REVIEW

Translational regulation by uORFs and start codon selection stringency

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In addition to the main, protein-coding, open reading frame (mORF), many eukaryotic mRNAs contain upstream ORFs (uORFs) initiated at AUG or near-cognate codons residing 5′ of the mORF start site. Whereas translation of uORFs generally represses translation of the mORFs, a subset of uORFs serves as a nexus for regulating translation of the mORF. In this review, we summarize the mechanisms by which uORFs can repress or stimulate mRNA translation, highlight uORF-mediated translational repression involving ribosome queuing, and critically evaluate recently described alternatives to the delayed reinitiation model for uORF-mediated regulation of the *GCN4*/*ATF4* mRNAs.

While the basic mechanism of protein synthesis has been understood for >50 yr, new insights into the mechanism and regulation of translation continue to emerge. The rapid increase in genome sequences and the development of ribosomal profiling, which maps the positions of initiating and elongating ribosomes on mRNAs, have increased interest in the mechanism and regulation of translation start site selection and utilization of alternative translational start sites. Here, we describe the mechanism of translation start site selection in eukaryotes and the impact of alternative translation start sites on main open reading frame (mORF) translation. Using well-characterized examples, we highlight varied means by which upstream open reading frames (uORFs)—reading frames that initiate 5′ to the mORF—regulate mORF translation.

Mechanism of translation start site selection

Both mRNA features and the translational apparatus (the ribosome and translation factors) contribute to translation start site selection. While in bacteria ribosomes bind to mRNAs close to the mORF start codon, a distinguishing feature of eukaryotic translation initiation is ribosomal scanning. The small (40S) ribosomal subunit with associated translation factors binds an mRNA near the 5′ cap and then progresses down the mRNA while scanning the nucleotide sequence for a start codon. Recent genetic, biochemical, and structural studies have provided new insights into the mechanisms of scanning and start codon selection.

[*Keywords*: ATF4; GCN4; eIF2 phosphorylation; ribosome queuing; stringency; uORF]

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Article published online ahead of print. Article and publication date are online at http://www.genesdev.org/cgi/doi/10.1101/gad.350752.123. Freely available online through the *Genes & Development* Open Access option.

Overview of eukaryotic translation initiation

Here, we highlight the findings most relevant to uORF control of translation and direct the reader to other reviews and the primary literature for more detailed descriptions of the mechanisms of scanning and start site selection (Hinnebusch 2011, 2014) as well as the impact of uORF translation on nonsense-mediated mRNA decay (NMD) (Gaba et al. 2005; Arribere and Gilbert 2013).

Assembly of a translationally competent 80S ribosome on the start codon is a complex process requiring the assistance of multiple eukaryotic translation initiation factors (eIFs), many of which impact start codon selection (for reviews, see Hinnebusch 2011, 2014; Dever et al. 2016). The factor eIF2 bound to GTP is competent to bind initiator methionyl tRNA (Met-tRNA$_i^{Met}$) to form an eIF2–GTP–Met-tRNA$_i^{Met}$ ternary complex (TC). Binding of eIF1A and eIF1 to the A site and near the P site, respectively, of the small (40S) ribosomal subunit opens the 40S mRNA binding channel and facilitates TC binding in the P site, with eIF1 contacting the eIF2β subunit. The multisubunit factor eIF3 binds across the back of the 40S subunit with contacts at both the mRNA entry and exit channels and with certain eIFs in the decoding center; the eIF3 also promotes TC binding to the 40S. Whereas an isolated 40S subunit is unable to bind an mRNA (with a few rare exceptions [CrPV IRES]) (Wilson et al. 2000), binding of the TC, eIF1, eIF1A, and eIF3 to the 40S subunit generates a 43S preinitiation complex (PIC) and licenses the complex to bind mRNA.

Binding of the 43S PIC to an mRNA is promoted by the eIF4F complex, which binds near the 5′ end of the mRNA owing to direct interaction of its cap-binding subunit

eIF4E with the m^7GTP mRNA cap. eIF4E also binds to the scaffolding subunit eIF4G, which additionally binds RNA, the mRNA poly(A) tail-binding protein (PABP or PABPC1), and the RNA helicase eIF4A. eIF4G also participates in recruiting the 43S PIC to mRNA via interactions with factors bound to the PIC (LeFebvre et al. 2006; Villa et al. 2013; Kumar et al. 2016; Brito Querido et al. 2020). eIF4A is thought to unwind cap-proximal secondary structures in mRNAs to prepare a binding site for the 43S PIC and also interacts with the PIC to enhance mRNA binding (Yourik et al. 2017). Following attachment near the cap, the PIC rapidly inspects the mRNA sequence (scans) for a start codon as it slides down the mRNA (Hinnebusch 2014; Wang et al. 2022).

The key feature directing selection of the translation start codon is the anticodon of the Met-$tRNA_i^{Met}$ (Cigan et al. 1988). The Met-$tRNA_i^{Met}$ in the PIC is thought to oscillate between P_{out} (not engaged with a codon) and P_{in} (the anticodon base-paired with an mRNA codon) conformations as the PIC scans the mRNA (Hinnebusch 2014). eIF1, bound adjacent to the P site, clashes with $tRNA_i^{Met}$ in the P_{in} state to impede codon–anticodon pairing at non-AUG codons (Hussain et al. 2014; Thakur and Hinnebusch 2018). Movement of Met-$tRNA_i^{Met}$ into the P_{in} conformation on pairing with an AUG codon triggers release of eIF1 (Maag et al. 2005). The N-terminal domain of eIF5, the GTPase-activating protein (GAP) for eIF2, binds the site vacated by eIF1 and stabilizes rather than clashes with $tRNA_i^{Met}$, with concurrent completion of GTP hydrolysis by eIF2 and release of the liberated P_i (Llácer et al. 2018) to finalize selection of the start site. Conformational changes in the PIC constrict the mRNA binding channel to arrest scanning, and additional eIFs are released. The eIF2-GDP is released, and guanine nucleotide exchange factor (GEF) eIF2B recycles it to functional eIF2–GTP for additional rounds of initiation (Pavitt 2018). The eIF1A remains bound in the A site and helps recruit the GTPase eIF5B, which reorients the position of the acceptor arm of the Met-$tRNA_i^{Met}$ to enable joining of the large (60S) ribosomal subunit (Lapointe et al. 2022). Subsequent hydrolysis of GTP by eIF5B enables release of the factor, and the resulting 80S IC can proceed into the elongation phase of protein synthesis.

mRNA features contributing to translation start site selection

Given the vectorial nature of scanning, translation typically initiates at the AUG codon closest to the 5′ end of the mRNA (Kozak 2002). However, several mRNA features influence the efficiency of start site selection. First, AUG codons residing within ~32 nt of the 5′ cap are inefficiently selected for initiation (Kozak 1991a). Second, as shown by Kozak (1999), the "context" nucleotides flanking a start codon influence the frequency of start codon selection. Sequence alignments of annotated start codons as well as mutational analyses of context nucleotides revealed the importance for purine nucleotides at the −3 position relative to the A of the AUG codon at position +1 and for G at position +4 (Kozak 1986b, 1987a,b; Nakagawa et al. 2008; Loughran et al. 2012; Noderer et al. 2014; Hernández et al. 2019). Mutational studies assessing translational output have established the optimum Kozak consensus sequence, 5′-GCCACCAUGG-3′ (Kozak 1986b, 1991b), with positions −3 and +4 playing the greatest roles in start site selection.

Whereas most ribosomes scanning an mRNA initiate translation at the first AUG codon they encounter, a fraction of ribosomes may continue scanning and initiate at a downstream start codon (see Kozak 2002). Such "leaky scanning" is more prominent when the first start codon is in poor context or under conditions of heightened global start codon selection stringency. As detailed below, elevated levels of eIF1 or reduced levels of eIF5 increase global stringency, while reduced eIF1 or elevated eIF5 levels decrease stringency and enhance initiation at start codons in poor context. In addition to eIF1 and eIF5, the factors eIF3 and eIF4G2 (DAP5) have been linked to start codon selection and uORF regulation. The studies on eIF4G2 have been recently reviewed (Shestakova et al. 2023), and it is unclear whether the impact of eIF3 and eIF4G2 on start codon selection is direct or indirect (She et al. 2023). Notably, lowered stringency was observed upon knockdown of ribosomal proteins, eIF3 subunits, or eIF4G2 and was correlated with the impact of the knockdowns on cell growth (She et al. 2023). Interestingly, reduced start codon selection stringency upon inhibition of general translation was recently correlated with altered relative levels of eIF1 and eIF5, possibly due to different half-lives of the two factors (Ivanov et al. 2022).

