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Molecular principles of translation reinitiation in mammals

Molekulární principy translační reiniciace v savčích buňkách

Doctoral thesis

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Declaration:

I declare that I prepared this thesis solely on my own with all information sources and literature cited. I did not use either this thesis or its substantial part as a background to obtain another or equivalent academic degree

Prague 27.3.2025

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Abstract

Translation is a fundamental biological mechanism that involves protein synthesis and its regulation at both the general and gene-specific levels. In eukaryotes, the initiation of messenger RNA (mRNA) translation is a key regulatory step, and understanding its molecular mechanism is crucial because this is the stage at which most regulation of translation occurs (Valášek, 2012). Translation initiation requires the interaction between eukaryotic initiation factors (eIFs), ribosome, and mRNA to ensure the formation of 80S ribosome complex ready for elongation.

In our laboratory, we investigate the molecular details of one of the gene-specific translational control mechanisms called reinitiation (REI), and, in particular, how this process is mediated by eIFs.

Translation REI is a molecular mechanism whereby only the large ribosomal subunit and deacylated tRNA get recycled past the translation of short uORF (upstream open reading frame) and subsequent termination, but the mRNA stays bound to the small ribosomal subunit to allow REI at the downstream start codon. The most extensively studied examples of REI are mammalian activating transcription factor 4 (*ATF4*) and yeast transcription factor general control nonderepressible 4 (*GCN4*), which are functional homologues. *ATF4* has two uORFs in its sequence, short uORF1 and longer uORF2 that partially overlaps the main coding sequence (CDS) of *ATF4*, *GCN4* has four short uORFs (Gunišová et al., 2018).

Different stress conditions, such as endoplasmic reticulum (ER) stress, activate translational reprogramming of mRNAs including *ATF4*, which is of particular interest because during stress-induced global translational attenuation, *ATF4* becomes upregulated in cells and drives a conserved program of integrated stress response (ISR) that is essential for cell adaptation and survival.

Although the original model of *ATF4* control *via* delayed REI was reported for the first time in 2004 (Lu et al., 2004; Vattem and Wek, 2004), mounting evidence from published data raised the question of exactly how *ATF4* translational regulation is governed under various stresses. Several discrepancies have emerged that are inconsistent with postulated theory, and it was later suggested that other layers of regulation of *ATF4* translation besides the delayed REI mechanism may be involved (Andreev et al., 2015; Starck et al., 2016; Wagner et al., 2020; Zhou et al., 2018).

Therefore, as a part of my PhD project, we decided to revisit the original REI theory and based on new knowledge of this particular problem, we attempted to decipher the molecular details of the control of human *ATF4* mRNA translation under ER stress *versus* non-stress conditions.

Using a comprehensive set of *ATF4*-HA tag reporter constructs and establishing the automated quantitative western blotting JessTM assay, we demonstrated a road block effect of a previously unexplored stem-loop in the uORF2/*ATF4* overlap on ribosome processivity and a role for this stem-loop in provoking ribosome queuing. We also showed that *ATF4* canonical start site is highly leaky scanned, and therefore the near-cognate CUG codon situated further downstream can be used as an alternative start site to further expand control of *ATF4* translation. We also explored the regulatory effects of two conserved methylated adenines in the *ATF4* sequence on *ATF4* translation.

Another study in which I was involved in investigated the mechanism of REI in the yeast *S. cerevisiae* model of *GCN4* mRNA. Using the previously established RNA-Protein Ni²⁺-Pull down (Rap-Nip) assay, we have demonstrated that eIF4G and eIF4A, but not eIF4E (all part of the eIF4F complex), are retained on ribosomes elongating and terminating on short REI-permissive uORFs.

Abstrakt

Translace představuje základní biologický mechanismus, který zahrnuje syntézu proteinů a její regulaci na obecné i specifické genové úrovni. U eukaryot je iniciace translace mRNA důležitým regulačním krokem a pochopení jejího molekulárního mechanismu je klíčové, protože v této fázi dochází k většině regulací translace (Valášek, 2012). Iniciace translace vyžaduje interakci mezi eukaryotickými iniciačními faktory (eIFs), ribozomem a mRNA, která zajistí vytvoření komplexu 80S ribozomu připraveného k elongaci.

V naší laboratoři zkoumáme molekulární detaily jednoho z genově specifických mechanismů kontroly translace, tzv. reiniciace translace (REI), a zejména to, jak je tento proces zprostředkován eIFs.

