms *et al.* 2014).

eIF4B is also implicated in 43S PIC recruitment. *In vitro* eIF4B significantly stimulated the recruitment of the 43S PIC to *RPL41A* and *DAD4* mRNAs in the presence of eIF4F (Mitchell *et al.* 2010). In addition, eIF4B binds directly to eIF3 (K_d = 380 nM) (Mitchell *et al.* 2010) and interacts with single-stranded RNA (K_d = 2.2 μM) and the head region of the 40S subunit via an interaction with Rps20 (K_d = 360 nM) (Walker *et al.* 2013). Yeast eIF4B possesses an NTD, an RRM, which binds mRNA and stimulates RNA duplex annealing (Altmann *et al.* 1993; Niederberger *et al.* 1998), and a 20- to 26-residue module that is repeated seven times forming the "7-repeats" domain (Figure 6B). The 7-repeats are divergent from mammalian eIF4B, but are conserved among fungi (Zhou *et al.* 2014). The 7-repeats contribute to mRNA binding, as an allele missing both the NTD and 7-repeats fails to bind the 40S or to stimulate mRNA recruitment to the 43S PIC and impairs translation *in vivo*, whereas all of these activities are largely intact when only the RRM is missing (Walker *et al.* 2013). Interaction of eIF4B with Rps20, a protein within the 40S head region, may indicate that eIF4B helps load mRNAs into the 40S entry channel to promote scanning (Zhou *et al.* 2014). A speculative model for eIF4G domain interactions with multiple eIFs and the mRNA 5′ UTR to recruit the 43S PIC and promote scanning is shown in Figure 6C.

Ded1: Ded1 is a second DEAD-box RNA helicase implicated in 43S PIC recruitment and scanning during translation initiation. *DED1* is essential and is a close homolog of mammalian DDX3. Ded1, like eIF4A, contains two RecA-homology domains, but with a distinct NTD and CTD (Linder and Jankowsky 2011). In addition to translation initiation, Ded1 has roles in pre-mRNA splicing, mRNA export from the nucleus, and mRNA decay. While Ded1 is predominantly cytoplasmic (Chuang *et al.* 1997), it interacts with both nuclear and cytoplasmic mRNA 5′ cap complexes and moves between the nucleus and cytoplasm, suggesting that Ded1 is a general RNA chaperone (Senissar *et al.* 2014). Because Ded1 is an active helicase by itself, it is an excellent model for biochemical studies of RNA helicase activities (Iost *et al.* 1999; Yang and Jankowsky 2005). A recent comprehensive review of Ded1 provides a detailed summary of RNA helicase activities and wider roles of Ded1 and its homologs (Sharma and Jankowsky 2014).

Ded1 binds the RNA3 motif at the C terminus of eIF4G1 (Hilliker *et al.* 2011). While mutational inactivation of Ded1 inhibits translation initiation (Chuang *et al.* 1997), its overexpression rescues eIF4E *ts* alleles (de la Cruz *et al.* 1997). However, greater overexpression of Ded1 from an inducible *GAL1* promoter represses translation, causing sequestration of eIF4E, eIF4G, and Pab1 in cytoplasmic granules (Hilliker *et al.* 2011). Ded1 can form very stable complexes with RNA *in vitro* (Liu *et al.* 2014) and it is important for resolving misfolded RNA structures and preventing higher-order structural contacts that would otherwise destabilize RNA architecture (Pan *et al.* 2014).

Ribosome profiling of *DED1* mutants has shown differential requirements for Ded1 across different mRNAs. Inactivation of a *ded1* cold-sensitive mutant impaired global translation rates, and greater than average reductions in translational efficiency were observed for only ∼600 genes (Sen *et al.* 2015). The Ded1 hyperdependent mRNAs had longer than average 5′ UTR lengths and greater propensity for secondary structure, thereby implicating Ded1 as critical for scanning through structured 5′ UTRs (Sen *et al.* 2015). Consistent with these findings, reporter mRNAs bearing long or structured 5′ UTRs exhibit heightened dependence on Ded1 (Berthelot *et al.* 2004; Sen *et al.* 2015).

AUG selection

Following binding of the 43S PIC near the 5′ end of the mRNA, it traverses in a 3′ direction inspecting for a start codon. Elegant experiments by Donahue and colleagues established that the anticodon of the Met-tRNA$_i^{Met}$ in the 43S complex is primarily responsible for start codon selection. Mutation of the tRNA$_i^{Met}$ anticodon from 5′-CAU-3′ to 5′-CCU-3′ enabled ribosomes to synthesize His4 when the *HIS4* mRNA start codon was mutated from 5′-AUG-3′ to 5′-AGG-3′ (Cigan *et al.* 1988a). Moreover, insertion of a 5′-AGG-3′ codon in the *HIS4* mRNA leader upstream and out-of-frame with the 5′-AGG-3′ codon at the *HIS4* start site blocked His4 production (Cigan *et al.* 1988a). This latter result supports the model that the anticodon of Met-tRNA$_i^{Met}$ in the scanning 43S complex inspects the mRNA in a base-by-base manner to select the translation start site.

This importance of codon–anticodon match in start codon selection was further supported by studies examining the kinetics and thermodynamics of 48S PIC formation in reconstituted yeast *in vitro* translation assays. Point mutations that altered the second or third positions of the AUG start codon on the mRNA dramatically lowered the affinity of Met-tRNA$_i^{Met}$ binding in the 48S PIC. This binding defect was suppressed by mutations in the tRNA$_i^{Met}$ anticodon that restored base-pairing interactions with the mRNA. As the start codon mutations in the mRNA mainly affected the on rate for Met-tRNA$_i^{Met}$ binding, and not the off rate, it was proposed that 48S PIC formation is accompanied by a conformational change that locks in Met-tRNA$_i^{Met}$ binding. Accordingly, in this closed state, Met-tRNA$_i^{Met}$ is stably bound to the 40S subunit and fixed on the translation start codon of the mRNA (Kolitz *et al.* 2009).

In addition to the Met-tRNA$_i^{Met}$, translation factors play key roles in the transition of the 40S subunit from its open, scanning-competent state to the closed, scanning-arrested state following start codon selection (reviewed in Hinnebusch 2011, 2014). Genetic screens in yeast have provided key insights into the factors contributing to start codon selection. Spontaneous Sui$^-$ mutations that enhance initiation from a UUG codon were isolated in eIF1 (Yoon and Donahue 1992), all three subunits of eIF2 (Donahue *et al.* 1988; Cigan *et al.* 1989; Huang *et al.* 1997), and in eIF5 (encoded by *TIF5*) (Huang *et al.* 1997). In subsequent directed screens Sui$^-$

mutations have also been isolated in eIF1A (Fekete *et al.* 2007), eIF3 subunits (Valasek *et al.* 2004; Chiu *et al.* 2010; Elantak *et al.* 2010; Karaskova *et al.* 2012), and in 18S rRNA (Nemoto *et al.* 2010). In contrast to the Sui$^-$ mutations, which relax the stringency for start codon selection, a second class of mutations enhances start codon selectivity. The Ssu$^-$ (suppressor of Sui$^-$) mutations block the ability of Sui$^-$ mutations to enhance initiation at a UUG codon in a mutant *HIS4* allele (Asano *et al.* 2001; Fekete *et al.* 2007; Saini *et al.* 2010). In general, Sui$^-$ mutations are thought to block scanning and promote conversion of the 40S subunit to its closed, scanning arrested conformation (P_{in}). In contrast, the Ssu$^-$ mutations promote scanning and stabilize the open conformation of the 40S subunit (P_{out}).

Biochemical analyses of the Sui$^-$ and Ssu$^-$ mutant forms of initiation factors have provided insights into the mechanism of start codon selection. The dominant *SUI5-G31R* mutation in eIF5 was reported to alter the release of Pi from GTP following its hydrolysis by eIF2 (Saini *et al.* 2014), and the *SUI3-2* (S264Y) mutation in eIF2β and the *SUI4* (*GCD11-N135K*) mutation in eIF2γ were reported to increase the intrinsic (eIF5 independent) GTPase activity of eIF2 (Huang *et al.* 1997). Thus, it was proposed that premature GTP hydrolysis by eIF2 would enable release of Met-tRNA$_i^{Met}$ to the P site in the absence of perfect codon–anticodon base pairing. The eIF5-G31R and eIF2β-S264Y mutations also stabilize 48S PICs at UUG codons in the presence of nonhydrolyzable GTP, indicating these mutations stabilize the closed P_{in} state of the 48S PIC (Martin-Marcos *et al.* 2014; Saini *et al.* 2014). The identification of Sui$^-$ mutations in the CTT and Ssu$^-$ mutations in the NTT of eIF1A likewise implicate these segments in stabilizing the P_{out} or P_{in} states of Met-tRNA$_i^{Met}$ binding, respectively (Fekete *et al.* 2007; Saini *et al.* 2010).

Sui$^-$ mutations in eIF1 have been found to weaken eIF1 binding to the 40S subunit, consistent with their recessive phenotype and with the notion that eIF1 dissociation from the 48S complex is required for start codon selection (Cheung *et al.* 2007; Martin-Marcos *et al.* 2013). Moreover, overexpression of eIF1 suppresses Sui$^-$ mutations in other translation factors, indicating that eIF1 dissociation from the 48S complex is a key commitment step in start codon selection (Valasek *et al.* 2004; Cheung *et al.* 2007; Martin-Marcos *et al.* 2011; Martin-Marcos *et al.* 2013; Martin-Marcos *et al.* 2014). Cryo-EM structures of 48S PICs in open and closed states revealed a clash between eIF1 and Met-tRNA$_i^{Met}$ in the P_{in} state (Llacer *et al.* 2015). This clash likely underlies the role of eIF1 in blocking initiation at non-AUG codons and evokes eIF1 release on AUG codon recognition (Figure 4, A and B). In addition to these genetic and structural studies, kinetic experiments have highlighted the critical gatekeeper function of eIF1 in regulating start codon selection. Recognition of the AUG start codon by the 48S PIC induces a conformational change that accelerates eIF1 dissociation, which in turn enables release of Pi from eIF2–GDP (Figure 4B) (Algire *et al.* 2005; Maag *et al.* 2005). Thus, eIF1 dissociation and the attendant release of Pi, and not simply GTP hydrolysis by eIF2 (Maag *et al.* 2005), is the irreversible step that commits the ribosome to initiate at the selected codon.

The 405-residue factor eIF5 folds into functionally distinct N- and C-terminal domains (Figure 3B). The N-terminal domain of eIF5 resembles eIF1 and like the C-terminal domain of eIF2β possesses a Zn-finger element (Conte *et al.* 2006). In contrast, the α-helical C-terminal domain of eIF5 folds into a HEAT repeat with structural similarity to the HEAT domains in the C terminus of eIF2Bε and in eIF4G (Wei *et al.* 2010). The N-terminal domain of eIF5 directly binds the G domain of eIF2γ (Alone and Dever 2006), and mutation of Arg15 in eIF5 confers a lethal phenotype and significantly impairs the ability of eIF5 to stimulate GTP hydrolysis by eIF2 (Das *et al.* 2001; Algire *et al.* 2005), supporting the notion that eIF5 functions as a GTPase activating protein (GAP) for eIF2. In accord with the gatekeeper function of eIF1, it is proposed that eIF1 release following start codon recognition enables eIF5 to move into the vacated space and thereby stimulate Pi release from eIF2–GDP+Pi (Nanda *et al.* 2009). Consistent with this proposed movement of eIF5 into the space previously occupied by eIF1, and thus closer to eIF1A, the *SUI5-G31R* mutation in eIF5 was found to strengthen eIF1A interaction with the PIC at a UUG codon (Maag *et al.* 2006). Moreover, mutations in the eIF1A CTT uncouple Pi release from eIF1 dissociation (Nanda *et al.* 2013). Thus the eIF5 N-terminal domain appears to be intimately involved in the structural rearrangements in the scanning ribosome upon start codon selection.

The C-terminal HEAT domain in eIF5 binds to eIF1, the NTT (K-boxes) of eIF2β, and to eIF3c/Nip1 (Yamamoto *et al.* 2005). Mutations in the eIF5 C-terminal domain that disrupted its interaction with both eIF1 and eIF2β conferred an Ssu$^-$ phenotype and destabilized the closed state of the 48S complex (Luna *et al.* 2012). Importantly, this mutation did not affect the ability of eIF5 to promote GTP hydrolysis by eIF2 (Luna *et al.* 2012), further strengthening the notion that GTP hydrolysis occurs prior to the step controlling start codon selection (Algire *et al.* 2005; Maag *et al.* 2005).