Near-cognate start codons that differ from AUG by a single nucleotide can also serve as initiation codons, albeit with lower efficiency than an AUG codon (Kozak 1989; Peabody 1989). Reporter assays established a hierarchy of near-cognate initiation codons in human cells with CUG (9.9% of AUG initiation efficiency) > ACG (4.1%) > GUG (2.5%) > AUU (1.3%) ≈ AUA (1.1%) > AUC (0.5%) ≈ UUG (0.4%) (Loughran et al. 2012). Similar preferences were seen in *Saccharomyces cerevisiae* and *Neurospora crassa* (Kolitz et al. 2009; Wei et al. 2013), and, notably, a purine at the +2 position has the greatest inhibitory effect on initiation. In human cells, initiation at a CUG codon in perfect context (9.9% of AUG) was more efficient than initiation at an AUG codon in poorest context (3.17%; with U at −6 to −1 and C at +4) (Loughran et al. 2012). Initiation at near-cognate start codons is more sensitive to mutations in the context nucleotides flanking the start codon (Diaz de Arce et al. 2018), such that near-cognate start codons in poor context are not generally used for initiation at significant levels.

In addition to *trans*-acting eIFs, mRNA secondary structures can act in *cis* to control leaky scanning and start site selection. While secondary structures near the 5′ end of an mRNA impair ribosome loading, and secondary structures within the leader impair scanning and lower translational yields (for review, see Hinnebusch 2011), secondary structures downstream from start codons can enhance initiation. Kozak (1990) showed that a stem–loop structure (−19 kcal/mol) placed 14 nt downstream from a weak start

codon enhanced initiation and nearly abolished leaky scanning in vitro. Altering the distance between the stem–loop and the upstream start codon to 8 or 32 nt dampened this stimulatory effect, indicating a need for precise positioning. As the length of the mRNA entry channel from the edge of the ribosome to the P site is 15 nt, the downstream stem–loop would position the scanning ribosome over the start codon and provide a greater chance for its selection. Similarly, a properly positioned downstream hairpin enhanced initiation at a near-cognate start codon in yeast (Wang et al. 2022) or at an AUG codon in poor context in mammalian cells (Clyde and Harris 2006). In accord with these findings, binding of the sex-lethal (SXL) protein downstream from the start codon of a uORF in the *msl-2* mRNA impaired mORF translation in a manner dependent on the uORF start codon (Medenbach et al. 2011), suggesting that SXL binding enhanced uORF translation. Thus, properly positioned impediments to scanning can enhance selection of a weak start site by positioning a 40S PIC in the vicinity of the start codon.

uORF attributes that affect downstream translation

While uORFs generally impair translation of the mORF, a variety of attributes contribute to the impact of the uORF on mORF translation (Fig. 1). An AUG start codon in poor context or a near-cognate start codon will lead to higher rates of leaky scanning, diminishing any inhibitory impact of the uORF. If the uORF stop codon is 3′ of the mORF start codon such that the uORF overlaps the mORF, ribosomes translating the uORF are unlikely to translate the mORF. While ribosomes primarily scan in the 3′ direction, modest backward scanning in the 5′ direction has been reported (Matsuda and Dreher 2006; Li et al. 2022; Wang et al. 2022). An early study suggested efficient reinitiation at AUG codons located 13 nt upstream of an overlapping uORF stop codon (Peabody and Berg 1986);

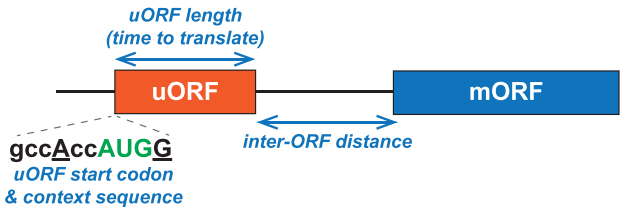

Figure 1. uORF attributes that affect mORF translation include the following: (1) The efficiency of initiation at the uORF start codon, impacted by the start codon context sequence, controls leaky scanning. (2) The length of the uORF and the time it takes to translate the uORF affect the ability of a ribosome to resume scanning and reinitiate at a downstream start site following translation termination at the uORF stop codon. (3) The distance between the uORF stop codon and downstream start codon impacts the ability of a ribosome sliding down the mRNA after termination at the uORF stop codon to reacquire the initiation factors and Met-tRNA$_i^{Met}$ required to initiate at a downstream start site.

however, later studies showed that reinitiation was strongly impaired when the AUG start codon was 1, 7, or 13 nt upstream of the uORF stop codon (Kozak 1987c, 2001). Thus, it appears that following uORF translation, very few ribosomes will scan in the 5′ direction to reinitiate at an upstream start codon.

For uORFs that terminate upstream of the mORF start codon, ribosomes that translate the uORF must terminate, remain bound to the mRNA, reacquire Met-tRNA$_i^{Met}$ and eIFs, and then scan to the mORF start codon to reinitiate translation and synthesize the mORF polypeptide. Each of these steps has the potential to impose control on mORF translation.

The efficiency of reinitiation increases as the distance between the uORF stop codon and the downstream start codon increases (Fig. 1). In mammalian cells, the efficiency of reinitiation ranged from <10% when the intercistronic distance was <11 nt to ~70% when the distance between the uORF and mORF was ≥79 nt (Kozak 1987c). Similarly, in yeast, increasing the intercistronic distance between a short uORF and downstream reporter mORF from 50 to 100 nt increased reporter expression over twofold (Grant et al. 1994). This dependency of reinitiation on the spacing between the uORF and mORF is attributed to the time-dependent reacquisition of translation factors and especially Met-tRNA$_i^{Met}$ by ribosomes that have translated the uORF (see *GCN4* discussion below).

The length of a uORF is inversely correlated with the efficiency of reinitiation (Fig. 1). Studies in mammalian cells using mutated HIV *tat* mRNAs indicated that increasing the length of a uORF lead to stepwise decreases in reinitiation efficiency, with 40 codons eliminating reinitiation and 28 codons triggering ~50% inhibition (Luukkonen et al. 1995). Similarly, increasing the length of a uORF in an altered hepatitis B virus mRNA from 13 codons (no inhibition of reinitiation) to 19 (~50%) or 29 (~80%) codons inhibited reinitiation (Hwang and Su 1998). In rabbit reticulocyte lysates, increasing the length of a uORF from three to 13 codons did not impact reinitiation; however, increasing the uORF length to 33 codons led to a threefold reduction in reinitiation efficiency (Kozak 2001). In yeast, mORF translation was inhibited by ~65% to ~95% when the length of a uORF increased from 13 to 35 codons (Rajkowitsch et al. 2004). While the precise impacts on reinitiation varied among the three studies, perhaps due to other attributes of the uORFs, uORF length was inversely correlated with reinitiation efficiency, and lengthening a uORF to >40 codons severely impaired reinitiation.

The inverse relationship between uORF length and reinitiation efficiency is commonly attributed to the time-dependent loss of eIFs from ribosomes elongating on the uORF. The notion is that reinitiation is enhanced if key initiation factors remain associated with the ribosome when it terminates uORF translation. Three lines of evidence support this model. First, slowing the rate of elongation either locally on the uORF or globally impaired reinitiation. Inserting a structured pseudoknot in a uORF to slow elongation increased the inhibitory impact of the

uORF in rabbit reticulocyte lysates (Kozak 2001). In yeast, the inhibitory impact of a uORF containing rare codons was suppressed by overexpression of the complementary tRNA, consistent with the notion that slow elongation through the uORF inhibited reinitiation (Lin et al. 2019). Likewise, uORFs encoding stalling peptides that block elongation or termination on the uORF are strongly inhibitory (Dever et al. 2020). While these examples support the notion that slow elongation impairs reinitiation, the slowly elongating ribosomes could also be impairing mORF translation by blocking leaky scanning of the uORF. Consistent with slow elongation impairing reinitiation, globally impairing tRNA aminoacylation by mutating Cca1, the tRNA nucleotidyltransferase (CCA-adding enzyme) in yeast, exacerbated the inhibitory effect of a uORF on mORF translation (Rajkowitsch et al. 2004), though impaired reinitiation in this mutant might also reflect reduced levels of Met-tRNA$_i^{Met}$. Second, the mechanism of initiation on the uORF impacted reinitiation. uORFs translated by viral internal ribosome entry site (IRES) mechanisms that do not require the cap-binding complex eIF4F (classic swine fever virus IRES) or any factors (cricket paralysis virus IRES) were defective for reinitiation when compared with uORFs translated by the traditional scanning mechanism (Pöyry et al. 2004), as expected if eIFs used in initiating at the uORF must be retained and reused for reinitiation at the mORF. Third, eIF3 was found to remain associated with elongating ribosomes at the start of ORFs (Bohlen et al. 2020a; Lin et al. 2020; Wagner et al. 2020) and to dissociate from the elongating ribosomes with a half-life (length) of ~12 translated codons (Bohlen et al. 2020a). Consistent with these findings, eIF3 association with uORFs decreased as the uORF length increased (Mohammad et al. 2017).