REI je molekulární mechanismus, při kterém se po translaci krátkého uORF a následné terminaci recykluje pouze velká ribozomální podjednotka a deacylovaná tRNA, ale mRNA zůstává navázána na malou ribozomální podjednotku, aby umožnila REI na následném start kodonu. Nejprozkoumanějšími příklady REI jsou savčí mRNA *ATF4* a kvasinková mRNA *GCN4*, které jsou funkčními homology. *ATF4* má ve své sekvenci dva uORF, krátký uORF1 a delší uORF2, který částečně překrývá hlavní kódující sekvenci (CDS) *ATF4*, *GCN4* má čtyři krátké uORF (Gunišová et al., 2018).

Stresové podmínky, jako stres endoplazmatického retikula, aktivují přeprogramování translace mRNA včetně *ATF4*, což je obzvláště zajímavé, protože během celkového translačního útlumu vyvolaného stresem je exprese *ATF4* v buňkách up-regulována a řídí konzervovaný program integrované odpovědi na stres, který je nezbytný pro adaptaci a přežití buněk.

Ačkoli původní model řízení translace *ATF4* mRNA prostřednictvím zpožděné REI byl poprvé popsán v roce 2004 (Lu et al., 2004; Vattem and Wek, 2004), přibývající důkazy z publikovaných údajů vyvolaly otázku, jak přesně je regulace translace *ATF4* mRNA řízena při různých stresových podmínkách. Objevilo se několik neshod, které jsou v protikladu s postulovanou teorií, a později bylo navrženo, že kromě mechanismu zpožděné REI mohou být zapojeny i další úrovně regulace translace *ATF4* (Andreev et al., 2015; Starck et al., 2016; Wagner et al., 2020; Zhou et al., 2018).

Proto jsme se v rámci mého doktorského projektu rozhodli přezkoumat původní teorii REI a na základě nových znalostí tohoto konkrétního tématu jsme se pokusili popsat

molekulární detaily kontroly translace lidského *ATF4* v podmínkách stresu endoplazmatického retikula.

Pomocí komplexní sady *ATF4*-HA tag reportérových konstruktů a pomocí automatizované kvantitativní western blottingové JessTM eseje, kterou jsem v naší laboratoři pro tento účel optimalizovala, jsme prokázali inhibiční efekt dosud neprozkoumané smyčky ve struktuře mRNA v překryvné oblasti uORF2/*ATF4* na procesivitu ribozomů a roli této smyčky při řazení ribozomů. Dále jsme prokázali, že kanonické startovací místo pro translaci *ATF4* je vysoce tzv. “leaky scanned” (tj. často není ribozomem rozpoznáno), a proto lze CUG kodon, který se nachází dále po směru translace použít jako alternativní startovací místo pro další kontrolu translace *ATF4*. Kromě toho jsme zkoumali vliv dvou konzervovaných metylovaných adeninů v sekvenci *ATF4* na regulaci translace *ATF4*.

Během další studie, na které jsem se podílela, byl zkoumán mechanismus REI na modelu mRNA *GCN4* u kvasinek *S. cerevisiae*. Pomocí dříve vypracované RNA–Protein Ni²⁺ -Pull down (Rap-Nip) metody jsme prokázali, že eIF4G a eIF4A, ale nikoli eIF4E (všechny jsou součástí komplexu eIF4F), jsou zadržovány na ribozomech účastnících se elongace a terminace na krátkých uORF, které jsou permissivní pro REI.

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Abbreviations

3' UTR – 3' untranslated region

3' SL – 3' stem-loop

40S subunit – eukaryotic small ribosomal subunit

43S PIC – preinitiation complex comprising 40S subunit, TC, eIF1, eIF1A, eIF3 and eIF5