Taking into account the results from the various genetic and biochemical studies on the translation factors that participate in start codon selection, a model can be proposed wherein the factors eIF1, eIF1A, eIF3, eIF5, and the eIF2 TC are bound to the 40S subunit as it scans the mRNA (Figure 4B). In this open, scanning-competent complex the Met-tRNA$_i^{Met}$ resides in the P_{out} state with eIF1 bound adjacent to the P site and eIF1A bound in the A site with its N- and C-terminal tails projecting into the P site. Both eIF1 and the eIF1A CTT prevent full accommodation of Met-tRNA$_i^{Met}$ into the P_{in} state. When the scanning complex encounters a start codon, base-pairing interactions between the anticodon of Met-tRNA$_i^{Met}$ and the start codon triggers entry of the Met-tRNA$_i^{Met}$ to the P_{in} state. This movement is accompanied by displacement of eIF1 and movement of the eIF1A CTT toward eIF5. These factor movements trigger Pi release from eIF2, a critical commitment step in start codon selection (Algire *et al.* 2005), and conversion of the 43S PIC to its

closed, scanning-arrested state (Hinnebusch 2011, 2014). In accord with this model, recent cryo-EM structures of 48S complexes in the open and closed states have revealed conformational changes in the 40S subunit as well as interaction of the eIF1A NTT with the Met-tRNA$_i^{Met}$–AUG codon duplex, and eIF2, eIF1A, and ribosomal proteins with the mRNA and start codon context nucleotides (Hussain *et al.* 2014; Llacer *et al.* 2015). Upon start codon selection, constriction of the mRNA channel and tightening of the P site are thought to block further scanning by the PIC.

Subunit joining

Following eIF1 and Pi release, the 48S PIC is in a closed conformation with Met-tRNA$_i^{Met}$ fully accommodated in the P site. Accompanying these changes, eIF1A binding to the 48S complex becomes tighter. It is unclear when the eIF2–GDP complex, eIF5 and eIF3 dissociate from the PIC; however, it is clear that based on its binding site on the intersubunit face of the 40S subunit, eIF2 must dissociate prior to 60S subunit joining.

The factor eIF5B, encoded by *FUN12* and an ortholog of the bacterial translation factor IF2, promotes 60S subunit joining (Choi *et al.* 1998; Pestova *et al.* 2000). eIF5B is 1002 amino acids in length and contains a GTP-binding domain near the center of the protein. Deletion of *FUN12* severely impairs yeast cell growth and causes a loss of polysomes, consistent with a defect in translation initiation (Choi *et al.* 1998). Removal of the N-terminal ~400 residues of eIF5B confers no growth defect *in vivo* and the truncated protein catalyzes subunit joining *in vitro* (Lee *et al.* 1999; Shin *et al.* 2002). The crystal structure of aIF5B, the archaeal ortholog of eIF5B, revealed a chalice-shaped protein with the G domain, domain II, and domain III, forming the cup of the chalice which is connected to the base, domain IV, by a long α-helix (Roll-Mecak *et al.* 2000; Kuhle and Ficner 2014a). Directed hydroxyl radical mapping studies of eIF5B–80S complexes placed domain II of yeast eIF5B near 18S rRNA helix h5 of the 40S subunit (Shin *et al.* 2009), consistent with results of a recent cryo-EM structure of eIF5B bound to an 80S initiation complex (Fernandez *et al.* 2013). This binding site is compatible with the eIF5B G domain binding to the GTPase activation center on the 60S subunit, similar to the binding sites of the bacterial translational GTPases IF2, EF-Tu, and EF-G on 70S ribosomes. Whereas the C-terminal domain of bacterial IF2, which corresponds to domain IV of eIF5B, directly binds the formylmethionine (fMet) on fMet-tRNA$_i^{Met}$, direct binding of eIF5B with Met-tRNA$_i^{Met}$ has not been observed. However, based on the ribosomal binding site of eIF5B and the dimensions of aIF5B, domain IV of eIF5B is thought to project across the A site to interact with Met-tRNA$_i^{Met}$ in the P site. This proposed contact of eIF5B with Met-tRNA$_i^{Met}$ in the P site is consistent with cryo-EM structures of the initiation complex (Fernandez *et al.* 2013; Kuhle and Ficner 2014b) and with the instability of 48S PICs and decreased recognition of an inhibitory upstream AUG codon ("leaky scanning") in yeast lacking eIF5B (Lee *et al.* 2002). It is proposed that eIF5B binding to the closed 48S complex at an AUG codon stabilizes Met-tRNA$_i^{Met}$ binding following eIF2–GDP release and promotes 60S subunit joining. In the absence of eIF5B, the Met-tRNA$_i^{Met}$ is not stably bound, causing some 48S complexes to dissociate from the mRNA and others to resume scanning to downstream start sites.

Domain IV of eIF5B binds to eIF1A (Choi *et al.* 2000) via interaction with the last five residues at the C terminus of eIF1A (Olsen *et al.* 2003; Acker *et al.* 2006; Fringer *et al.* 2007). Mutation of the eIF1A C terminus impairs subunit joining and full activation of eIF5B GTPase activity *in vitro* (Acker *et al.* 2006) and impairs yeast cell growth and eIF5B binding to 40S complexes *in vivo*. The growth and translation initiation defects of this eIF1A mutant are suppressed by overexpression of eIF5B, indicating that eIF1A helps recruit eIF5B to 40S complexes prior to subunit joining and thereby accelerates ribosomal subunit joining (Acker *et al.* 2009).

Both eIF1A and eIF5B are bound to the 80S ribosome following subunit joining, and GTP hydrolysis by eIF5B is required for their release (Shin *et al.* 2002; Fringer *et al.* 2007; Acker *et al.* 2009). Blocking eIF5B GTPase activity, either by inclusion of nonhydrolyzable GTP analogs or by mutation of the eIF5B G domain, does not impair subunit joining (Shin *et al.* 2002, 2007, 2009; Acker *et al.* 2009); however, it does impede eIF1A release from 80S ribosomes both *in vivo* and *in vitro* (Fringer *et al.* 2007; Acker *et al.* 2009). Mutations that disrupt the GTPase activity of eIF5B severely impair yeast cell growth (Shin *et al.* 2002, 2007, 2009). Suppressor mutations of these eIF5B mutants either restore the factor's GTPase activity (Shin *et al.* 2007) or decrease the binding affinity of eIF5B for the 80S ribosome (Shin *et al.* 2002, 2009), consistent with GTP hydrolysis lowering eIF5B affinity for the ribosome. It is proposed that GTP hydrolysis by eIF5B alters the conformation of the 80S to promote eIF1A release (Acker *et al.* 2009). As the eIF5B suppressor mutants that bypass the requirement for GTP hydrolysis show enhanced levels of leaky scanning (Shin *et al.* 2002), GTP hydrolysis by eIF5B might serve as a checkpoint to ensure the fidelity of subunit joining (Shin *et al.* 2002). Following release of eIF5B and eIF1A, the ribosome is poised with Met-tRNA$_i^{Met}$ in the P site and a vacant A site available to receive the first elongator aa-tRNA. It is possible that some initiation factors including eIF3 (Szamecz *et al.* 2008) remain associated with the ribosome through the first few steps of elongation.

Recycling eIF2–GDP to eIF2–GTP

eIF2–GDP released from the 48S PIC following start codon selection must be converted to an active GTP-bound form to promote Met-tRNA$_i^{Met}$ binding and continued rounds of translation initiation (Figure 1 and Figure 7A). This is an important step as phosphorylation of eIF2α converts eIF2 into an inhibitor of its GEF eIF2B, thereby lowering TC levels. eIF2 recycling was thought to be a single reaction involving eIF2B; however, eIF5 antagonizes eIF2B and must be

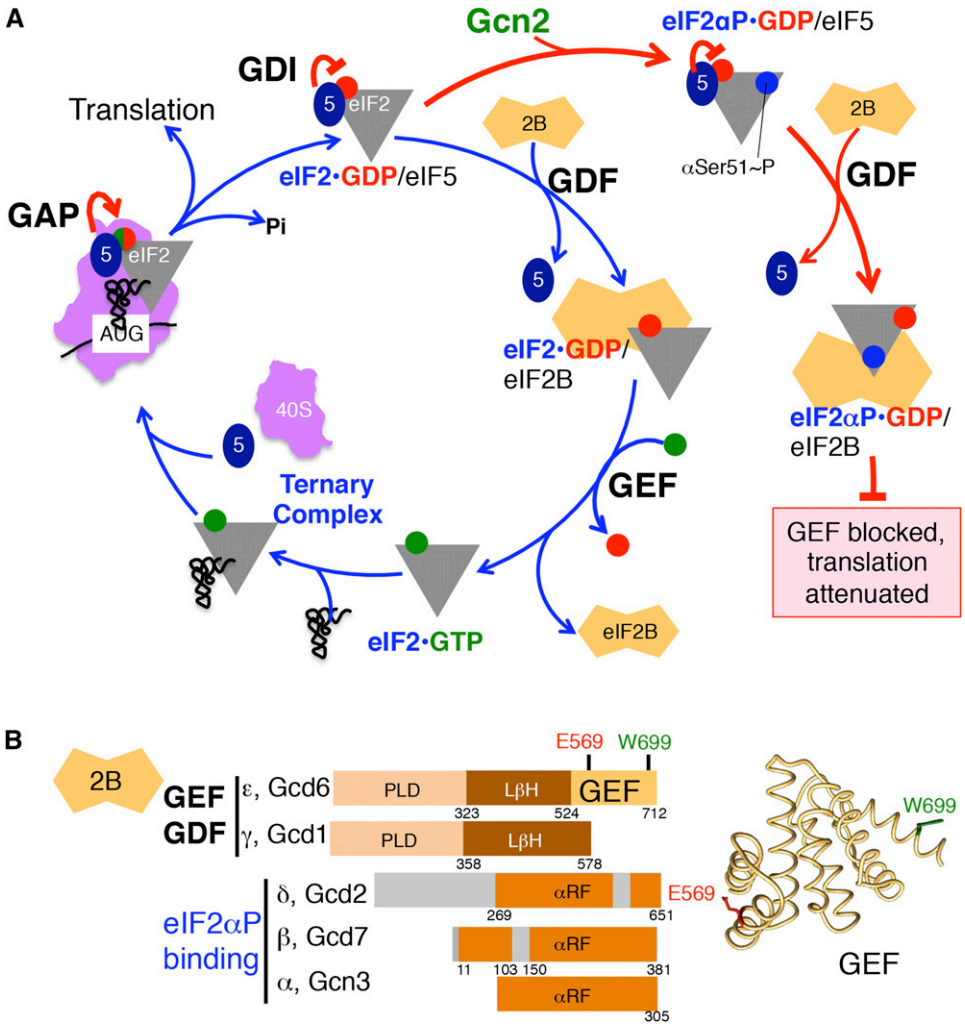

Figure 7 Recycling and regulation of eIF2 by eIF2B. (A) Pathway of eIF2 nucleotide cycle and its regulation by eIF2α phosphorylation, adapted from Figure 1. GDI function of eIF5, GDF and GEF activities of eIF2B, and GAP function of eIF5 (5) are described in the text. Phosphorylation of eIF2α on Ser51 by GCN2 is represented by the blue circle; GDP, red circle; and GTP, green circle. (B) Schematics of eIF2B subunits and domain organization (left) and structure of the eIF2Bε GEF domain (right, pdb 1PAQ) (Boesen *et al.* 2004). Homologous domains are shown in identical color shades. PLD and LβH indicate the pyrophosphorylase-like and the left-handed β-helical domains, respectively (Reid *et al.* 2012). αRF indicates the α-helical domain followed by a Rossmann-like fold shared by the α-, β-, and δ-subunits. Structural models were drawn using Chimera software (UCSF).

displaced from eIF2 prior to nucleotide exchange (Figure 1 and Figure 7A) (Jennings and Pavitt 2014).