Following translation termination and peptide release, an 80S ribosome with deacylated tRNA in the P site is situated on the stop codon. The ribosome recycling factor ABCE1 (or Rli1 in yeast) dissociates the 60S subunit from this complex. The factor eIF2D (ligatin; Tma64 in yeast) or the combination of DENR and MCT-1 (Tma20 and Tma22, respectively, in yeast) releases the deacylated tRNA from the resulting 40S–tRNA–mRNA complex, enabling the 40S subunit to dissociate from the mRNA (for review, see Hellen 2018). Efficient reinitiation requires retention of the 40S subunit on the mRNA following termination and the first steps of recycling. How ribosome recycling factors contribute to translation reinitiation is not fully understood. In mammalian cells, MCT-1 and DENR promote reinitiation downstream from uORFs, most notably for uORFs encoding Met-stop (Schleich et al. 2014, 2017; Ahmed et al. 2018; Castelo-Szekely et al. 2019; Vasudevan et al. 2020). As discussed below, loss of these recycling factors in yeast increased translation in 3′ UTRs but did not significantly affect reinitiation following translation of the short uORFs on the *GCN4* mRNA (Young et al. 2018; Gaikwad et al. 2021). Notably, mRNA sequences upstream of and downstream from *GCN4* uORFs control permissiveness for reinitiation (Grant and Hinnebusch 1994; Grant et al. 1995; Munzarová et al. 2011; Gunišová and Valášek 2014; Gunišová et al. 2016). While the role of ribosome recycling in reinitiation requires more study, one possible model is that following uORF termination, the 60S subunit and deacylated tRNA are released from the 40S subunit. The persistence of eIF3 and/or eIF4G on the ribosome following translation of a short uORF enables retention of the postterination 40S subunit on the mRNA. The 40S subunit then migrates down the mRNA, acquires an eIF2 TC, and then reinitiates at a downstream AUG codon.

Distinction between regulatory uORFs and inhibitory uORFs

It is important to differentiate between uORFs that simply impair ribosome access to the mORF (and thereby throttle down the translational capacity of an mRNA) and uORFs that enable translational regulation in response to changes in conditions. Not all uORFs are regulatory. A vast number of translated uORFs have been identified in ribosome profiling experiments (for example, see Ingolia et al. 2009, 2011; Chew et al. 2016; Johnstone et al. 2016; Zhang et al. 2018; Takahashi et al. 2020; Chothani et al. 2022; Liu et al. 2023); however, it is likely that many of these uORFs simply reduce the number of ribosomes that translate the mORF but do not alter translation of the mORF in response to changing conditions. Here, we focus on several well-characterized examples of regulatory uORFs and describe the varied ways by which they control mORF translation. Owing to space limitations, we cannot comprehensively discuss regulatory uORFs and we direct the reader to other reviews and primary literature for discussions of plant uORFs (von Arnim et al. 2014; van der Horst et al. 2020; Zhang et al. 2020) and other regulatory uORFs in metazoans (Zhang et al. 2019).

Examples of translational control by uORFs and stringency

The delayed reinitiation model of translational control of GCN4 *and* ATF4 *mRNAs*

Defining features of delayed reinitiation (REI) One of the best-understood examples of translational regulation by uORFs applies to yeast *GCN4* and mammalian *ATF4* mRNAs, involving phosphorylation of the α subunit of eIF2 (eIF2α). Gcn4 and Atf4 are transcription factors that activate many genes enabling cells to adapt to starvation or stress, including genes promoting amino acid biosynthesis or tRNA aminoacylation. Gcn2, the sole eIF2α kinase in *S. cerevisiae*, is activated by amino acid starvation and mediates "general amino acid control" (GAAC) (for review, see Hinnebusch 2005). Mammalian cells contain multiple eIF2α kinases besides Gcn2 (each activated by different stresses but all regulating translation initiation by similar mechanisms) to comprise the "integrated stress response" (ISR) (Harding et al. 2000).

Phosphorylation of eIF2α on Ser51 by the eIF2α kinases converts eIF2–GDP from substrate to inhibitor of its GEF

eIF2B, and the ensuing depletion of eIF2–GTP reduces TC assembly. This broadly reduces protein synthesis as one leg of the GAAC/ISR while activating translation of particular mRNAs (e.g., *GCN4*/*ATF4*) whose encoded products act to alleviate stress. The reduction in TC assembly preferentially impairs translation of mRNAs of lower translational efficiencies, presumably reflecting their inability to compete effectively for limiting 43S PICs (Gaikwad et al. 2021). *GCN4* and *ATF4* mRNAs are very inefficiently translated in nonstressed cells, owing to the presence of multiple uORFs, but their translation is paradoxically induced by eIF2α phosphorylation because, at low TC levels, ribosomes that have translated the first or second uORFs and resumed migrating downstream (as 40S subunits following termination) can bypass the start codons of the more inhibitory 5′-distal uORFs and reinitiate at the mORF AUG codons instead (Fig. 2).

The *GCN4* mRNA leader is unusually long (~600 nt) and contains four short uORFs of only two or three codons (uORF1 to uORF4, from 5′ to 3′) (Figs. 2, 3) whose AUG codons have optimal sequence contexts for yeast. The uORF1 of mouse *ATF4* is also three codons long, whereas uORF2 is much longer and overlaps the mORF, and both uORF AUGs are in favorable Kozak context. According to the delayed REI model, uORF1 is translated by nearly all PICs scanning from the 5′ end, and a sizable fraction of the 40S subunits remaining at the uORF1 stop codon after termination and recycling of the 60S subunit resume migration downstream. Under nonstress conditions of abundant TC, the majority of these 40S subunits rebind TC and reinitiate translation at one of the distal uORFs (*GCN4* uORF3 or uORF4 and *ATF4* uORF2), after which essentially none of the resulting posttermination 40S subunits goes on to reinitiate at the *GCN4* or *ATF4* mORFs. When TC levels are reduced by eIF2α phosphorylation, rebinding of TC is delayed, and a fraction of migrating 40S subunits reacquires TC only after bypassing the inhibitory distal uORFs and initiates at the mORF instead (Fig. 2). Similar to uORF1, *GCN4* uORF2 exhibits a high propensity for reinitiation and serves as a fail-safe for PICs scanning from the cap that leaky-scan uORF1 during the primary initiation event (Fig. 3). This confers stronger repression of *GCN4* under nonstress conditions—but greater derepression in starved cells owing to a larger fraction of scanning PICs put into "reinitiation mode" that are thus able to reach *GCN4*—than occurs with uORF1 alone (Gunišová and Valášek 2014).

A defining feature of the delayed REI model is that the 5′-proximal uORFs promote, rather than inhibit, translation of the mORF under conditions of limiting TC. This positive effect is observed only in the presence of distal uORFs, as removing uORF1 from an otherwise uORF-less leader increases rather than decreases *GCN4* translation. This finding indicated that uORF1 stimulates *GCN4* translation indirectly by overcoming inhibition by the distal uORFs. In addition to *GCN4* uORF1 and uORF2 (Mueller and Hinnebusch 1986; Gunišová and Valášek 2014), this behavior applies to uORF1 of mouse *ATF4* (Lu et al. 2004; Vattem and Wek 2004) and the mRNAs encoding mammalian transcription factor Atf5 (Zhou et al. 2008) and the *N. crassa* Gcn4 ortholog *cpc-1* (Ivanov et al. 2017)—all regulated by delayed REI. The positive function of uORF1 was attributed to its ability to allow retention of 40S posttermination complexes at its stop codon that resume migrating and bypass the distal inhibitory uORFs when TC levels are reduced (Abastado et al. 1991). In the absence of the 5′-proximal uORFs, 48S PICs harboring TC will scan directly from the cap to the distal uORFs, whose translation and termination eliminate virtually all scanning ribosomes from the mRNA.

A second defining feature of delayed REI, shown for *GCN4*, *ATF4*, *ATF5*, and *cpc-1* in the studies cited above, is that removing the distal uORFs elevates mORF translation when TC is abundant, regardless of the presence of the proximal uORFs. This finding indicates that the distal uORFs are potent barriers to reinitiation (Figs. 2, 3). Strong evidence that the distal uORFs are bypassed by 40S subunits migrating from the proximal uORFs was that mutations that removed multiple in-frame stop codons of the distal *GCN4* uORF4, rendering it a much longer uORF that overlaps the mORF, had no effect on *GCN4* expression at limiting TC levels. As this extension would preclude reinitiation, the elongated uORF4 must be skipped

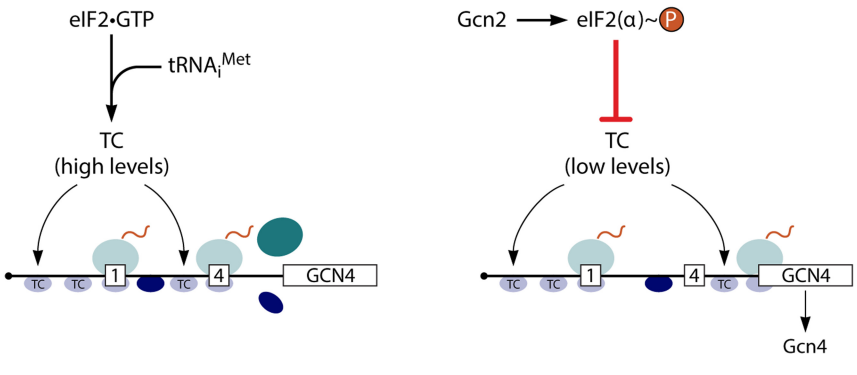

Figure 2. Schematic model of *GCN4* translational control, simplified to show only uORF1 and uORF4, which are sufficient for nearly wild-type regulation. Following translation of uORF1 (boxed 1), posttermination 40S subunits remain attached to the *GCN4* mRNA and resume scanning. (*Left*) Under nonstarvation conditions, they quickly rebind TC and reinitiate at uORF4 (boxed 4), and the 80S ribosome dissociates after terminating at uORF4. (*Right*) Under amino acid starvation conditions, the concentration of TC is reduced by eIF2α phosphorylation, such that many 40S ribosomes fail to rebind TC until after scanning past uORF4 and can thereby reinitiate at the *GCN4* ORF instead. (Reproduced from Hinnebusch 2011 with permission from American Society for Microbiology).

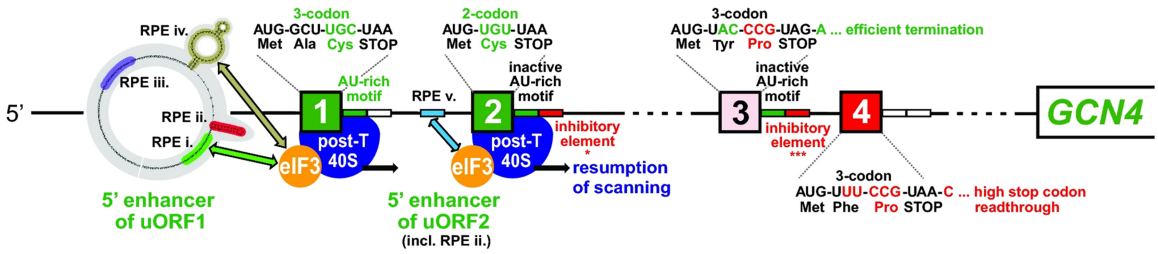

Figure 3. Schematic summary of the mRNA leader of *GCN4* mRNA with its four short uORFs (uORF1 to uORF4), summarizing the positions and functions of elements surrounding uORF1 and uORF2 that promote reinitiation following their translation, including RPE(i) to RPE(iv) in the enhancer region upstream of uORF1 and the AU-rich motif following uORF1, as well as RPE(v) upstream of uORF2. RPE(i), RPE(iv), and RPE(v) interact with eIF3 (arrows) at the exit channel of the 40S subunit to enhance resumption of scanning by 40S posttermination complexes at the uORF stop codons. uORF3 and uORF4 allow much less reinitiation because they are closer to the *GCN4* mORF, lack functional stimulatory elements found at uORF1 and uORF2, and contain the CCG Pro codon that impairs reinitiation. Additionally, the inefficient termination codon at uORF4 allows stop codon readthrough, placing posttermination 40S complexes even closer to the *GCN4* mORF, thus rendering them less able to reinitiate there. (Reprinted with permission from Gunisova et al. 2016).

by the 40S subunits migrating from the proximal uORFs en route to the *GCN4* start codon (Abastado et al. 1991). Consistent with this, the native inhibitory uORF2s of both *ATF4* and *ATF5* extensively overlap the mORFs, and the inhibitory *cpc-1* uORF2 is ~10-fold longer than the three-codon inhibitory uORF3 and uORF4 of *GCN4*. When uORF1 was similarly elongated to overlap the *GCN4* ORF in an allele lacking uORF2–uORF4, it became a strong barrier to *GCN4* translation, demonstrating that most 40S subunits scanning from the 5′ end translate uORF1 and that its permissiveness to downstream translation does not arise from leaky scanning of its start codon but from frequent reinitiation (Grant et al. 1994).

A third defining feature of delayed REI is that mORF translation is highly sensitive to the distances separating the proximal and distal uORFs. Lengthening the uORF1–uORF4 interval in *GCN4* mRNA by inserting spacer sequences of increasing length progressively reduced *GCN4* translation under limiting TC, with derepression nearly eliminated when the expanded uORF1–uORF4 distance was close to that normally separating uORF1 from the mORF. This is the outcome expected if the expanded uORF1–uORF4 separation provides sufficient time for TC binding by all 40S subunits migrating from uORF1 before they encounter uORF4, such that none can bypass the uORF4 AUG codon. It also explains why (1) a heterologous short uORF was not bypassed under conditions of limiting TC when inserted just upstream of the *GCN4* AUG, (2) *GCN4* uORF1 as a solitary uORF becomes more inhibitory when moved closer to the *GCN4* start codon (Grant et al. 1994), and (3) shortening the spacing between uORF1 and uORF4 in *GCN4* (Grant et al. 1994) or between uORF1 and uORF2 of *ATF4* (Lu et al. 2004) leads to greater bypass of the distal inhibitory uORFs in nonstress conditions of abundant TC. As noted, Kozak (1987c) first demonstrated that reinitiation declines as the separation between an uORF and the downstream mORF is reduced in mammalian cell-free extracts and further showed that an inhibitory uORF could be overcome by inserting a short uORF further upstream—a key principle of the delayed REI mechanism.

A fourth defining feature of delayed REI is that inhibiting scanning by insertions of stem–loops of strong predicted stability between uORF1 and the distal inhibitory uORFs blocks induction of mORF translation under conditions of reduced TCs, as shown for *GCN4* (Abastado et al. 1991) and *ATF4* (Vattem and Wek 2004). These experiments exploited knowledge about the inhibitory effects of stem–loops on PIC attachment or scanning (Pelletier and Sonenberg 1985; Kozak 1986a) to provide evidence that ribosomes do not bypass the inhibitory distal uORFs by hopping or internal initiation.

The key tenet of delayed REI is that migrating 40S subunits generated by the proximal uORF bypass the start codons of the inhibitory distal uORFs when TC becomes limiting and reinitiate further downstream at the mORF instead. This tenet was supported by observing reductions in rates of *GCN4* uORF4 or *ATF4* uORF2 translation on limiting TC and quantified by immunoprecipitation of pulse-labeled reporter polypeptides fused to these uORFs (Abastado et al. 1991; Lu et al. 2004). A moderate reduction in initiation at these inhibitory uORFs should be sufficient to account for a large increase in initiation at the downstream mORFs because almost none of the migrating 40S subunits generated by uORF1 translation bypasses the distal uORFs when TC is abundant. The increased fraction that bypasses when TC is limiting can be predicted as ~20%–40% by comparing *GCN4* translation with or without the distal uORFs (Mueller and Hinnebusch 1986), which is consistent with the observed approximately twofold reduction in synthesis of an uORF4-β-galactosidase fusion (Abastado et al. 1991). The inferred reduction in *ATF4* uORF2 translation on eIF2α phosphorylation by PERK corresponds to only ~18% (Lu et al. 2004; Vattem and Wek 2004) and to only 8% for *ATF5* (Zhou et al. 2008). Indeed, a small reduction in *ATF4* uORF2 expression in response to eIF2α phosphorylation induced by arsenite was observed by quantifying a tracer peptide encoded by a modified version of uORF2 (Starck et al. 2016), though it was unjustifiably interpreted as being contradictory to the delayed REI model because of its small magnitude. Because the steady-state level rather than rate of

synthesis of the uORF2 tracer was measured, an even larger decrease in synthesis could have been obscured by the pre-existing tracer synthesized prior to arsenite treatment. Increased *ATF4* mRNA expression on eIF2α phosphorylation (Dey et al. 2010; Zhou et al. 2018) could also have obscured a larger decrease in uORF2 translation at limiting TC levels.