GDP dissociation inhibitor function of eIF5: In addition to its roles in PIC formation, AUG codon recognition and stimulation of eIF2–GTP hydrolysis, eIF5 functions as a GDP dissociation inhibitor (GDI) to prevent unregulated release of GDP from eIF2. eIF5 binds both eIF2–GDP and TC with identical high affinity (eIF2–GDP K_d = 23 ± 9 nM, TC K_d = 23 ± 5 nM) (Algire *et al.* 2005), and cells contain an abundant fraction of inactive eIF2–GDP/eIF5 complexes that are thought to be released from the 48S PIC following AUG recognition (Singh *et al.* 2006). eIF5 lowers the rate of spontaneous GDP release from eIF2 over a range of Mg^{2+} concentrations (Jennings and Pavitt 2010a), and this GDI activity requires the eIF5 CTD and the region linking it to the NTD. Thus, GDI and GAP activities of eIF5 are distinct (Jennings and Pavitt 2010a). Mutation of a conserved tryptophan (W391F) in the CTD, or seven substitutions within a conserved "DWEAR" motif in the linker region (termed L7A) (Figure 3B), eliminates GDI activity (Jennings and Pavitt 2010a,b). Though neither GDI mutation significantly alters growth of yeast on rich or minimal medium, they dramatically impair responses to eIF2 phosphorylation, indicating an important role of eIF5 in tight regulation of eIF2B GEF activity.

eIF2B displaces eIF5 from eIF2–GDP: Because the CTD of eIF5 and the GEF domain of eIF2Bε (see Figure 3, C and D) share a common HEAT repeat structure (Boesen *et al.* 2004; Bieniossek *et al.* 2006) necessary for binding to eIF2β (Asano *et al.* 1999), binding of each factor to eIF2 is mutually exclusive (Jennings and Pavitt 2010b). eIF5 must dissociate from the stable eIF2–GDP/eIF5 complex to enable eIF2B GEF action; however, eIF2B itself can displace eIF5 (Jennings *et al.* 2013). In common with other G protein regulator nomenclature, eIF2B is a GDI displacement factor (GDF) (Figure 7A).

eIF2B consists of five subunits α–ε (encoded by *GCN3*, *GCD7*, *GCD1*, *GCD2*, and *GCD6*, respectively; Table 1) in equimolar stoichiometry (Figure 7B) (Cigan *et al.* 1993; Kito *et al.* 2007). The subunits are subdivided into two functionally and structurally distinct αβδ and γε subcomplexes (Figure 7B). The eIF2B GEF domain is within the γε subcomplex, lodged at the eIF2Bε C terminus (Pavitt *et al.* 1998; Gomez and Pavitt 2000; Boesen *et al.* 2004). This subcomplex is as effective as intact eIF2B for eIF5 displacement (GDF) activity; however, neither γ nor ε alone have GDF

activity, suggesting that these subunits cooperate in GDF function (Jennings *et al.* 2013). Importantly, missense mutations in eIF2Bγ (*gcd1-G12V* and *gcd1-L480Q*) specifically impair GDF function (release of eIF5 from eIF2–GDP) and only affect nucleotide exchange function when eIF2–GDP is prebound by eIF5 (Jennings *et al.* 2013). As the *gcd1* mutations confer slow-growth and Gcd$^-$ phenotypes, the GDF activity of eIF2B is critical *in vivo*.

eIF2B catalysis of guanine nucleotide exchange: A long-established function of eIF2B is to recycle inactive eIF2–GDP complexes to functional eIF2–GTP complexes (Figure 7A) (Cigan *et al.* 1993). Similar to GEFs for other G proteins, eIF2B likely catalyzes exchange by decreasing the binding affinity of eIF2 for GDP (Sprang and Coleman 1998). Given the greater abundance of GTP *vs.* GDP in growing cells (Rudoni *et al.* 2001), release of GDP from eIF2 is likely sufficient to allow recharging of eIF2 with GTP. Yeast eIF2B was discovered through studies examining the translational control of the *GCN4* mRNA. Mutations in the eIF2B subunits cause Gcd$^-$ phenotypes (Harashima and Hinnebusch 1986) and also confer slow-growth and reduced rates of translation initiation (Hannig *et al.* 1990; Foiani *et al.* 1991; Bushman *et al.* 1993a).

Yeast eIF2B promotes release of GDP from eIF2 (V_{max} = 250.7 fmol of GDP released per minute, at 0°) at rates similar to values reported for mammalian eIF2B (Nika *et al.* 2000). eIF2Bε alone is ~5- to 10-fold less effective than intact eIF2B at promoting nucleotide exchange, while eIF2Bγε subcomplexes have the same activity as intact eIF2B (Gomez and Pavitt 2000; Jennings *et al.* 2013). The GEF domain comprises the C-terminal ~200 residues (eIF2Bε$^{518–712}$) (Gomez and Pavitt 2000; Gomez *et al.* 2002) and adopts a HEAT repeats structure with conserved residues important for GEF activity on one face (Boesen *et al.* 2004). Conserved residue E569 is critical for GEF function (Boesen *et al.* 2004) and cell viability (Mohammad-Qureshi *et al.* 2007), and residue W699 is important for binding to eIF2β and γ (Figure 7D) (Mohammad-Qureshi *et al.* 2007).

Regulation of eIF2B activity: eIF2α is phosphorylated at Ser51 by Gcn2 (Dever *et al.* 1992) causing inhibition of eIF2B GEF activity (Pavitt *et al.* 1998; Jennings *et al.* 2013). Genetic and biochemical experiments implicate the homologous eIF2Bα, β, and δ subunits in mediating translational control. Multiple missense mutations in each of these subunits impair the regulatory response to phosphorylated eIF2 (Vazquez de Aldana and Hinnebusch 1994; Pavitt *et al.* 1997) and deletion of *GCN3*, encoding nonessential eIF2Bα, blocks the Gcn2-dependent induction of *GCN4* expression (Hannig and Hinnebusch 1988). The mutations cluster in two regions of sequence similarity shared by the subunits (Pavitt *et al.* 1997), and based on the structure of human eIF2Bα, the mutations are largely distributed across one surface of each subunit (Hiyama *et al.* 2009). Each subunit appears to independently contribute to the regulatory mechanism, rather than being redundant. Mutations in eIF2Bβ weaken interactions with phosphorylated eIF2α (Pavitt *et al.* 1997; Krishnamoorthy *et al.* 2001), and direct interactions between eIF2α and eIF2B are supported by allele-specific genetic interactions between eIF2α and eIF2Bβ (Dev *et al.* 2010) and by lysine-specific cross-links between eIF2α and eIF2Bδ (Gordiyenko *et al.* 2014). The current model for inhibition of eIF2B activity proposes that phosphorylation causes tight binding of eIF2α to an eIF2Bαβδ surface such that eIF2Bε can no longer bind productively to eIF2γ to promote nucleotide exchange. In addition, binding of one molecule of eIF2 to eIF2Bαβδ likely prevents simultaneous interaction of a second eIF2 molecule with the eIF2Bγε subunits (Pavitt *et al.* 1998; Krishnamoorthy *et al.* 2001; Jennings *et al.* 2013).

Recent evidence reveals that eIF2B is a dimer of pentamers and thus a decamer of 590 kDa bearing two copies of each subunit (Gordiyenko *et al.* 2014). The precise arrangement of these subunits is not yet clear. eIF2Bγ and eIF2Bε both share significant sequence homology with apparently functionally unrelated enzymes containing a pyrophosphorylase-like domain (PLD) and a left-handed β-helix (LβH) that are important for intersubunit interactions (Figure 7B) (Koonin 1995; Reid *et al.* 2012; Gordiyenko *et al.* 2014). The remaining three subunits, eIF2Bα, eIF2Bβ, and eIF2Bδ, also share sequence/structural similarity with each other and with closely related protein families in Archaea: ribose-1,5-bisphosphate isomerases, methylthioribose-1-phosphate isomerases, and a final group that are proposed archaeal eIF2B homologs (Dev *et al.* 2009). All of these proteins share a α-helical domain followed by a Rossmann-like fold (αRF) (Figure 7B). Lysine-specific cross-links indicate a strong network of connections among all the essential eIF2Bβ/γ/δ/ε subunits (Gordiyenko *et al.* 2014). Genetic observations suggest interactions between eIF2Bβ and eIF2Bγ (Dev *et al.* 2010) and between eIF2Bα and both eIF2Bγ and eIF2Bε (Bushman *et al.* 1993b). Although the complete eIF2B structure is not yet available, a recent structural study of eIF2Bβδ from the thermophilic filamentous fungus *Chaetomium thermophilum* (Kuhle *et al.* 2015) and modeling and biochemical studies of mammalian eIF2B subunits (Bogorad *et al.* 2014) suggest that the eIF2Bαβδ may form the dimer core of eIF2B.

A significant proportion of eIF2B forms higher order structures in cells referred to as a large "body" or "filament" (Campbell *et al.* 2005; Noree *et al.* 2010) that can diffuse through the cytoplasm (Taylor *et al.* 2010). In the absence of stress, GFP fusions of most translation factors exhibit a diffuse cytoplasmic localization, whereas eIF2B is present in both diffuse and localized forms (Campbell *et al.* 2005). Fluorescence recovery after photobleaching experiments showed that eIF2B is resident in the body, while eIF2 shuttles through it. eIF2 movement is reduced by translation inhibitors, eIF2 phosphorylation, or eIF2B mutation, suggesting that the eIF2B body is a major site of eIF2B function and may contribute to translational control (Campbell *et al.* 2005).

Mechanism of Translation Elongation, Termination, and Recycling

Relative to bacterial systems, many mechanistic and structural aspects of yeast elongation are well conserved. The elongation factors eEF1A and eEF2 in yeast (Table 2) are structural and functional homologs of the bacterial factors EF-Tu and EF-G, respectively, and the basic pathway of elongation is also conserved (Figure 8). An eEF1A–GTP–aa-tRNA TC binds to the A site of the ribosome. Codon recognition by the tRNA triggers GTP hydrolysis and release of eEF1A–GDP, which allows the aa-tRNA to be accommodated in the A site. The ribosomal peptidyl transferase center (PTC) positions the aa-tRNA in the A site and the peptidyl-tRNA in the P site to allow rapid peptide bond formation. Ratcheting of the ribosome following peptide bond formation moves the tRNAs into hybrid P/E and A/P states with the acceptor ends of the tRNAs in the E and P sites and the anticodon loops remaining in the P and A sites, respectively. Binding and GTP hydrolysis by eEF2–GTP promotes translocation of the tRNA anticodon loops into the E and P sites, respectively. The deacylated tRNA is released from the E site and the next eEF1A–GTP–aa-tRNA binds to the A site in a codon-dependent manner. The cycle continues until a nonsense codon is reached. Recycling of eEF1A–GDP to eEF1A–GTP between each cycle requires the GEF eEF1B. Distinctive features in yeast include the subunit composition of the GEF and the mode of interaction of its catalytic subunit with eEF1A, unique and functionally important post-translational modifications on several elongation factors, and most prominently, the requirement for the essential eukaryotic elongation factor 3 (eEF3). A comprehensive review of the structures of the yeast translation elongation factors and of many mutants of these factors was previously published (Taylor *et al.* 2007a). As described below, molecular analyses of translation elongation factors have provided additional insights into the accuracy of translation elongation (Valente and Kinzy 2003) and helped elucidate the function of post-translational modifications of elongation factors (Greganova *et al.* 2011). These genetic studies have been complemented by structural studies of the yeast elongation factors eEF1A and eEF1Bα (Andersen *et al.* 2000), eEF1Bγ (Jeppesen *et al.* 2003), eEF2 (Jørgensen *et al.* 2003), and eEF3 (Andersen *et al.* 2006). As the kinetic mechanisms of translation elongation have been extensively studied in bacteria (Wintermeyer *et al.* 2004), and also recently reviewed for eukaryotes (Rodnina and Wintermeyer 2009; Dever and Green 2012), this section will focus on insights obtained using the yeast system.

aa-tRNA delivery by eEF1

The eEF1A–eEF1Bαγ complex in yeast, like the analogous EF-Tu–EF-Ts complex of bacteria, provides both aa-tRNA delivery (eEF1A) and GEF (eEF1Bαγ) functions. Consistent with their strong sequence and structural similarity, the canonical translation functions and kinetic mechanisms of G proteins eEF1A and EF-Tu are very similar. In addition, studies in yeast have illuminated roles for elongation factors outside translation elongation.

Table 2 Translation elongation and termination factors

Factor	Gene	Systematic name	Length (AA)
eEF1A (EF1α)	*TEF1*	YPR080w	458
	TEF2	YBR118w	458
eEF1Bα (EF1β)	*EFB1/TEF5*	YAL003w	206
eEF1Bγ (EF1γ)	*TEF4*	YKL081w	412
	CAM1	YPL048w	415
eEF2	*EFT1*	YOR133w	842
	EFT2	YDR385w	842
eEF3	*YEF3/TEF3*	YLR249w	1044
	HEF3	YNL014w	1044
eIF5A	*HYP2*	YEL034w	157
	ANB1	YJR047c	157
eRF1	*SUP45*	YBR143c	437
eRF3	*SUP35*	YDR172w	685
Rli1 (ABCE1)	*RLI1*	YDR091c	608

eEF1A: Mutations in *TEF2* (Sandbaken and Culbertson 1988), one of two genes encoding eEF1A (Table 2), and altered levels of eEF1A (Song *et al.* 1989) affect translation fidelity. Dominant *TEF2* mutants selected to suppress non-programmed +1 ribosomal frameshifting (Sandbaken and Culbertson 1988) were found to have differential effects on +1 and −1 frameshifting and missense suppression when present as the only form of eEF1A in the cell (Dinman and Kinzy 1997; Plant *et al.* 2007). An N153T mutation in the guanine-specificity element of the GTP-binding domain increased the intrinsic GTPase activity of eEF1A, enhanced amino acid misincorporation rates *in vitro* (Cavallius and Merrick 1998), and promoted nonsense suppression while generally not affecting missense suppression *in vivo* (Carr-Schmid *et al.* 1999a). In support of the notion that eEF1A–GTP–aa-tRNA ternary complexes and termination factors compete for binding to the ribosomal A site, deleting the *TEF2* gene to lower eEF1A levels decreased nonsense suppression *in vivo* (Song *et al.* 1989). Overall, the studies on eEF1A reveal a diversity of effects on A-site events when eEF1A activity is altered.

eEF1A is subject to multiple post-translational modifications, many of which occur across eukaryotes. In yeast, these include di- and trimethyl lysine, but do not include phosphoglycerol ethanolamine as observed in the mammalian factor (Cavallius *et al.* 1993). The functional role of these modifications, however, remains unclear as mutation of the methylation sites does not affect cell growth or general protein synthesis (Cavallius *et al.* 1997). The C-terminal lysine of yeast eEF1A is methyl esterified; however, the role of this modification remains unclear (Zobel-Thropp *et al.* 2000).

eEF1Bαγ and guanine nucleotide exchange: Following delivery of aa-tRNA to the ribosomal A site, GTP hydrolysis by eEF1A results in release of an eEF1A–GDP complex. The eEF1B complex that catalyzes guanine nucleotide exchange

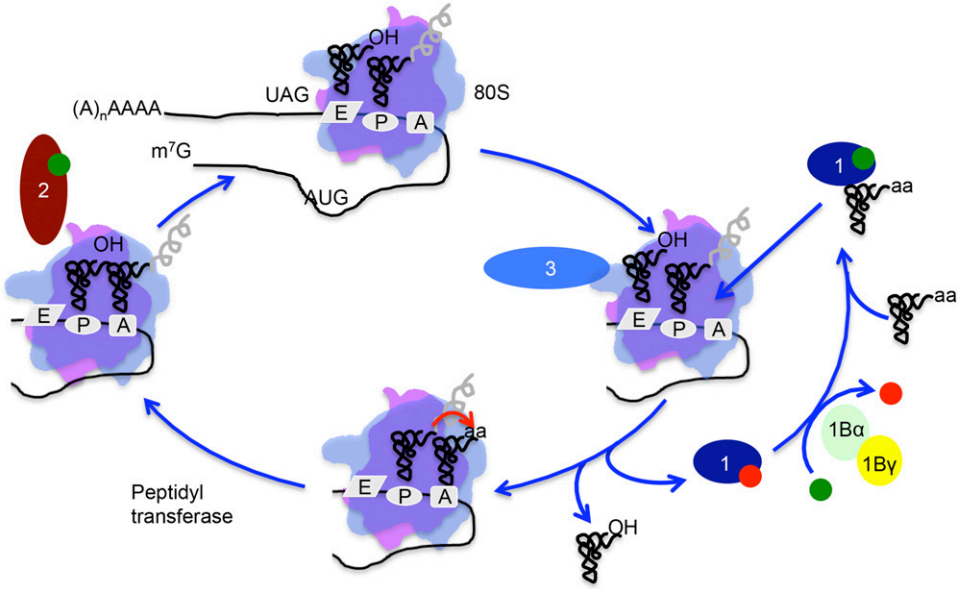

Figure 8 Model of yeast translation elongation. Starting at the top, an elongating ribosome contains a peptidyl-tRNA in the P site and a deacylated tRNA in the E site. eEF1A(1)–GTP (green circle) binds aa-tRNA for delivery to the cognate codon in the A site. The codon–anticodon match in the A site triggers conformational changes in eEF1A, GTP hydrolysis, and release of eEF1A–GDP (red circle), leaving the aa-tRNA in the A site. Guanine nucleotide exchange on eEF1A is catalyzed by the α-subunit of the eEF1Bαγ complex. Following ribosome-catalyzed formation of the peptide bind, the ribosomal translocase eEF2 (2)–GTP stimulates movement of the A-site peptidyl-tRNA to the P site and of the now deacylated tRNA in the P site to the E site. The fungal-specific and essential factor eEF3 (3) interacts with eEF1A and is proposed to assist in the release of the E-site tRNA to allow continued cycles of elongation.

on eEF1A is more complex than the larger, but functionally homologous, single polypeptide EF-Ts protein, the GEF for EF-Tu in bacteria. While both eEF1B and EF-Ts catalyze the release of GDP from the G protein, eEF1B has two subunits with the essential exchange function lodged in eEF1Bα. Unlike the two subunits in yeast eEF1B, other eukaryotic eEF1B is composed of three subunits, including the additional polypeptides eEF1Bβ in mammals or eEF1Bδ in plants. Whereas the critical function of eEF1Bα is dispensable when eEF1A is overexpressed, mutations impairing eEF1Bα alter the efficiency of nonsense and missense suppression in yeast (Carr-Schmid *et al.* 1999b; Plant *et al.* 2007). The eEF1Bα mutations likely result in a decreased pool of active eEF1A–GTP complexes, which in turn enables termination factors to effectively compete with eEF1A ternary complexes at stop codons, leading to decreased nonsense suppression. Despite their similar functions EF-Ts and eEF1Bα interact with their G-protein substrates in distinct manners. Whereas EF-Ts binds to domains I and III of EF-Tu (Kawashima *et al.* 1996), eEF1Bα interacts with domains I and II of eEF1A (Andersen *et al.* 2000, 2001). This different mode of binding may reflect an evolutionary difference in the cofactors of eEF1A, which include a conserved interaction with actin across eukaryotic species and, in yeast, an association with eEF3 (Anand *et al.* 2003, 2006). Notably, eEF1Bα competes with actin for binding to eEF1A (Pittman *et al.* 2009).

eEF2 and ribosomal translocation

eEF2 function: The role of G protein eEF2 is functionally comparable to bacterial translocase EF-G: translocation of the mRNA and peptidyl-tRNA from the A site to the P site of the ribosome. Structurally, bacterial EF-G and *S. cerevisiae* eEF2 are highly conserved. Genetic analysis of yeast eEF2 has demonstrated the key role of the tip of domain IV of eEF2 that enters the ribosomal A site (Taylor *et al.* 2007b). Much analysis has focused on His699, the modified residue (see below) at the tip. Viable mutants that alter His699 or other residues within the tip of domain IV cause modest defects in translational fidelity, including increased programmed −1 frameshifting (Ortiz *et al.* 2006). Analysis of the site of binding of Sordarin, a natural product inhibitor of eEF2, via mutational and structural analyses, has demonstrated that the compound binds between domains I, III, and V of eEF2, matching the sites of Sordarin-resistant mutations (Justice *et al.* 1998; Soe *et al.* 2007). These findings highlight the movements of domains I and II relative to III, IV, and V during translocation. Interestingly, a P596H mutation in human eEF2 has been linked to the neurodegenerative disorder, dominant spinocerebellar ataxia. The yeast equivalent, eEF2-P580H, exhibits an increased rate of −1 programmed ribosomal frameshifting (Hekman *et al.* 2012). It is noteworthy that cells carefully maintain the levels of eEF2. Any mutations that inactivate eEF2, yet produce a stable protein, cause a dominant-negative phenotype, apparently due to reduced levels of active WT eEF2 in the cell (Ortiz and Kinzy 2005), suggesting negative autoregulation of eEF2 levels.

Post-translational modifications of eEF2: Conserved His699 in eEF2 is post-translationally modified to diphthamide. Named for the source of the bacterial toxin that ADP-ribosylates the residue upon infection with *Corynebacterium diphtheriae*, studies in yeast have been instrumental in identifying and characterizing the seven gene products (*DPH1-7*) required for synthesis of diphthamide (Schaffrath *et al.* 2014). Interestingly, neither His699 (Kimata and Kohno 1994) nor any of the *DPH* genes except *KTI11*, which is also a subunit of the tRNA-modification complex Elongator, is essential *in vivo*. eEF2 is also subject to methylation. The

enzyme Efm3, encoded by *YJR129c* and related to human FAM86A, trimethylates eEF2 on Lys509, causing an increase in ribosomal frameshifting and increased sensitivity to antibiotics including sordarin (Davydova *et al.* 2014; Dzialo *et al.* 2014). Efm2 also dimethylates Lys613 (Couttas *et al.* 2012). In addition to these methylations, Thr557 is phosphorylated by the kinase Rck2 (Teige *et al.* 2001); however, this residue is not essential for eEF2 activity *in vivo* (Bartish *et al.* 2007).

The unique elongation factor eEF3

Proposed role in promoting tRNA release from the E site: The translation elongation factor 3 (eEF3) was first described in the 1970s as a novel activity required for protein synthesis in yeast (Skogerson and Wakatama 1976). Further analysis of eEF3 and its gene *YEF3* showed that eEF3 is essential for fungal translation and cell viability, yet is not found in mammals (Dasmahapatra and Chakraburtty 1981; Hutchinson *et al.* 1984; Sandbaken *et al.* 1990). Biochemical studies suggested that eEF3 links the A- and E-site activities of the ribosome by facilitating release of deacylated tRNA from the E site, which enables eEF1A delivery of aa-tRNA to the A site (Triana-Alonso *et al.* 1995). This function of eEF3 is based, in part, on the proposed allosteric model of aa-tRNA binding to the three binding sites on the ribosome. However, as the role of the ribosomal E site and the allosteric model of elongation are not fully resolved (Petropoulos and Green 2012), the critical role for eEF3 in translation elongation remains unclear. Despite this uncertainty in eEF3 function, both genetic and biochemical studies have revealed physical links between eEF1A and eEF3 that are important for protein synthesis (Anand *et al.* 2003).

eEF3 binding site on ribosome: The association of eEF3 with the ribosome is of fundamental interest because the requirement for eEF3 is defined by the source of ribosomes. Using eEF1A and eEF2 from yeast or mammals, the requirement for eEF3 is observed with yeast, but not with mammalian, ribosomes (Skogerson and Engelhardt 1977). The availability of both a high-resolution crystal structure of most of eEF3 and a cryo-EM reconstruction of eEF3 on the yeast ribosome revealed that eEF3 binds near the ribosomal E site (Andersen *et al.* 2006). eEF3 contains an N-terminal HEAT repeat domain as well as two ABC-type ATPase domains with a chromodomain-like insertion (residues 761–869) in the second ATP-binding domain. The chromodomain interacts through multiple sites with the ribosome. While mutation of ribosome-contacting residues in the chromodomain did not affect ribosome binding, they did impair cell growth, elongation rate, and ribosome-stimulated ATPase activity (Sasikumar and Kinzy 2014). Interestingly, many of the eEF3 mutations isolated in screens based on different eEF3 functions cause inhibition of the ATPase activity, indicating the central role of this activity to eEF3 function on the ribosome.

eEF3 related proteins in yeast and other eukaryotes: While most work on eEF3 has focused on the *YEF3* product, the form of the protein expressed under laboratory conditions, yeast *HEF3* encodes a protein 84% identical to eEF3 (Maurice *et al.* 1998; Sarthy *et al.* 1998). While *HEF3* cannot complement the lack of *YEF3* when expressed from its own promoter, it can when expressed from the *YEF3* promoter. As the *HEF3* promoter is active under conditions of zinc deficiency, the inability of *HEF3* to substitute for *YEF3* may simply reflect the lack of expression of *HEF3* under normal growth conditions. The role of the *HEF3*-encoded form of eEF3 remains unknown.

Traditionally, eEF3 has been viewed as a "fungal-specific" factor, since it was not found in mammals or plants. A partial eEF3-like protein has been described in *Chlorella* virus CVK2 (Yamada *et al.* 1993), and genes very similar to *YEF3* were recently found in many single cell eukaryotes. Consistent with these findings, 2D gel electrophoresis and MS analysis identified a protein with sequence similarity to eEF3 in *Phytophthora infestans* (Ebstrup *et al.* 2005). While none of these eEF3-like proteins have been shown to function like eEF3, the identification of these apparent eEF3 orthologs in other eukaryotes raises questions as to the functional distribution and evolution of this essential yeast protein.

eIF5A promotion of peptide bond formation

The translation factor eIF5A was originally identified as an initiation factor based on its ability to promote methionyl–puromycin synthesis (Kemper *et al.* 1976; Schreier *et al.* 1977; Benne *et al.* 1978; Benne and Hershey 1978), an *in vitro* assay designed to monitor first peptide bond synthesis. However, this assay also monitors the peptidyl-transferase activity of the ribosome. Yeast eIF5A is encoded by *TIF51A* (*HYP2*) and *TIF51B* (*ANB1*). Whereas *TIF51A* is expressed in aerobically grown yeast, *TIF51B* expression is restricted to anaerobic conditions (Lowry *et al.* 1983; Schnier *et al.* 1991). It is not clear why yeast differentially express the two forms of eIF5A that differ at only 15 of their 157 residues.

eIF5A is post-translationally modified by the conversion of a lysine residue to hypusine (Wolff *et al.* 2007; Dever *et al.* 2014). An n-butylamine group is transferred from spermidine to the ε-amino group of Lys51 in eIF5A to generate deoxyhypusine. This reaction is catalyzed by deoxyhypusine synthase, encoded by the essential *DYS1* gene (Kang *et al.* 1995; Sasaki *et al.* 1996; Park *et al.* 1998). In a second step, the enzyme deoxyhypusine hydroxylase, encoded by nonessential *LIA1*, hydroxylates the 2-position of the added moiety to generate hypusine (Park *et al.* 2006a).