Consistent with delayed REI, ribosome profiling shows modest reductions in ribosome footprint (RFP) density in uORF2 of native *ATF4* and *ATF5* mRNAs in arsenite-treated cells (Andreev et al. 2015). The opposite conclusion was reached for *ATF4* uORF2 translation during amino acid starvation by calculating the uORF2/uORF1 ratio of RPFs (Zhou et al. 2018), which may be problematic because RPF densities in short uORFs (like three-codon uORF1) cannot be measured reliably (Gerashchenko and Gladyshev 2014). In this last study, an *uORF2-FLUC* reporter showed no change in steady-state expression in starved cells; however, an increase in reporter mRNA abundance that parallels native *ATF4*—or the complication of pre-existing luciferase made prior to starvation—could have obscured the predicted small decrease in *uORF2-FLUC* translation (Zhou et al. 2018). In fact, quantification of 80S ICs by ribosome profiling in the presence of lactimidomycin (QTI-seq) showed reduced initiation at the uORF2 start codon during starvation (Zhou et al. 2018), in agreement with the delayed REI model.

Surprisingly, the QTI-seq results just mentioned revealed no 80S ICs at the *ATF4* mORF AUG, leading to the conclusion that reinitiation following uORF1 translation involves scanning 80S versus 40S posttermination ribosomes. However, this conclusion was based on the ad hoc assumption that lactimidomycin cannot bind to an 80S IC formed by a scanning 80S ribosome (Zhou et al. 2018). This proposal is at odds with findings that reinitiation by 80S ribosomes in 3′ UTRs generally does not occur at AUG codons in yeast cells (Young et al. 2015a) or in a mammalian reconstituted system where it is dictated by complementarity to the elongator tRNA decoding the last codon of the uORF (Skabkin et al. 2013). It is also difficult to envision how a delay in reinitiation by 80S ribosomes could arise from reduced TC levels, as TC binding to the 40S subunit (Hussain et al. 2014) is incompatible with the 40S:60S interface of an 80S ribosome (Ben-Shem et al. 2011). Another line of evidence advanced to support reinitiation by scanning 80S ribosomes, of no increase in the 40S:60S ratio bound to *ATF4* mRNA on TC limitation (Zhou et al. 2018), is not compelling because delayed REI predicts that the number of 40S subunits scanning from the cap to uORF1 or migrating downstream from uORF1 is constitutive, and only ~20% of the latter bypass uORF2 and continue downstream to the mORF in starved cells rather than dissociating after uORF2 translation. The small increase in 40S subunits between uORF2 and the *ATF4* AUG on TC limitation predicted by delayed REI would be challenging to detect biochemically.

Other evidence from Zhou et al. (2018) led to the proposal that m^6A methylation of nucleotide A225 in uORF2 acts as a fail-safe to prevent translation of the *ATF4* mORF in nonstarved cells by promoting initiation at the ORF2 start codon such that demethylation of A225 is required to induce mORF translation in starved cells. This model is supported by the findings (1) of reduced methylation at uORF2 during starvation, (2) that knocking down m^6A demethylase ALKBH5 or FTO impaired induction of both Atf4 and *ATF4-FLUC* expression in starved cells, (3) of cross-linking of ALKBH5 to *ATF4* mRNA, and (4) that overexpressing FTO in the livers of transgenic mice derepressed Atf4 expression in nonstarvation conditions. However, the fail-safe model seems inconsistent with the findings that either overexpressing FTO, knocking down methylase METTL3 or mutating A225 in the *ATF4-LUC* reporter produced no increase in Atf4 protein or *ATF4-FLUC* reporter expression, respectively, in nonstarved cells. Additionally, A225G mutation did not increase 80S ICs at the *ATF4* AUG codon, even though it reduced ICs at the uORF2 AUG, when assayed in lysates by toeprint analysis. Overexpressing FTO, depleting METTL3, or mutating A225, however, did enhance induction of Atf4 or *ATF4-FLUC*, respectively, in starved cells, and the A225G mutation both eliminated the enhanced induction conferred by depleting METTL3 and restored induction of *ATF4-LUC* in cells depleted of demethylase ALKBH5 (Zhou et al. 2018). These last findings could indicate that A225 methylation dampens induction of the mORF in response to eIF2α phosphorylation rather than preventing its induction in nonstarved cells. The proposal that A225 methylation increases initiation at the uORF2 AUG predicts a reduction in ORF2 translation during starvation (when methylation is reduced) that will be diminished by the A225G mutation, which should be tested in the future.

Determinants of the differing reinitiation potentials of 5′-proximal vs. 5′-distal uORFs. The delayed REI mechanism depends on a high frequency of reinitiation following translation of the 5′-proximal uORFs. For *GCN4* uORF1, this depends partly on its short three-codon length (Stiles et al. 1981; Williams et al. 1988; Miller and Hinnebusch 1989) and its location far upstream of the mORF (350 nt) (Grant et al. 1994), both in line with determinants of reinitiation mentioned above. It also requires *cis*-acting sequences upstream of uORF1 (Grant et al. 1995), including four reinitiation-promoting elements (RPEs), of which RPE(ii) and RPE(iv) form stem–loop structures. The relatively lower reinitiation potential of uORF2 also depends on RPE(ii) plus an additional element, RPE(v), which is similar in sequence to uORF1 RPE(i) (Fig. 3; Munzarová et al. 2011; Gunišová and Valášek 2014). uORF1 contains an AU-rich element just downstream from its stop codon that also promotes reinitiation (Miller and Hinnebusch 1989; Grant and Hinnebusch 1994; Gunišová et al. 2016). uORF2 and uORF3 contain similar sequences of consensus $AU_{1\text{-}2}A/UUAU_2$ that can functionally replace the uORF1 sequence but are nullified by other sequences at these two uORFs (Fig. 3; Gunišová et al. 2016). Combining the functions of these various positive and negative elements helps to explain why the

reinitiation potential varies as uORF1 > uORF2 > uORF3 (Gunišová et al. 2016).

Genetic evidence indicates that RPE(i) and RPE(iv) of uORF1 and RPE(v) of uORF2 (Fig. 3) functionally cooperate with two segments in the N-terminal domain (NTD) of the largest subunit of eIF3 (a/Tif32), shown to be required in *trans* for efficient reinitiation at *GCN4* following translation of uORF1 and uORF2 (Szamecz et al. 2008; Munzarová et al. 2011; Gunišová and Valášek 2014). Located upstream of the uORFs, these RPEs will lie just outside of the exit channel of 40S posttermination complexes positioned at the uORF stop codons, in proximity to the eIF3a-NTD in the yeast 48S PIC (Llácer et al. 2015). It was proposed that eIF3 remains associated with the 40S subunit during elongation and termination at uORF1 and uORF2 and that the eIF3a-NTD interaction with RPE elements impedes dissociation of the 40S posttermination complex to enable reinitiation (Munzarová et al. 2011). Biochemical evidence supports the predicted association of eIF3 with *GCN4* mRNA sequence intervals containing uORF1 or uORF2 (with RPEs) versus uORF3 or uORF4 (lacking RPEs), dependent on the RPEs and key segments of the eIF3a-NTD (Mohammad et al. 2017). Importantly, the eIF3/uORF1 association was impaired by lengthening uORF1 by two or five alanine codons, which were shown previously to impair reinitiation by placing RPEs beyond the reach of eIF3a-NTD at the exit channel (two-codon insertion) or by increasing eIF3 dissociation during translation of a longer version of uORF1 (five-codon insertion) (Mohammad et al. 2017). Consistent with these last findings, selective ribosome profiling revealed high occupancies of 40S subunits bound to eIF3 and eIF2 selectively at *GCN4* uORF1 and uORF2 that were attributed to posttermination complexes at the proximal uORFs that retained eIF3, reacquired TC, and became competent to reinitiate downstream, whereas posttermination complexes at uORF3 and uORF4 are recycled from the mRNA. Even larger occupancies of 40S:eIF3 and 40S:eIF2 complexes were observed upstream of uORF1, which might serve as a reservoir of mRNA-bound PICs that can be mobilized for *GCN4* translation in response to stress (Wagner et al. 2020). There is evidence that sequences upstream of or downstream from the *ATF4* uORF1 similarly promote reinitiation, possibly via the h subunit of mammalian eIF3 (Hronová et al. 2017).