Yeast eIF5A was found to interact with the translational machinery (Jao and Chen 2006; Zanelli *et al.* 2006; Saini *et al.* 2009), and depletion of eIF5A impaired protein synthesis and yeast cell growth (Kang and Hershey 1994; Saini *et al.* 2009; Henderson and Hershey 2011). Analysis of polyribosome profiles of yeast depleted of eIF5A or following inactivation of a temperature-sensitive eIF5A mutant revealed retention of polysomes in the absence of cycloheximide (CHX), indicating a defect in translation elongation (Gregio *et al.* 2009; Saini *et al.* 2009). However, it has also been reported that depletion of eIF5A causes a loss of polysomes,

suggesting that eIF5A may have a function in translation initiation or perhaps a specialized function in translation elongation (Henderson and Hershey 2011). Consistent with the translation elongation defect observed in the eIF5A temperature-sensitive mutant, purified eIF5A stimulated tripeptide synthesis in reconstituted yeast *in vitro* translation assays (Saini *et al.* 2009). An eIF5A function in elongation is consistent with identification of an eIF5A mutant as a suppressor of nonsense-mediated mRNA decay (Zuk and Jacobson 1998), which depends on translation elongation (Peltz *et al.* 1992; Beelman and Parker 1994).

A prokaryotic ortholog of eIF5A termed EF-P was recently shown to stimulate translation of polyproline (Doerfel *et al.* 2013; Hersch *et al.* 2013; Ude *et al.* 2013), and a similar function was identified for yeast eIF5A (Gutierrez *et al.* 2013). Partial inactivation of a temperature-sensitive eIF5A variant confers reduced expression of reporter genes or authentic yeast ORFs containing homopolyproline stretches (Gutierrez *et al.* 2013), leading to defects in fertility and polarized cell growth (Li *et al.* 2014). The requirement for eIF5A for synthesis of polyproline sequences was also observed *in vitro* and shown to be dependent on the hypusine modification in eIF5A. Directed hydroxyl radical probing experiments revealed eIF5A binds near the ribosomal E site with the hypusine residue in the vicinity of the acceptor stem of the P-site tRNA and the peptidyl-transferase center of the ribosome. Thus, eIF5A is proposed to insert its hypusine residue into the active site of the ribosome and promote peptide bond formation especially for poor substrates like polyproline (Gutierrez *et al.* 2013).

Ribosomal frameshifting

The yeast system has proven a valuable genetic tool for dissecting the elongation pathway and in particular the fidelity of protein synthesis *in vivo*. The stoichiometry of structural and catalytic proteins produced by Ty retrotransposons of yeast is determined by a "programmed" +1 translational frameshift. For both Ty1 and Ty3 retrotransposons, a +1 ribosomal frameshifting event establishes the levels of Gag *vs.* Gag–Pol fusion proteins. While the precise mechanism of ribosomal frameshifting differs for Ty1 and Ty3, in both cases frameshifting is triggered by a codon that is decoded by a rare tRNA (Belcourt and Farabaugh 1990; Farabaugh *et al.* 1993). The L-A double-stranded RNA virus of yeast likewise encodes both Gag and Pol proteins with the catalytic Pol synthesized as a Gag–Pol fusion by −1 ribosomal frameshifting (Dinman *et al.* 1991). Mutations predicted to impair recycling of eEF1A–GDP to eEF1A–GTP and thus slow A-site tRNA binding enhance +1 ribosomal frameshifting that occurs on the P site, whereas the fungal eEF2-specific antibiotic sordarin as well as mutations that impair eEF2 function specifically inhibit +1 frameshifting (Dinman and Kinzy 1997; Harger *et al.* 2001, 2002). In contrast, mutations thought to impair GTP hydrolysis by eEF1A increase the probability of −1 frameshifting (Dinman and Kinzy 1997; Harger *et al.* 2001). These results are consistent with the notion that +1 frameshifting occurs by miscoding in the P site, while −1 frameshifting occurs on ribosomes in which both the A and P sites are occupied by tRNAs (Harger *et al.* 2002).

Ribosomal frameshifting is also used to control the production of cellular proteins in yeast. Polyamine-regulated +1 ribosomal frameshifting on the *OAZ1* mRNA governs the synthesis of antizyme, a regulator of polyamine synthesis in cells (Palanimurugan *et al.* 2004; Kurian *et al.* 2011), and +1 frameshifting is also used to synthesize Est3, a subunit of telomerase (Morris and Lundblad 1997; Taliaferro and Farabaugh 2007), and Abp140 (Trm140), a tRNA methyltransferase (Asakura *et al.* 1998; D'Silva *et al.* 2011; Noma *et al.* 2011). Whereas +1 frameshifting can be triggered when the A site contains a stop codon or a sense codon decoded by a rare tRNA, −1 frameshifting relies on a slippery sequence that allows repairing of the A- and P-site tRNAs with the mRNA and on a downstream secondary structure that impedes forward movement of the ribosome. Computational approaches to identify slippery sites and potential downstream pseudoknot structures predict that as many as 10% of yeast genes, including several genes controlling telomere maintenance (Table 3, Advani *et al.* 2013), contain a frameshift signal (Jacobs *et al.* 2007; Belew *et al.* 2008) and that frameshifting on these sites impacts mRNA levels by activating the nonsense-mediated decay pathway (Belew *et al.* 2011).

Actin bundling and nontranslation functions of eEF1A

eEF1A has activities outside its canonical role in translation elongation, including functions in several steps of viral life cycles, apoptosis, actin bundling, and others in metazoans (Mateyak and Kinzy 2010). The interaction of eEF1A with actin was first identified in the slime mold *Dictyostelium discoideum* (Yang *et al.* 1990). Using a combination of genetic and/or biochemical approaches, this ability of eEF1A to bind actin and promote actin bundling was demonstrated to be conserved in both budding (Munshi *et al.* 2001) and fission yeast (Suda *et al.* 1999). Subsequent genetic studies in *S. cerevisiae* enabled an analysis of the functional interaction between these two highly abundant proteins *in vivo*. Utilizing both overexpression and mutational analysis, the actin-bundling activity of eEF1A was found to reside in multiple regions of the protein, in particular the N terminus and the C-terminal domain. Overexpression of eEF1A results in altered cell size, disorganization of the actin cytoskeleton, and accumulation of cells in the G1 phase of the cell cycle (Munshi *et al.* 2001), while a genetic screen designed to capitalize on these phenotypes identified a series of mutations in eEF1A that when coupled with an altered C terminus result in similar cellular effects (Gross and Kinzy 2005, 2007). The eEF1A mutant proteins, in particular the F309L and S405P mutants, reduced actin bundling *in vitro*, supporting the original biochemical data from the Condeelis laboratory (Yang *et al.* 1990), and caused defects in the cell cytoskeleton and morphology, revealing a critical biological role for this interaction. Most surprisingly, these eEF1A mutants showed

Table 3 Selected examples of translationally controlled yeast mRNAs

mRNA	Mechanism of control	References
Initiation control		
ASH1	RNA structure elements in 3′ UTR and coding region repress translation of unlocalized mRNA	Chartrand *et al.* 2002; Irie *et al.* 2002; Olivier *et al.* 2005; Paquin *et al.* 2007; Deng *et al.* 2008
BOI1, *FLO8*, *GIC1*, *MSN1*, *NCE102*, *PAB1*, *TIF4632*, *YMR181c*	eIF4G dependent, cap-independent element in 5′ UTR directs translation	Gilbert *et al.* 2007
CLN3	Contains uORF, expression affected by eIF4E activity	Polymenis and Schmidt 1997
ENO1, *FBA1*, *TPI1*	Translation induced by amino acid starvation, 5′ UTR dependent, A-rich region implicated for *ENO1*, *TPI1*	Rachfall *et al.* 2011
ERS1, *STE12*	Caf20 dependent translational repression	Castelli *et al.* 2015; Park *et al.* 2006b
FOL1, *MKK1*, *RPC11*, *TPK1*, *WSC3*	uORF represses translation	Zhang and Dietrich 2005
GCN4	Four uORFs regulate translation in response to amino acid levels via eIF2 phosphorylation	Hinnebusch 2005
GRS1, *ALA1*	Non-AUG initiation produces mitochondrial tRNA synthetase, leaky scanning to downstream in-frame AUG produces cytoplasmic enzyme	Chang and Wang 2004; Tang *et al.* 2004; Chen *et al.* 2008
HAC1	Long-range base pairing between intron and 5′ UTR sequences represses translation	Ruegsegger *et al.* 2001; Sathe *et al.* 2015
INO2	uORF represses protein expression in presence of inositol and choline	Eiznhamer *et al.* 2001
MOD5	Leaky scanning of first AUG directs tRNA modifying enzyme Mod5 to cytoplasm instead of mitochondria	Slusher *et al.* 1991
POM34	Translationally repressed by a complex involving Eap1, Asc1, Scp160, and Smy2	Sezen *et al.* 2009
SUI1	Poor start codon context enables autoregulation of eIF1 levels	Martin-Marcos *et al.* 2011
URE2	5′ cap-independent translation	Komar *et al.* 2003; Reineke and Merrick 2009
YAP1	uORF represses translation via a leaky scanning mechanism	Vilela *et al.* 1998; Zhou *et al.* 2001
Elongation control		
CPA1	uORF stalls ribosomes and induces mRNA decay; regulated by arginine levels	Gaba *et al.* 2001; Gaba *et al.* 2005
OAZ1	Polyamine-regulated ribosome +1 frameshifting	Kurian *et al.* 2011
TYA-TYB	Ribosome +1 frameshifting to produce Gag–Pol fusion protein	Belcourt and Farabaugh 1990; Farabaugh *et al.* 1993; Harger *et al.* 2001
L-A	Ribosome −1 frameshifting to produce Gag–Pol fusion protein	Dinman *et al.* 1991
EST3, *TRM140*	Ribosome +1 frameshifting	Morris and Lundblad 1997; Asakura *et al.* 1998; Taliaferro and Farabaugh 2007; D'Silva *et al.* 2011; Noma *et al.* 2011
EST1, *EST2*, *STN1*, *CDC13*	Ribosome −1 frameshifting	Advani *et al.* 2013
Termination control		
BSC1–BSC6, *IMP3*, *ZDS1*, *PDE2*	3–25% read through of stop codon to create an extended protein	Namy *et al.* 2001; Namy *et al.* 2002; Namy *et al.* 2003; Beznoskova *et al.* 2015

Genes are grouped by publication/similar mechanism. Many examples given rely on a single publication and detailed mechanisms remain unknown.

translation defects at the level of initiation and increased phosphorylation of eIF2α (Perez and Kinzy 2014). Deletion of the eIF2 kinase *GCN2* eliminated the initiation defect, but revealed an elongation defect. Taken together, these studies reveal linkages between the cellular cytoskeleton and translation and are consistent with early work highlighting this important interaction (Howe and Hershey 1984) .

Termination and recycling

Termination and ribosome recycling are linked processes critical to release the completed polypeptide and to provide a pool of 40S and 60S subunits for additional rounds of translation. Termination begins with recognition of one of the 3 stop codons in the A site by the release factor eRF1 (Sup45), which binds to the ribosome together with the GTP-bound form of factor eRF3 (Sup35). The eRF1, composed of three domains, functionally mimics a tRNA with the N-terminal domain recognizing the stop codon (Bertram *et al.* 2000), the central domain with its methylated GGQ motif promotes hydrolysis of the peptidyl-tRNA bond (Heurgue-Hamard *et al.* 2005), and the C-terminal domain interacts with eRF3. Recognition of all three stop codons by eRF1 is mediated by the YxCxxxF and TASNIKS motifs, as well as by other binding pockets/cavities in the N domain

(Conard *et al.* 2012; Blanchet *et al.* 2015). The interaction between eRF1 and eRF3 is critical for stop codon recognition (Wada and Ito 2014), and GTP hydrolysis by eRF3 facilitates eRF1 discrimination of stop codons (Salas-Marco and Bedwell 2004) and accelerates peptide release (Eyler *et al.* 2013). Upon GTP hydrolysis eRF3 dissociates, leaving eRF1 in the A site. Binding of Rli1, an ABC-family ATPase, promotes eRF1-mediated hydrolysis of the aminoacyl bond linking the polypeptide to the peptidyl-tRNA (reviewed in Dever and Green 2012). Structural analysis of yeast eRF1 in complex with eRF3 or Rli1 on the ribosome revealed conformational changes in eRF1, while eRF3 and Rli1 were bound to the intersubunit space overlapping the binding sites for eEF1A and eEF2 (Preis *et al.* 2014). In complex with eRF3, eRF1 interacts with the stop codon; while in complex with Rli1, eRF1 no longer interacts with the stop codon but the GGQ motif is positioned toward the peptidyl-transferase center to promote hydrolysis. These structural changes reveal an uncoupling of stop codon recognition from peptide release by eRF1 (Preis *et al.* 2014). As would be expected, both eRF1 and eRF3 are essential for yeast viability; however, the N terminus of eRF3 can be deleted. This N-terminal prion domain of eRF3 is the basis of the [*PSI*$^+$] aggregation of eRF3 (reviewed in Liebman and Chernoff 2012), resulting in impaired translation termination (Baudin-Baillieu *et al.* 2014).

Following release of the completed polypeptide, an 80S ribosome is bound to the mRNA with a deacylated tRNA in the P site base paired to the penultimate codon. ATP hydrolysis by Rli1 promotes release of the 60S subunit (Shoemaker and Green 2011). Depletion of Rli1 leads to aberrant reinitiation near the stop codon, leading to translation of 3′ UTR sequences (Young *et al.* 2015). In mammals, the protein Ligatin, or the complex of MCT-1 and DENR, promote release of mRNA and deacylated tRNA from the 40S subunit in the final step of recycling (Skabkin *et al.* 2010). The yeast orthologs of these 40S recycling factors [termed Tma64 (Ligatin), Tma20 (MCT-1), and Tma22 (DENR)] were previously identified as ribosome-associated proteins, and expression of human MCT-1 complemented translation defects in a strain lacking *TMA20* (Fleischer *et al.* 2006). These results suggest that the mechanism of ribosome recycling is well conserved between yeast and mammals.

Translational Control in Yeast

Many studies have examined how protein synthesis is controlled in yeast. One premise is that certain mRNAs are efficiently translated only when the encoded proteins are required at a specific location (*e.g.*, the growing bud tip) or time during the cell cycle, or in response to a specific stress. Where transcriptional control alone cannot provide a sufficiently rapid response or precise localization of proteins, translational regulation can provide the needed additional control. Progress has been made in uncovering the scope of translational controls in yeast, signal transduction pathways involved, and mRNA-specific elements that enable mRNAs to respond to the perceived demand. These studies show that translation is rapidly reprogrammed when cells experience changes in their external environment and that the translational adjustments are important for cells to adapt to the altered environment. Translational changes involve both global repression of translation of many mRNAs and activation of translation of specific stress-response genes. Yeast studies have provided molecular insights into mechanisms of control operating across eukaryotes. Indeed, the detailed understanding of *GCN4* translation is now widely viewed as primary evidence to support the scanning mechanism of translation initiation. In the sections below, we first outline global approaches used to address translation changes and then describe translational control by the 4E-BPs as well as control of the *GCN4* and *CPA1* mRNAs via distinct mechanisms.

Global approaches to studying translation controls

Global translational activity in cell populations is often monitored by polysome profile analysis. "Freezing" ribosomes on mRNAs with CHX immediately prior to cell harvest provides a snapshot of translation. CHX binds to the ribosome E site, preventing tRNA release and trapping ribosome–mRNA complexes (Schneider-Poetsch *et al.* 2010). In recent years, formaldehyde cross-linking has also been used to preserve factor–ribosome interactions (Valasek *et al.* 2007), while for high-throughput sequencing applications, adding CHX at very high concentrations or only during cell lysis is now favored to avoid possible artifacts (Gerashchenko and Gladyshev 2014).

For polysome profile analyses, lysates are sedimented through sucrose gradients and then fractionated to generate an absorbance trace of rRNA that reveals a snapshot of the ribosome distribution (Pospisek and Valasek 2013). In actively growing cells, most ribosomes (85 $\pm$ 5%) are engaged in translation. mRNAs have a mean density of 0.64 $\pm$ 0.31 ribosomes per 100 nt coding region, with the density varying from $<$0.1 to $>$1.6 between mRNAs. Typically, longer ORFs have lower ribosome density (Arava *et al.* 2003). Stressed cells, or those with mutations that inhibit global translation initiation, exhibit an increased proportion of 80S ribosomes [monosomes and 80S couples (associated 40S and 60S subunits not bound to an mRNA)] relative to polysomes. Northern blotting or RT-PCR analysis of polysome gradient fractions have been used to study specific candidate genes, while microarray and high-throughput sequencing approaches such as ribosome footprint profiling (ribo-seq) have been used to assess changes in translation across multiple mRNAs simultaneously (Ingolia *et al.* 2009).

Stresses found to deplete bulk polysomes include the sudden withdrawal of glucose (Ashe *et al.* 2000; Arribere *et al.* 2011; Vaidyanathan *et al.* 2014) or amino acids (Smirnova *et al.* 2005); nutrient limitation that induces sporulation (Brar *et al.* 2012); temperature shift (Preiss *et al.* 2003); and the addition of cellular stress agents: hyperosmotic salt (Melamed *et al.* 2008), hydrogen peroxide (Shenton *et al.*

2006), fusel alcohols (Smirnova *et al.* 2005), or drugs: rapamycin (Preiss *et al.* 2003), calcofluor-white (Halbeisen and Gerber 2009), and chlorpromazine (Deloche *et al.* 2004). These studies have shown that there is widespread reprogramming of translation following stress with diminished ribosome association of some mRNAs hypersensitive to stress, while the translation of other mRNAs is relatively resistant to the stress. Moreover, the mechanisms of translational reprogramming are stress specific. For example, the global response to amino acid starvation is dependent on Gcn2 and eIF2α phosphorylation, while the response to hydrogen peroxide involves inhibiting both initiation via Gcn2 and translation elongation (Shenton *et al.* 2006), causing pausing at aspartic acid and serine codons according to ribo-seq experiments (Pelechano *et al.* 2015); however, antioxidant-response mRNAs become well translated (Shenton *et al.* 2006; Kershaw *et al.* 2015). The response to glucose depletion is rapid and does not require eIF2α phosphorylation, with almost all ribosomes being lost from mRNAs within 1–2 min (Ashe *et al.* 2000), perhaps via alterations in eIF4A function (Castelli *et al.* 2011).

Unexpectedly, some mRNAs, translationally activated 15 min after glucose withdrawal, share the same promoter sequence that binds Hsf1 (Zid and O'Shea 2014), suggesting that transcription and mRNA nuclear history might contribute to active translation during stress (Zid and O'Shea 2014). The fate of translationally repressed mRNAs following glucose depletion has also been analyzed. mRNAs enter cytoplasmic foci termed P bodies or stress granules or are degraded (Hoyle *et al.* 2007; Buchan *et al.* 2008; Arribere *et al.* 2011). P bodies and stress granules form with different kinetics and contain different protein components (Hoyle *et al.* 2007; Buchan *et al.* 2008) and repressed mRNAs enter P bodies at different times after stress (Simpson *et al.* 2014). After several hours of starvation, growth resumes with a distinct pattern of highly translated mRNAs, allowing enhanced synthesis of mitochondrial-targeted proteins (Vaidyanathan *et al.* 2014). Altered protein kinase A signaling contributes to the translational reprograming (Ashe *et al.* 2000; Tudisca *et al.* 2012; Vaidyanathan *et al.* 2014). Hence diverse cellular stress conditions can rapidly perturb global and mRNA-specific protein synthesis via multiple mechanisms to achieve stress-specific translational reprogramming. In addition to revealing translation changes under stress conditions, ribosomal profiling techniques have provided mechanistic insights into elongation through the use of distinct inhibitors (Lareau *et al.* 2014) as well as by identifying specific pauses during elongation (Pelechano *et al.* 2015).

Regulating eIF4E–eIF4G interactions by 4E-BPs

In higher eukaryotes, 4E-BPs play a prominent role in controlling eIF4E function and cellular translation (Richter and Sonenberg 2005). The 4E-BPs are a diverse set of proteins that share a common, albeit rather degenerate, motif YxxxxLΦ (where x is any residue and Φ is hydrophobic) that enables them to compete with eIF4G for binding to the surface of the cap-binding protein eIF4E. The 4E-BPs regulate a variety of cellular and developmental processes in higher eukaryotes (Kong and Lasko 2012). Yeast has two characterized 4E-BPs that contain the consensus eIF4E-binding motif, Caf20 (also called p20) and Eap1, which are 18 and 70 kDa, respectively (Altmann *et al.* 1997; Cosentino *et al.* 2000). Caf20 and Eap1 share no sequence similarity outside the eIF4E-binding motif, mutation of which abrogates eIF4E interactions (Altmann *et al.* 1997; Cosentino *et al.* 2000; Ibrahimo *et al.* 2006). Because mutations in eIF4E have differential impacts on the binding of eIF4G and Caf20, the eIF4E-interaction interfaces with eIF4G and Caf20 are likely overlapping, but distinct (Ptushkina *et al.* 1998). This potentially enables Caf20 to displace eIF4G from eIF4E. Recent structural studies show higher eukaryote 4E-BPs share similar eIF4E-binding properties (Peter *et al.* 2015). These data suggest the yeast proteins are parallels of the higher eukaryote 4E-BPs.

Both 4E-BPs are nonessential and deletion mutants display normal polysome profiles in optimum growth conditions (Cridge *et al.* 2010). The 4E-BPs play nonredundant roles in the adaptive growth response to nitrogen limitation as deletion mutants prevent pseudohyphal development and invasive growth of the Σ1278b strain (Ibrahimo *et al.* 2006), while S288c deletion strains are sensitive to growth on alternative nitrogen sources (Cridge *et al.* 2010). Translational repression upon cell treatment with the antipsychotic drug chlorpromazine or the oxidants cadmium and diamide are partially dependent upon Eap1 (Deloche *et al.* 2004; Mascarenhas *et al.* 2008), while *caf20Δ* displays synthetic growth phenotypes when combined with *tif3Δ* (eIF4B deletion) (de la Cruz *et al.* 1997) or the *tif4631-459* allele that disrupts eIF4G1 binding to eIF4E (Hershey *et al.* 1999). These phenotypes are consistent with both 4E-BPs being translational repressors.

Targeted and global studies have been used to identify mRNA targets of Caf20 and Eap1 regulation. For example, the expression of *STE12*, *GPA2*, and *CLN1* was shown to be translationally upregulated in *caf20Δ* cells during filamentous growth, and *STE12* regulation was dependent on Caf20 (Park *et al.* 2006b). Eap1 (together with Scp160, Asc1, and Smy2) is involved in translational repression of the *POM34* mRNA (Sezen *et al.* 2009). These data support the idea that the 4E-BPs can interact with and repress the translation of particular mRNA targets, possibly via interactions with other sequence-specific mRNA binding proteins, similar to examples from higher eukaryotes (Kong and Lasko 2012).

Microarray analysis of polysome-associated mRNAs identified >1000 genes whose polysome association was affected by deletion of either 4E-BP (Cridge *et al.* 2010). A computational analysis suggested that Caf20 binds mRNAs with structured 5′ UTRs (Cawley and Warwicker 2012). High-throughput sequencing of mRNAs associated with TAP-tagged Caf20 or Eap1 revealed that the two 4E-BPs bind mainly the same set (>1000) of longer than average mRNAs, suggesting that translation of these mRNAs is dampened or that their translation is poised for repression by 4E-BP binding (Costello *et al.*

2015). Most 4E-BP-bound mRNAs were also enriched in eIF4E interaction, as expected if the 4E-BPs repress translation via their interaction with eIF4E. However, some 4E-BP-bound mRNAs were not enriched for eIF4E, suggesting that the 4E-BPs can act independently of eIF4E (Costello *et al.* 2015). Over 100 mRNAs were found to interact with Caf20 independently of its ability to bind eIF4E (Castelli *et al.* 2015). The 3′ UTR of one mRNA tested (*ERS1*) directed Caf20-mediated repression of translation, and this regulation could be transplanted to a heterologous reporter (Castelli *et al.* 2015). In summary, both 4E-BPs can compete with eIF4G for binding to eIF4E on many mRNAs, resulting in impaired translational efficiency. However, the 4E-BPs may also act as repressors independently of eIF4E on some mRNAs.

Translationally regulated mRNAs

There are many examples of translationally controlled mRNAs in yeast. General features of translational control at the initiation phase include regulated recognition of uORFs by scanning ribosomes (*GCN4*), inhibition of scanning by mRNA secondary structure (*HAC1*), leaky scanning to initiate internally (*MOD5* and *GRS1*), or translational repression during mRNA localization through recognition of mRNA structural elements by RNA-binding proteins (*ASH1*). In addition, the elongation and termination phases of translation can be regulated. For example, ribosome stalling (*CPA1*) as well as frameshifting (retrotransposons, L-A virus, and *OAZ1*) and stop codon read-through can lead to the production of alternate proteins. Programmed frameshifting can further alter the mRNA levels by triggering decay such as for *EST1*, *EST2*, *STN1*, and *CDC13* (Advani *et al.* 2013).

In addition to the cap-dependent scanning model of translation initiation, an alternative cap-independent mode of translation has been proposed for some mRNAs in higher organisms (Jackson 2013). While this alternate mode of translation initiation is best characterized for viral mRNAs, some cellular mRNAs may also employ this alternate means of translation initiation (Gilbert 2010; Jackson 2013). In general, cap-independent initiation is thought to rely on special secondary structure elements in the mRNAs to recruit translation factors and/or the 40S subunit (or PIC). In experiments employing *in vitro* translation assays and mRNA electroporation experiments, the translation of several yeast mRNAs encoding proteins required for invasive growth was maintained when the canonical m^7GTP cap was replaced by an ApppG cap structure (Gilbert *et al.* 2007). This cap-independent translation was dependent on eIF4G and was attributed to a poly(A) element in the 5′ UTR of the mRNAs (Gilbert *et al.* 2007). Similarly, the *URE2* mRNA was reported to direct the synthesis of both full-length Ure2 and an N-terminally truncated Ure2 that initiates from an internal AUG codon (Komar *et al.* 2003). Interestingly, synthesis of the truncated Ure2 was maintained upon genetic inactivation of the mRNA cap-binding protein eIF4E and when sequences with high secondary structure were inserted in the 5′ UTR to block scanning ribosomes (Komar *et al.* 2003). This putative internal initiation of translation on the *URE2* mRNA was enhanced in cells lacking the protein eIF2A (YGR054w) (Komar *et al.* 2005) and was found to depend on a 104-nt A-rich stem-loop element that includes the internal AUG start codon (Reineke *et al.* 2008; Reineke and Merrick 2009). Currently it is unclear under what conditions cap-independent translation will be important in yeast and whether the mRNA elements that support cap-independent translation might function as general translational "enhancers" to help the mRNAs compete for the translational apparatus by the conventional cap-dependent pathway (Gilbert 2010).