The low reinitiation potential of the *ATF4* uORF2 can probably be explained by the combination of its extended length and extensive overlap with the mORF; however, other explanations are required for the three-codon inhibitory uORF3 and uORF4 at *GCN4*. Being closer to the *GCN4* AUG codon and lacking both REI elements that retain eIF3 and stimulatory AU-rich sequences downstream from uORF3 and uORF4 stop codons (Fig. 3) helps to explain their lower reinitiation potential versus uORF1 and uORF2. The latter also depends on the third CCG Pro codons at both uORF3 and uORF4 (Fig. 3), conserved among related yeasts (Gunišová et al. 2016), which can be partially mimicked by the other three Pro codons (Miller and Hinnebusch 1989; Grant and Hinnebusch 1994; Gunišová et al. 2016). This effect could involve the known slow decoding of Pro codons or inefficient peptide release at stop codons following a Pro codon (Doerfel et al. 2015), which would mimic the time required to translate a longer uORF.

The lower reinitiation permitted by uORF4 versus uORF3 was attributed partly to (1) their different second codons, but in a manner that likely involves their different sequences rather than differing cognate tRNAs, and (2) their different stop codons and the adjacent nucleotide. The latter tetranucleotide sequence at uORF4 (TAA-C), but not that at uORF3 (TAG-A), confers a high frequency (>30%) of stop codon readthrough, dependent on the +4 C residue (Fig. 3; Gunišová et al. 2016). Readthrough might be associated with slow decoding of the stop codon by release factor, and when readthrough occurs, it will effectively increase the length of uORF4. Either effect would increase the time required to complete uORF4 translation and reduce reinitiation frequency accordingly. The important *cis*-acting elements identified at the four *GCN4* uORFs that collaborate to establish the reinitiation hierarchy uORF1 > uORF2 > uORF3 > uORF4 are summarized in Figure 3.

Role of 40S recycling factors in reinitiation on human ATF4 mRNA In contrast to the third (penultimate) CCG Pro codon at *GCN4* uORF3 and uORF4, which contributes to the low reinitiation potential of these uORFs, the penultimate UGC and UGU Cys codons of uORF1 and uORF2 (Fig. 3) appear to be dispensable, as substitutions with many other triplets was compatible with high reinitiation downstream (Grant et al. 1995; Gunišová et al. 2016). Analysis of human *ATF4* uORF1 and short uORFs in other human mRNAs indicated that certain codons at either the penultimate or antepenultimate codons preceding the uORF stop codons, including the antepenultimate GCG Ala codon in *ATF4* uORF1, confer a requirement for 40S recycling factors (the DENR/MCT-1 heterodimer and related eIF2D protein) for efficient reinitiation at the mORFs in response to eIF2α phosphorylation. As penultimate codons, these triplets also generally increase dependence on these factors for recycling of posttermination 40S subunits at uORFs and main CDSs. It was proposed that efficient recycling is required to dissociate the tRNAs decoding the sensitive triplets in 40S posttermination complexes to allow the vacant 40S subunits to resume migrating and reinitiate downstream (Bohlen et al. 2020b). It seems difficult to explain by this model the observed effects in reinitiation of antepenultimate codons, which should occupy the empty E site in 40S posttermination complexes. Translation of *GCN4* is unaffected by simultaneously eliminating the yeast homologs of the DENR/MCT-1 heterodimer and eIF2D (Gaikwad et al. 2021). It remains to be seen whether replacing the WT triplets of uORF1 with others that confer a greater dependence on these factors for 40S recycling at stop codons (Young et al. 2018) confers a similar dependence on these proteins for efficient reinitiation and induction of *GCN4* translation.

Single uORFs that regulate translation in response to changes in TC levels

A key hallmark of the delayed REI model is the presence of multiple uORFs in the mRNA. However, eIF2α phosphorylation also regulates translation of select mRNAs containing a single regulatory uORF, though the mechanism is less well understood. Treating mammalian cells with arsenite triggers eIF2α phosphorylation and translationally derepresses numerous mRNAs containing a single uORF (Andreev et al. 2015), including GADD34 (*PPP1R15A*), which together with the protein phosphatase 1 (PP1c) directs dephosphorylation of eIF2α-P to restore protein synthesis. Although the GADD34 mRNA leader contains two uORFs, the second uORF is sufficient to confer derepression under conditions of high eIF2α phosphorylation (Lee et al. 2009; Young et al. 2015b). The regulatory uORF encodes a peptide that is under purifying selection near its C terminus, which ends with the sequence PPG (Young et al. 2015b). Translation of the uORF peptide directs ribosomes to stall during decoding of its termination codon (Fig. 4A, panel ii; Young et al. 2015b). The C-terminal PPG motif is critical for the regulatory properties of the uORF and is thought to impede termination. The slow termination could lead to greater loss of initiation factors from the ribosomes translating the uORF, leading to ribosome dissociation from the mRNA following uORF translation and thus preventing reinitiation. Ribosomes are thought to leaky-scan the regulatory uORF when eIF2α is phosphorylated, facilitated by the imperfect context of its AUG codon (Fig. 4A, panel i); however, the imperfect context is not essential for the regulation. Mutating the start codon of the regulatory uORF of GADD34 to a noninitiating codon or mutating the sequence of the encoded peptide derepresses mORF translation even under conditions of high TC (Young et al. 2015b).

CHOP (*DDIT3*), like *GCN4*/*ATF4*, is a bZIP transcription factor that modulates gene expression programs during cellular stress. The leader of the CHOP mRNA contains a single uORF that confers derepression under conditions of high eIF2α phosphorylation. Like GADD34, the uORF of the CHOP mRNA inhibits mORF translation under conditions of high TC. Also, like GADD34, the C terminus of the encoded uORF peptide is under purifying selection, though the conserved sequences are dissimilar to each other. Unlike GADD34, the CHOP uORF peptide sequence appears to pause ribosomes during elongation rather than termination (Fig. 4A, panel ii). Mutations that alter the uORF peptide sequence affect the level of repression of the mORF and impair translational regulation. Interestingly, the uORF is initiated by two in-frame AUG codons, though both are in suboptimal contexts. Introducing the optimal context at the uORF start codons increases repression of the downstream mORF and dampens, but does not eliminate, the eIF2α phosphorylation-mediated regulation (Young et al. 2016). Current models suggest a common mechanism for GADD34 and CHOP regulation based on peptide sequence-dependent impairment of reinitiation that is relieved by increased leaky scanning of the uORF following eIF2α phosphorylation (Fig. 4A, panel i); however, the mechanism whereby eIF2α phosphorylation increases leaky scanning remains unclear.

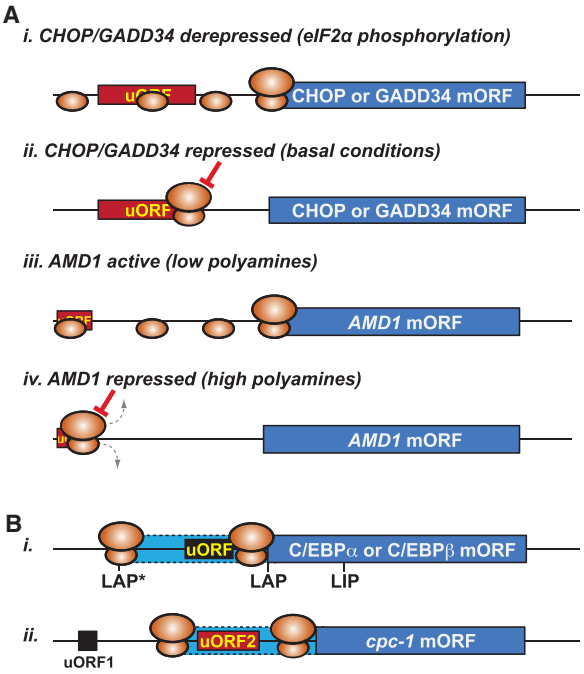

Figure 4. uORF and stringency regulation of translation. (*A*) CHOP, GADD34, and *AMD1* regulation. (Panels *i*,*ii*) Elongation/termination pause impairs leaky scanning over the uORF and represses CHOP and GADD34 translation, and eIF2α phosphorylation enhances leaky scanning to derepress mORF translation. (Panels *iii*,*iv*) Ribosomes access the vertebrate *AMD1* mORF by leaky scanning over the cap-proximal uORF. Polyamine-triggered pausing of a ribosome on the uORF precludes additional ribosomes from loading, repressing mORF translation. (*B*) Alternative initiation enables bypass of a regulatory uORF. (Panel *i*) Initiation at upstream weak start sites in C/EBP mRNAs, generating LAP*, leads to ribosomes bypassing the short regulatory uORF that controls synthesis of LAP or LIP isoforms in response to eIF2α phosphorylation. (Panel *ii*) Reinitiation at the near-cognate start codon upstream of inhibitory uORF2 generates N-terminally extended *cpc-1*.