Selected examples of translationally controlled mRNAs and associated references are given in Table 3. Detailed discussions of *GCN4* and *CPA1* control mechanisms are presented below.

GCN4

Probably the best-characterized example of regulation of protein synthesis in yeast is *GCN4*. Environmental signals are transduced to modulate start codon selection on the *GCN4* mRNA and limit production of the Gcn4 protein to specific cellular stress conditions. Gcn4 is a basic leucine zipper (bZIP) transcriptional activator of amino acid and related biosynthetic genes (Natarajan *et al.* 2001). *GCN4* mRNA translation is controlled by a reinitiation mechanism that requires an interplay of sequences in its 5′ leader including uORFs with translation initiation factors and ribosomes. Under nonstarvation conditions the flow of ribosomes to the *GCN4* AUG start codon is limited by up to ~100-fold and *GCN4* translation is repressed unless cells are starved (Hinnebusch 2005).

Following starvation for one or more amino acids, a signaling pathway, termed general amino acid control, is deployed, which activates Gcn2 to phosphorylate eIF2α (Dever *et al.* 1992) causing inhibition of eIF2B (Pavitt *et al.* 1998) and a reduction in eIF2 TC levels (Dever *et al.* 1995). The lower level of TC leads to reduced ribosome engagement with most mRNAs; however, paradoxically, more ribosomes reach the *GCN4* AUG codon and Gcn4 levels increase by up to 10-fold (Albrecht *et al.* 1998). Because *GCN4* translation is acutely sensitive to the levels of active TC, it has proved an extremely useful tool to probe the role of translation factors in the scanning mechanism of protein synthesis as well as many of the details of translational control by eIF2 kinases (Hinnebusch 2005; Hinnebusch 2011).

Translation reinitiation at uORFs represses GCN4 expression: The 5′ leader of the *GCN4* mRNA is unusually long (591 nucleotides) and contains four short uORFs (uORF1–uORF4), each encoding either a di- or tripeptide product (Figure 9A) (Hinnebusch 1984). Extensive mutational analyses of the 5′ leader have shown that the uORFs are essential for mediating both the repression under replete conditions and for induction of Gcn4 levels under stress (Mueller and Hinnebusch 1986). uORFs 1 and 4 are both critical and have opposing roles (Mueller *et al.* 1988). Exquisite genetic

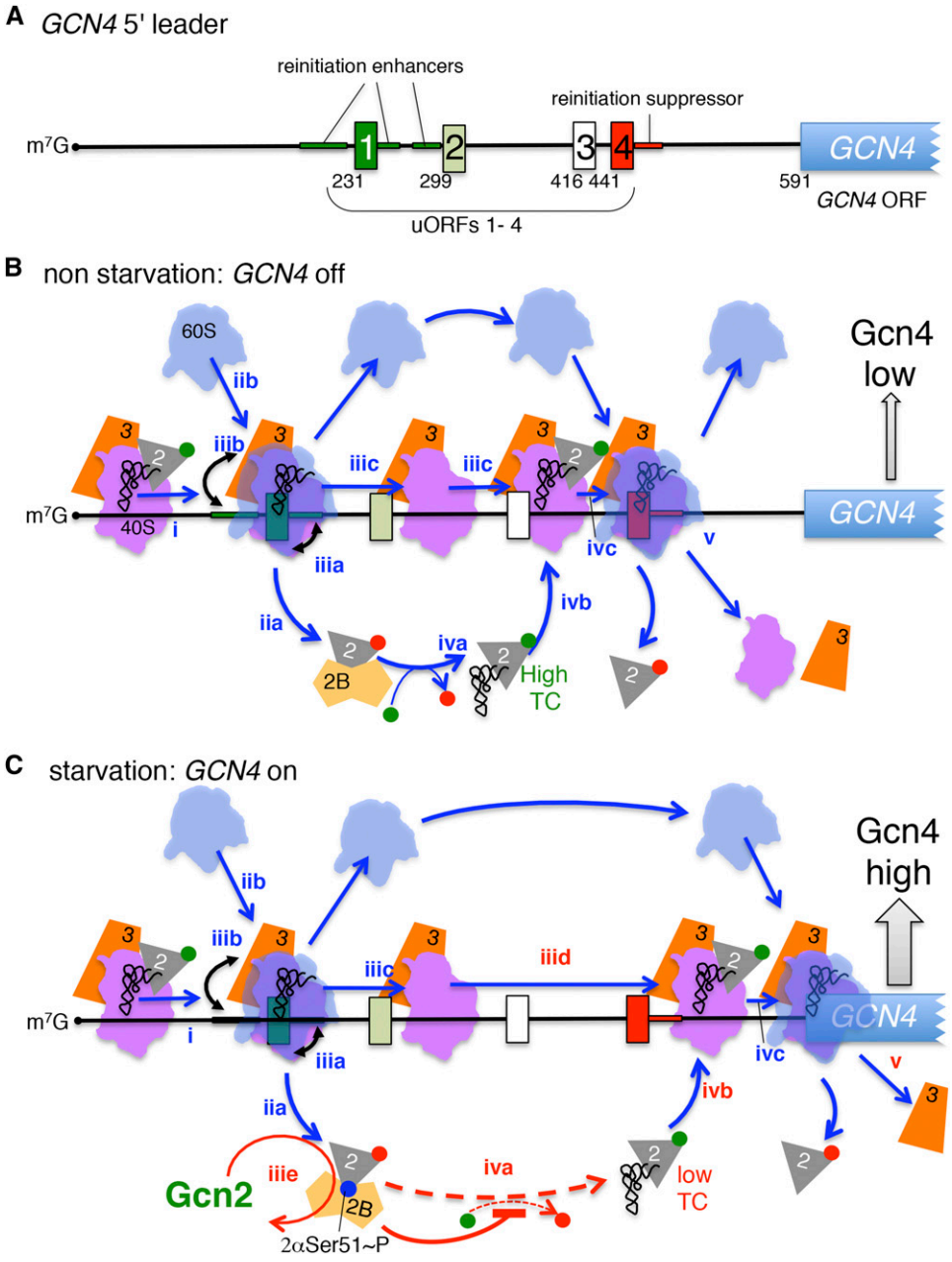

Figure 9 Translational regulation by reinitiation on *GCN4* mRNA. (A) The *GCN4* 5′ leader sequences showing uORFs 1–4 and the start of the *GCN4* ORF as filled boxes in their relative positions. The nucleotide positions of each AUG codon are shown relative to the transcription start site. The approximate location of reinitiation enhancer and suppressor sequences is indicated. (B) Reinitiation model in nonstarvation conditions with stepwise depiction of ribosomes and key factor interactions with the *GCN4* leader sequence (cartoons as per Figure 1). Blue arrows depict the movement and blue numbered steps (i–v) are explained in the main text. Note: uORF spacing has been altered to accommodate the ribosome cartoons. As depicted, following uORF1 translation high TC levels enable reinitiation at uORF4 leading to ribosome disengagement from the mRNA and *GCN4* expression is repressed. (C) Reinitiation model under amino acid starvation conditions. Initial steps through translation of uORF1 (blue numbered i–iiic) are unchanged from nonstarvation conditions. Subsequent steps (red numbered iiid–v) are altered by activation of the eIF2α kinase Gcn2 (step iiie) resulting in low levels of TC. Ribosomes traverse past uORF4 without initiating and then reacquire TC (step ivb). The scanning ribosome (step ivc) recognizes the *GCN4* start codon and *GCN4* expression is derepressed.

experiments coupled with biochemistry have established and tested a model for control (Figure 9 and described here).

Ribosomes bind the *GCN4* mRNA close to the 5′ cap and follow the normal scanning mechanism for ribosome recruitment and AUG recognition to translate uORF1 (Figure 9B, step i). Rather than completely dissociating following translation of the uORF1 tripeptide, a portion of ribosomes (estimated as ~50%) remains attached to the mRNA. This aberrant termination/ribosome-release cycle permits reinitiation to occur at a subsequent downstream AUG codon. It is likely that only the 40S ribosome remains attached to the mRNA following termination at uORF1 and that the 40S retains some bound translation factors, including eIF3 (Szamecz *et al.* 2008; Munzarova *et al.* 2011; Peter *et al.* 2015). The 40S lacks eIF2, as this factor was released at AUG recognition (Figure 9B, step iia) and prior to 60S joining (Figure 9B, step iib). This release of eIF2 is important for the mechanism of control. As uORF1 encodes only a tripeptide, the ribosome has not cleared the AUG codon before encountering the stop codon. Reporter analyses suggest that reinitiation can be efficient when uORFs are shorter than 35 codons (Poyry *et al.* 2004; Rajkowitsch *et al.* 2004).

To reinitiate at a downstream ORF, the 40S subunit must resume migration along the 5′ leader sequence. The precise factor requirements for resumed ribosome movement are not clear. The eIF2–GTP–Met-tRNA$_i^{Met}$ TC is not necessary for 40S movement, but is required for AUG recognition by the tRNA$_i^{Met}$ anticodon. Ribosomes migrating downstream of

uORF1 must reacquire TC before reinitiating translation. Under nonstarvation conditions, TC levels are not limiting and reinitiation is efficient. Translation of uORF4 (or uORF3) is sufficient to prevent almost all ribosomes from reaching the *GCN4* AUG codon because translation of uORF4, also encoding a tripeptide, does not favor reinitiation. In contrast to uORF1, uORF4 favors release of translating ribosomes and prevents resumed scanning/reinitiation events. These differences in reinitiation properties following translation of uORF1 *vs.* uORF4 are determined by the sequences flanking the uORFs (Figure 9A), which for uORFs 1 and 2, favor 40S and eIF3 retention.

Enhancer elements both 5′ and 3′ of uORF1 act to promote retention of 40S ribosomes (Grant and Hinnebusch 1994; Grant *et al.* 1995). The 5′ reinitiation enhancer region (5′ RER) was shown to interact with eIF3a bound to the 40S (Szamecz *et al.* 2008). The 5′ RER contains four distinct elements, two of which are important for eIF3a binding (Munzarova *et al.* 2011). Based on its ribosome-binding properties, eIF3a bridges the mRNA interaction to the 40S protein Rps0A located near the 40S mRNA exit channel (Szamecz *et al.* 2008). The 5′ RER acts in concert with the AU-rich sequences immediately downstream of the uORF1 stop codon to promote reinitiation (Miller and Hinnebusch 1989; Grant and Hinnebusch 1994; Rajkowitsch *et al.* 2004). On encountering the uORF1 stop codon, the 3′ enhancer element interacts with the terminating ribosome mRNA entry channel retaining the 40S (Figure 9B, step iiia). The 5′ RER, which has emerged from the mRNA exit channel of the ribosome, contacts eIF3a (Figure 9B, step iiib) stabilizing eIF3 and the 40S on the *GCN4* mRNA (Szamecz *et al.* 2008). To facilitate 40S migration downstream, presumably eIF1 and eIF1A are recruited (or retained) to promote ribosome movement in a 3′ direction (Figure 9B, step iiic) (Passmore *et al.* 2007).

Although uORFs 2 and 3 are not necessary for regulated control of *GCN4*, uORF2 functions similarly to uORF1 and promotes reinitiation by retaining eIF3–40S interactions. Thus uORF2 is suggested to act as a "fail safe" to catch any scanning ribosomes that do not initiate at uORF1 (Gunisova and Valasek 2014). Ribo-seq experiments identified two non-AUG initiating uORFs positioned upstream of uORF1 (Ingolia *et al.* 2009). Although translation of one these elements was confirmed with reporter constructs, these noncanonical uORFs are not necessary for *GCN4* translational control (Zhang and Hinnebusch 2011).

Efficient recycling of eIF2–GDP to eIF2–GTP by eIF2B (Figure 9B, step iva) favors reacquisition of TC and eIF5 by 40S ribosomes engaged with the *GCN4* mRNA (Figure 9B, step ivb) and thus will promote reinitiation at uORF3 or uORF4 (Figure 9B, step ivc). The sequences 3′ of uORF4 are GC rich and do not favor reinitiation (Grant and Hinnebusch 1994). Following translation of uORF4, ribosomes terminate and disengage from the mRNA, the *GCN4* ORF is not translated, and Gcn4 levels are repressed (Figure 9B, step v).

Delayed reinitiation activates GCN4 translation in starved cells: Under amino acid starvation, the mechanism of cap-dependent initiation at uORF 1 and resumed 40S movement are as described for nonstarvation cells (Figure 9C, steps iiia–c). An important distinction in starved cells is that following translation of uORF1 40S ribosomes migrate for a longer time and further along the *GCN4* leader than in unstarved cells (Figure 9C, step iiid). This is caused by activation of the eIF2α kinase Gcn2, which phosphorylates eIF2α on Ser51 (Figure 9C step iiie). As eIF2α phosphorylation inhibits the activity of its GEF eIF2B (Figure 9C step iva), reduced TC levels and impaired global translation initiation ensue (Figure 7). This slows the rate of reacquisition of TC by ribosomes migrating along the *GCN4* mRNA, enabling 40S bypass of uORFs 2–4 before TC binding (Figure 9C, step iiid). TC binding to the 40S in the interval between uORF4 and the *GCN4* AUG codon, enables ribosomal scanning and recognition of the *GCN4* start codon. Subsequent joining of the 60S subunit (Figure 9C, step ivc) permits translation of *GCN4*. As translation elongation proceeds on the *GCN4* ORF, eIF3 will be lost (Figure 9C, step v). Hence, the balance between reinitiation at the uORF4 and *GCN4* AUG codons facilitates control of *GCN4* translation (Hinnebusch 2005, 2011). Importantly, this model of *GCN4* translational control is supported by an extensive series of experiments employing mutated *GCN4* leader sequences with altered secondary structure, codons, uORF lengths, and uORF spacings (Miller and Hinnebusch 1989; Abastado *et al.* 1991; Grant and Hinnebusch 1994; Grant *et al.* 1994, 1995). In addition, ribosomal toe printing on *in vitro* translated *GCN4* mRNAs (Gaba *et al.* 2001) provide further support for the model and confirm the role of eIF2 levels in regulating reinitiation at uORFs 3 and 4 *vs.* the *GCN4* start codon.

Gcn1 and Gcn20 sense amino acid levels to activate Gcn2: *GCN4* translation is regulated physiologically by eIF2α phosphorylation, primarily in response to amino acid limitation. Aminoacyl tRNA synthetases bind specific free amino acids and deacylated tRNAs to "charge" the latter for use in protein synthesis. When one or more amino acids becomes limiting, cells accumulate higher levels of deacylated tRNAs (Zaborske *et al.* 2009). Uncharged (deacylated) tRNAs directly bind and activate Gcn2 (Dong *et al.* 2000). Gcn2 is one of a family of protein kinases that phosphorylate eIF2α on Ser51 to regulate global protein synthesis across eukaryotes (Wek *et al.* 2006; Dever *et al.* 2007). In its basal state, the Gcn2 kinase is inactive, and in response to an activation signal, conformational changes and altered interactions within the multidomain protein enable its eIF2α kinase activity (Qiu *et al.* 2001, 2002; Padyana *et al.* 2005; Garriz *et al.* 2009; Lageix *et al.* 2014).

Gcn2 is a 190 kDa protein composed of a central kinase domain (KD) surrounded by domains necessary to regulate its function and interactions, including a Gcn1-binding domain and pseudokinase domain N terminal to the KD, and a histidyl-tRNA synthetase-related (HisRS) domain followed by a ∼160 residue CTD that binds 60S subunits (ribosome

binding domain, RBD). Gcn2 is a dimer with multiple intermolecular interactions between monomers as well as intramolecular interactions between adjacent domains and longer range interactions between the KD and the RBD within Gcn2 monomers (Ramirez *et al.* 1992; Zhu *et al.* 1996; Qiu *et al.* 1998, 2001; Padyana *et al.* 2005; Garriz *et al.* 2009; Lageix *et al.* 2014). Binding of uncharged tRNA to the HisRS domain (Zhu *et al.* 1996; Dong *et al.* 2000) stimulates autophosphorylation of the activation loop in the KD on Thr882 and Thr887 (Romano *et al.* 1998) enabling phosphorylation of its only known substrate eIF2α (Dey *et al.* 2005, 2007; Padyana *et al.* 2005).

Inactivating mutations in Gcn1 or Gcn20, an ABC-type ATPase, prevent Gcn2 activation in response to amino acid starvation (Marton *et al.* 1993; Vazquez de Aldana *et al.* 1995). Both Gcn1 and Gcn20 bind elongating ribosomes (Marton *et al.* 1997) via the Gcn1 NTD, and this binding is required for Gcn2 activation (Sattlegger and Hinnebusch 2005). A Gcn2–Gcn1–Gcn20 complex forms by interactions between the Gcn1 central region, which is homologous to eEF3, and the Gcn20 NTD, as well as by the Gcn1 CTD binding the Gcn2 NTD (Sattlegger and Hinnebusch 2000; Kubota *et al.* 2001). A model for Gcn2 activation is based in part on Gcn1–Gcn20 homology with eEF3. It proposes that deacylated (uncharged) tRNA binding to the ribosomal A site is the amino acid starvation signal sensed by Gcn1–Gcn20. They pass deacylated tRNA from the A site to the Gcn2 HisRS domain, promoting Gcn2 activation and the chain of regulatory events that lead to *GCN4* translation (Hinnebusch 2005).

Other regulators of Gcn2: Gcn2 engages in cross-talk with other nutrient-sensing regulatory pathways. The TOR complex 1 (TORC1), important for controlling cell growth and rRNA synthesis among other targets (Martin *et al.* 2006), senses nitrogen and carbon sufficiency (Beck and Hall 1999; Loewith and Hall 2011). TORC1 deploys a complex phosphatase-signaling network involving Tap42 and Sit4 (Loewith *et al.* 2002) and indirectly mediates the inhibitory phosphorylation of Gcn2 on Ser577, providing a link between nitrogen and amino acid signaling (Cherkasova and Hinnebusch 2003). Inhibition of TORC1 activates Sit4 leading to dephosphorylation of Ser577 and constitutive activation of Gcn2 via enhanced binding of uncharged tRNAs to the Gcn2 HisRS domain (Garcia-Barrio *et al.* 2002; Kubota *et al.* 2003). Genome-wide studies confirm that Gcn4 targets are activated by rapamycin treatment, an allosteric TORC1 inhibitor (Staschke *et al.* 2010).

Snf1 senses low glucose levels in yeast and also promotes Gcn2 activity upon amino acid starvation (Cherkasova *et al.* 2010). In addition to nutrient-sensing kinases, the protein Yih1 regulates Gcn2 activity. Yih1 resembles the Gcn2 NTD and, when overexpressed, interacts with Gcn1 and prevents it from activating Gcn2 (Sattlegger *et al.* 2004, 2011). It is not yet clear how Yih1 interactions with Gcn1 are regulated to control Gcn2 activation.

Dephosphorylation of eIF2α to reset GCN4 control: When amino acids are no longer scarce, high levels of *GCN4* translation and Gcn2 activity are not required and eIF2α phosphorylation levels fall. The essential type 1 protein phosphatase Glc7 has a broad range of substrates including eIF2α (Wek *et al.* 1992). Typically, Glc7 is targeted to its substrates via interactions with dedicated regulatory (targeting) subunits (Cannon 2010). However an N-terminal extension unique to budding yeast eIF2γ (Gcd11) contains a PP1-docking motif that targets Glc7 to eIF2 to dephosphorylate eIF2α (Rojas *et al.* 2014). Glc7 may not be the sole eIF2 phosphatase. Like Glc7 mutants, PP2A/Sit4 and PP2C/Ptc2 mutants also increase eIF2α phosphorylation levels (Taylor *et al.* 2010), and Sit4 can interact with eIF2α (Cherkasova *et al.* 2010). Thus, multiple phosphatases may contribute to eIF2α dephosphorylation.

Arginine-regulated ribosome stalling controls CPA1 translation

CPA1 encodes the small subunit of carbamoyl phosphate synthetase, an enzyme that catalyzes a step in the synthesis of citrulline, an intermediate in the arginine biosynthesis pathway. *CPA1* mRNA translation is regulated by a uORF, and by Arg, via a ribosome-stalling mechanism that is conserved across fungi (Hood *et al.* 2007). The *CPA1* uORF, *YOR302W*, encodes a 25 amino acid arginine attenuator peptide (AAP). The peptide sequence, especially residues 6–23, is critical for translational repression by Arg (Werner *et al.* 1987; Delbecq *et al.* 2000; Hood *et al.* 2007). Many single missense mutations in the uORF eliminate Arg-controlled ribosome stalling (Delbecq *et al.* 2000). Of these, the D13N mutant has been used widely to inform mechanistic understanding (Wang *et al.* 1999; Gaba *et al.* 2001). In contrast to *GCN4*, most nucleotide sequences around the *CPA1* uORF are not important (Delbecq *et al.* 1994).

In the absence of Arg, both the uORF and *CPA1* are translated. The uORF AUG context is poor, ensuring that some scanning PICs bypass the uORF AUG and instead initiate translation at *CPA1* (Werner *et al.* 1987). This was confirmed by ribosome toe print analyses using a uORF-regulated luciferase reporter and yeast translation extracts (Gaba *et al.* 2001), as well as via ribo-seq experiments (Ingolia *et al.* 2009). There is no evidence supporting ribosome reinitiation after uORF translation. The presence of high Arg concentrations induces ribosome stalling at the uORF stop codon, with 80S complexes retaining a P-site tRNA-linked nascent peptide within the ribosome exit tunnel. The stalled 80S prevents any PICs that leaky scan through the uORF AUG codon from progressing to the *CPA1* AUG codon (Wang *et al.* 1999; Gaba *et al.* 2001). This ensures Cpa1 levels drop when Arg is abundant.

CPA1 control is not mediated by monitoring tRNA Arg charging levels and the *Saccharomyces* AAP sequence does not contain Arg residues (Wang *et al.* 1999). Hence it is mechanistically distinct from both *GCN4* uORF control and the ribosome stalling associated with Trp attenuation in

bacteria. Studies performed using the orthologous *Neurospora crassa Arg-2* locus have helped inform the mechanism of peptide and Arg-induced stalling. Control requires Arg itself, which alters interactions between the P-site tRNA/nascent AAP and both rRNA and ribosomal proteins within the PTC and the peptide exit tunnel of the 60S. Although ribosome stalling naturally occurs at the end of the AAP sequence, it was shown that Arg-dependent stalling occurs during translation elongation as removing the stop codon to extend the peptide or transferring the AAP sequence to the middle of a reporter gene generated novel Arg-mediated ribosome-stalling contexts (Wang *et al.* 1998; Fang *et al.* 2004). Structural analysis of the 80S-bound stalled nascent peptide by cryo-EM revealed that residues 10–24 of the *N. crassa* AAP (equivalent to residues 11–25 of yeast AAP) forms an α-helix within the exit tunnel and that AAP makes a series of contacts with tunnel-exposed conserved 28S rRNA bases in the upper tunnel and with residues of Rpl4 and Rpl17 at the exit tunnel constriction point (Bhushan *et al.* 2010). Complementary analyses with photo-cross-linked amino acids suggest that Arg alters the AAP conformation within the exit tunnel, affecting its interactions with both Rpl4 and Rpl17 (Wu *et al.* 2012). The cryo-EM data also suggest that Arg stabilizes a distinct conformation of 28S rRNA residue A2062 such that the AAP-linked P-site tRNA conformation within the PTC could prevent eRF1 action (Bhushan *et al.* 2010). An *in vitro* translation puromycin release assay confirmed that Arg-mediated stalling inhibits peptidyl transfer activity, thereby preventing normal translation termination and ribosome recycling (Wei *et al.* 2012). How Arg interferes with PTC function is not yet clear.

As stalling occurs at the uORF stop codon, Cpa1 levels are further controlled by the NMD quality control pathway that recognizes mRNAs with aberrant premature stop codons (Kervestin and Jacobson 2012). NMD requires three proteins: Upf1, Nmd2, and Upf3. When 80S complexes translating the uORF stall in the presence of Arg, the *CPA1* mRNA is destabilized, dependent on Upf1 (Messenguy *et al.* 2002) and Nmd2 (Gaba *et al.* 2005). Consistent with these findings, mutating each NMD gene enhances Cpa1 activity in the presence of Arg (Messenguy *et al.* 2002). Translation of the AAP and ribosome stalling over the uORF stop codon are necessary for NMD as mutations altering the AUG start codon or the D13N mutation in the AAP eliminate NMD (Gaba *et al.* 2005).

Perspective

The use of *S. cerevisiae* to study protein synthesis has provided novel insights into both the mechanism and regulation of translation that are shared among all eukaryotic organisms. Molecular genetic, biochemical, and structural studies in yeast have been especially useful in deciphering the functions of translation factors in recruiting an mRNA to the ribosome and in selecting the start codon by the scanning ribosome. Moreover, the elegant *GCN4* translation control system in yeast has not only provided novel insights into the functions of a variety of translation factors, but this mechanism of gene-specific translational control has served as a paradigm for the integrated stress response in mammalian cells. In addition, studies of translation in yeast have led to the development of the ribosomal profiling technique to monitor genome-wide protein synthesis. By combining the new techniques of ribosomal profiling, high-resolution cryo-EM imaging, and single-molecule biochemistry with traditional, yet powerful, molecular genetic approaches, yeast is an ideal system to study protein synthesis and the translational control processes operating in all eukaryotes.

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