The *GCN4* homolog in *Candida albicans* contains multiple uORFs; however, the single uORF3 is sufficient for translational regulation in response to eIF2α phosphorylation (Sundaram and Grant 2014), raising the possibility that it is regulated in a manner similar to CHOP and GADD34. While *Schizosaccharomyces pombe* lacks a *GCN4* homolog, the Fil1 protein performs a similar function, regulating expression of amino acid biosynthetic enzyme genes (Duncan et al. 2018). The fission yeast *fil1* mRNA contains multiple uORFs that enable eIF2α phosphorylation control, but it is unclear whether the regulation is via delayed REI or a distinct mechanism (Duncan and Mata 2022).

uORFs responding to small metabolites

A rate-limiting step in the synthesis of spermidine and spermine is decarboxylation of S-adenosyl-L-methionine,

a reaction catalyzed by the enzyme S-adenosylmethionine decarboxylase (AdoMetDC) encoded by the gene *AMD1*. The vertebrate *AMD1* mRNA contains a short conserved uORF that starts 13–14 nt downstream from the 5′ cap and encodes the peptide sequence MAGDIS (Hill and Morris 1993; Ruan et al. 1994, 1996; Ivanov et al. 2010a). The uORF in the *AMD1* mRNA confers polyamine-dependent translational regulation of AdoMetDC synthesis with high-level synthesis in low-polyamine conditions (Fig. 4A, panel iii) and repression of AdoMetDC synthesis in high-polyamine conditions (Fig. 4A, panel iv). Mutagenesis of the uORF codons showed the importance of Asp in the fourth position and Ile in the penultimate position for polyamine regulation (Mize et al. 1998). Increased polyamine concentrations cause ribosomal stalling during termination on the uORF stop codon (Raney et al. 2000; Law et al. 2001), dependent on the DI codons. However, elevated polyamine levels also increase stalling at the termination codon of a mutant peptide that is less inhibitory on downstream translation, indicating that polyamines might have a general inhibitory effect on translation with exacerbating impacts on inefficient terminators (Law et al. 2001). The inefficient termination of the uORF might impair reinitiation. Alternatively, a ribosome stalled at the stop codon of the MAGDIS uORF could, by virtue of its size and distance from the 5′ end, interfere with loading of 40S PICs at the cap (Ivanov et al. 2010a). Together, these two effects of polyamines on uORF translation termination—reduced reinitiation and impaired ribosome loading—impair AdoMetDC synthesis (Fig. 4A, panel iv).

Additional examples of uORFs conferring translational control in response to metabolites include the *N. crassa arg-2* and mammalian *PTP4A2* (*PRL-2*) mRNAs. Arginine-triggered stalling of a translating ribosome on a conserved uORF in the *arg-2* mRNA and its *S. cerevisiae* homolog, *CPA1* mRNA, represses mORF translation, and, likewise, apparent magnesium-induced ribosome stalling on a conserved uORF in the *PTP4A2* mRNA represses mORF translation (for review, see Dever et al. 2020).

uORFs regulated in response to changes in global stringency of start codon selection

Although the stringency of start codon selection in cells was originally thought to be static, with perhaps variations in stringency existing only between different organisms or different cell types, the levels of eIF1 and eIF5 modulate the stringency of start codon selection. Whereas eIF1 binding to the 43S PIC favors scanning, eIF1 dissociation and eIF5 binding favor initiation (Hinnebusch 2011). The genes encoding eIF1 and eIF5 in eukaryotes have evolved features that mediate cybernetic feedback regulation and cross-regulation to maintain the stringency of start codon selection and homeostasis of eIF1 and eIF5 expression (Fig. 5; Ivanov et al. 2010c; Martin-Marcos et al. 2011; Loughran et al. 2012). The eIF1 CDS is initiated by an AUG codon in conserved poor context (Miyasaka et al. 2010; Martin-Marcos et al. 2011). High levels of

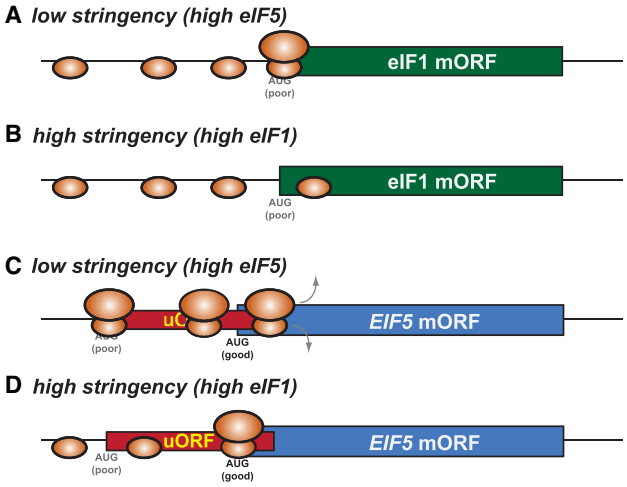

Figure 5. Stringency control of eIF1 and eIF5 expression. Suboptimal AUG start codons of *EIF1* mORF and inhibitory uORF in *EIF5* mRNA sense global stringency of start codon selection: Low stringency enhances *EIF1* mORF and *EIF5* uORF translation, thereby increasing eIF1 and repressing eIF5 synthesis. High stringency increases leaky scanning over poor AUG codons, repressing *EIF1* mORF and *EIF5* uORF translation, thereby repressing eIF1 and increasing eIF5 synthesis.

eIF1 confer high stringency, leading to repression of eIF1 mRNA translation for a negative autoregulatory loop (Fig. 5B; Ivanov et al. 2010c; Martin-Marcos et al. 2011). The leader of the eIF5 mRNA contains inhibitory uORFs initiated by AUG codons in conserved poor context (Loughran et al. 2012). High levels of eIF5 lower stringency, which enhances initiation at the inhibitory uORFs in the eIF5 mRNA and impairs eIF5 synthesis—a second negative autoregulatory loop (Fig. 5C; Loughran et al. 2012). In parallel, high-level eIF5 increases initiation at the poor context AUG codon of eIF1 mRNA to increase eIF1 synthesis (Fig. 5A), whereas high-level eIF1 decreases initiation at the poor context AUG of the eIF5 mRNA uORF to elevate eIF5 synthesis (Fig. 5D). These cross-regulatory effects combine with the autoregulatory controls to maintain a constant ratio of eIF1:eIF5 and level of global stringency. The cellular levels of BZW (or eIF5-mimic protein) also modulate global stringency of start codon selection in cells by apparently counteracting the activity of eIF5 (Tang et al. 2017; Loughran et al. 2018).

The existence of the eIF1/eIF5 homeostatic autoregulatory and cross-regulatory system and its conservation over a vast evolutionary time scale strongly suggest that perturbations of global stringency occur under some physiological conditions (see discussion on *cpc-1* and C/EBP below) and that conserved uORFs in the leaders of some mRNAs have evolved to sense changes in global stringency. Such uORFs, like the one in eIF5 mRNA, should inhibit expression of the mORF (i.e., either be long or overlap the mORF) and should be initiated by inefficient start codons (i.e., poor context AUGs or near-cognate start codons). Likely examples are the conserved uORFs in the leaders of the mouse *Hoxa1*, *Hoxa9*, and *Hoxa11* mRNAs

that have been shown to mediate differential mORF expression in response to global changes in the stringency of start codon selection (Ivanov et al. 2022). The total number of mammalian genes with similar characteristics is unknown, as is the number of uORFs that fit the eIF5 paradigm and mediate translational control in response to changes in global stringency.

uORF-mediated ribosomal queuing controls local stringency of start codon selection

Human antizyme inhibitor (AZIN1) is a key component of another cybernetic mechanism that maintains intracellular polyamine homeostasis. AZIN1 binds to and inhibits the protein antizyme, which in turn inhibits the enzyme ornithine decarboxylase (ODC) catalyzing the first step in the biosynthesis of polyamines. The mRNAs encoding all three proteins are translationally regulated by polyamines in mammals (Dever and Ivanov 2018). AZIN1 and ODC are homologs that started diverging from each other in early vertebrate evolution (Ivanov et al. 2010b). The leader of the *AZIN1* mRNA contains three conserved AUG-initiated short uORFs that do not have known regulatory functions. However, the mRNA also contains an upstream conserved coding region (uCC) that has two defining features: a conserved AUU near-cognate start codon and a conserved C terminus that often encodes the amino acid sequence PPW (Fig. 6; Ivanov et al. 2008). Both features are ancient and predate the divergence of AZIN1 and ODC but have been lost in mammalian ODC. Similar sequences have also evolved apparently independently in fungi. The uCC of *AZIN1* directs polyamine-dependent regulation, with high polyamine levels inhibiting mORF translation and low polyamine levels stimulating translation (Ivanov et al. 2008). Both the near-cognate AUU start codon of the uCC and the conserved C-terminal sequence of the encoded peptide are essential for polyamine regulation. Polyamines were found to enhance the efficiency of initiation on the near-cognate AUU start codon of the uCC. While high polyamine levels were initially proposed to reduce the global stringency of start codon selection (Ivanov et al. 2008) and by this mechanism account for enhanced *AZIN1* translation, subsequent studies showed that the enhanced translation of the *AZIN1* uCC in the presence of high polyamine levels is strictly dependent on the conserved uCC peptide sequence (Ivanov et al. 2018). High polyamine levels were also shown to inhibit the activity of the translation factor eIF5A, which is required for translating the PPW motif in the uCC, resulting in ribosomal stalling. Interestingly, substitution of the uCC PPW motif with the arginine-regulated stalling peptide from the *N. crassa arg-2* mRNA (Wang and Sachs 1997; Fang et al. 2004) conferred enhanced initiation at the AUU near-cognate start codon in the presence of high arginine levels (Ivanov et al. 2018).

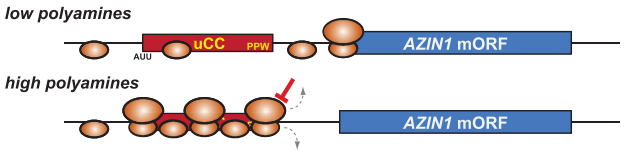

Figure 6. Schematic model of *AZIN1* translational control. (*Top*) Under low-polyamine conditions, most ribosomes leaky-scan over the near-cognate AUU start codon of the uCC uORF and translate the mORF. (*Bottom*) Polyamine-triggered pausing of a ribosome on the PPW motif in the uCC causes ribosomes to queue and enhances initiation at the near-cognate uCC start codon, repressing AZIN1 synthesis.

Taken together, these results suggested a model in which a ribosome elongating on the uCC pauses in response to high concentration of polyamines. The paused ribosome triggers ribosomal queuing on the uCC that causes a trailing 40S ribosome to dwell in the vicinity of the near-cognate AUU start codon, enhancing the efficiency of initiation at this site (Fig. 6). At low polyamine levels, ribosome pausing in the uCC is diminished, dissipating the queue and allowing trailing 40S subunits to leaky-scan the uCC and initiate at the mORF instead (Fig. 6). Thus, the stringency of selecting the poor uCC start codon is modulated locally by expanding or contracting the queue of scanning 40S PICs (and 80S elongating ribosomes) to make it either include or exclude the uCC start codon. This model is strengthened by the observation that lowering PIC loading on mRNA, either by phosphorylation of eIF2α or by disrupting the interaction between eIF4G and eIF4E, reduces uCC translation and derepresses AZIN1 synthesis in the presence of high polyamine levels (Ivanov et al. 2018). The decreased PIC loading will diminish the queue despite persistence of polyamine-induced ribosomal pausing within the uCC. It is currently unknown whether uCC-like elements in other mRNAs confer similar regulation involving ribosomal queuing in response to different metabolites. However, the uORFs described above controlling GADD34 and CHOP mRNA translation and the ascorbate-mediated translational regulation of the *GGP* mRNA in plants (Laing et al. 2015) are good candidates in containing uORFs initiated at a suboptimal or near-cognate start codon.

Upstream initiation enables bypass of regulatory uORFs

As noted earlier, the uORF architecture of *N. crassa cpc-1* and its orthologs in other filamentous fungi is similar to the uORF architecture in mammalian *ATF4* with a short uORF1 (three to six codons long) and a longer uORF2 (35–70 codons), except that *cpc-1* uORF2 does not overlap the mORF. *cpc-1* and all its >100 known orthologs in *Pezizomycotina* share a feature that distinguishes them from *GCN4*/*ATF4* and suggests an additional mode of regulation. No in-frame stop codons are present in the >500 nt preceding the *cpc-1* mORF and extending beyond the beginning of uORF2. Thus, translation initiating upstream of uORF2 and in-frame with the mORF will generate an N-terminally extended CPC1 protein and bypass the inhibitory consequences of translating uORF2 (Fig. 4B, panel ii; Ivanov et al. 2017). The *cpc-1* mRNA sequence between uORF2 and the mORF shows evidence of

purifying selection in the mORF, providing evidence that the region is translated, which is supported by ribosome profiling data. Moreover, phylogenetic analysis identified conserved near-cognate start codons in-frame with the mORF and upstream of uORF2 that could initiate N-terminal extensions of CPC1. The *cpc-1* homologs in *Basidiomycota* appear to have independently acquired an mRNA architecture analogous to *cpc-1* (Ivanov et al. 2017). These findings suggest that, in addition to translational control by delayed REI, the *Pezizomycotina* and *Basidiomycota cpc-1* homologs are also translationally regulated in response to altered global or local stringency of start codon selection.

CCAAT enhancer-binding proteins (C/EBPs) are a family of bZIP transcription factors in vertebrates that regulate gene expression in response to developmental or environmental cues. C/EBPα and C/EBPβ, two members of this paralogous group, are important for adipogenesis and macrophage functioning, respectively, among other documented roles. The mRNAs of both C/EBPα and C/EBPβ contain a short uORF that generally inhibits downstream translation (Fig. 4B, panel i; Calkhoven et al. 1994; Lincoln et al. 1998). This uORF controls the production of two alternative isoforms of the proteins in response to eIF2α phosphorylation, though in a manner distinct from the delayed REI model for *GCN4* and *ATF4* (Calkhoven et al. 2000). In addition, the leaders of both C/EBPα and C/EBPβ mRNAs have conserved suboptimal initiation codons, a near-cognate CUG codon in C/EBPα and an AUG in poor context in C/EBPβ, located upstream of the short regulatory uORF and in-frame with the mORF. Like *cpc-1* in *N. crassa*, translation initiation at these suboptimal start codons will enable synthesis of N-terminally extended isoforms and will bypass the regulatory effects of the short uORF (Fig. 4B, panel ii; Calkhoven et al. 2000). Also, like *cpc-1*, this mRNA architecture suggests that the C/EBPα and C/EBPβ mRNAs have evolved features to sense changes in the global stringency of start codon selection, perhaps in response to specific physiological conditions.

In conclusion, uORFs are powerful elements of translational control that generally act to repress mORF translation. If the uORF contains the features required to allow reinitiation and is located 5′-proximal of another uORF, it can stimulate mORF translation indirectly by suppressing initiation at the 5′-distal uORF. This stimulation can be enhanced under conditions that reduce TC abundance. Repression by solitary uORFs frequently involves uORF coding sequences that stall ribosomes during elongation or termination, which can be modulated by metabolites to establish a negative regulatory loop. The negative impact of the elongation/termination pause can be amplified by formation of a ribosome queue in the uORF that delays migration of scanning PICs to elevate translation of the uORF, creating a positive feedback loop, which is crucial when the uORF has a poor context AUG or near-cognate start codon. Formation of the queue and hence translational repression by the uORF can be diminished by reducing the rate of PIC attachment to the mRNA, providing another layer of control over uORF repression. Alteration of global stringency can also modulate translational repression by altering the rate of uORF initiation. Translated uORFs are very frequent in eukaryotic mRNAs, making it likely that many new instances of translational control involving delayed REI, elongation/termination pausing with or without ribosome queuing, altered global stringency, or other yet to be discovered mechanisms will be uncovered in the future.

Competing interest statement

The authors declare no competing interests.

Acknowledgments

Research in our laboratories is supported by the Intramural Research Program of the National Institutes of Health.

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