48S PIC – preinitiation complex comprising 43S PIC bound to mRNA

4E-BPs – eIF4E-binding protein family of translational repressors

5' UTR – 5' untranslated region

5' TCH – 5' triple-circle hairpin

60S subunit – eukaryotic large ribosomal subunit

80S ribosome – eukaryotic ribosome consisting of a small and a large subunit

AARE – amino acid response element

ATF3 – activating transcription factor 3

ATF4 – activating transcription factor 4

ATF6 – activating transcription factor 6

BiP – binding immunoglobulin protein

bZIP – basic leucine zipper

C/EBP – CAAT/enhancer binding protein

CARE – C/EBP-ATF response element enhancers

CDS – coding sequence

CHOP – C/EBP homologous protein

CITEs – cap-independent translation enhancers

CRE – cAMP-responsive element

CREB-2 – cAMP-response element binding-2 transcription factor family

CR ϵ P – constitutive repressor of eIF2 α phosphorylation

DRACH – methylation motif, where D = A, G or U; R = G or A and H = A, C or U

eIF – eukaryotic initiation factor

eIF2 α – eIF2 subunit alpha

eIF2 β – eIF2 subunit beta

eIF2 γ – eIF2 subunit gamma

eIF2•GDP – eIF2•guanosine diphosphate

eIF2 α -P – phosphorylated eukaryotic translation initiation factor 2 α

eIF3d – eIF3 subunit d

eIF4E-BP – eIF4E-binding proteins

eIFs – eukaryotic translation initiation factors

ER stress – endoplasmic reticulum stress

GAAC – general amino acid control pathway

GADD34 – growth arrest and DNA damage-inducible protein

GCN2 – general control nonderepressible 2 kinase

GCN4 – general control nonderepressible 4; an amino acid biosynthesis transcription factor

GEF – guanine nucleotide exchange factor

GRP78 – 78-kDa glucose-regulated protein

GTP – guanosine triphosphate

HEAT domain – Huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1

HRI – haem regulated inhibitor

IC – initiation complex

IRE1 – inositol-requiring enzyme-1

ISR – integrated stress response

ISRIB – integrated stress response inhibitor

m⁶A – N⁶-methyladenosine modification

m⁷G-cap – ⁷-methyl guanosine cap

Met-tRNA_i^{Met} – methionyl initiator methionine tRNA

Mnk – MAP kinase-interacting kinase

mRNA – messenger RNA

mTOR – Mechanistic/ mammalian target of rapamycin

mTORC1 – mTOR complex 1

NaAsO₂ – sodium arsenite

NMD – nonsense-mediated mRNA decay surveillance pathway

NTD – N-terminal domain

PABP – poly(A) binding protein

PERK – protein kinase-like endoplasmic reticulum kinase
PIC – preinitiation complex
PKR – double-stranded RNA-activated protein kinase
PP1 – protein phosphatase 1
PTMs – post-translational modifications
QTI-seq – quantitative translation initiation sequencing
Rap-Nip – RNA-Protein Ni²⁺ -Pull down assay
RBDs – RNA binding domains
REI – reinitiation
RPEs – REI-promoting elements
RRACH – methylation motif, where R = G or A and H = A, C or U
S. cerevisiae – *Saccharomyces cerevisiae*
SERCA – sarco/ ER Ca²⁺-ATPase
TC – ternary complex
Tg – thapsigargin
uORF – upstream open reading frame
UPR – unfolded protein response

1. Introduction

Translation of mRNA is the process carried out on ribosomes in which the information stored in mRNA guides the incorporation of amino acids into the chain that constitutes the protein to be produced (Crick, 1970; Uzman, 2003). The whole process is cyclic and demands a lot of energy in the cell (Drummond and Wilke, 2009; Gunišová et al., 2018; Lane and Martin, 2010).

In total, four steps are involved in the translation process: initiation, elongation, termination and ribosome recycling (Gunišová et al., 2018; Valášek et al., 2017). Initiation of translation is a crucial step, as this is the stage where the most control of translation takes place. Recent studies indicate that inadequate regulation of protein synthesis and improperly balanced eIFs expression is not only a hallmark of cell transformation and cancer, but also contributes to other human diseases such as diabetes, prion and neurodegenerative diseases.

Phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α -P) by eIF2 α kinases under conditions of cellular stress activates the integrated stress response (ISR) mechanism allowing cells to cope with incoming stress stimuli by upregulating *ATF4*. Nevertheless, the time course and expression level of *ATF4* are important parameters that need to be well coordinated during ISR, as persistent overactivation of *ATF4* leads to continuous expression of downstream *ATF4* targets, the adaptive genes of ISR, which may eventually contribute to serious pathologies.

In this way, characterization of the stress response, including unfolded protein response (UPR), acute endoplasmic reticulum (ER) stress, and ISR, and the role of eIFs in it, would have profound implications for understanding the mechanisms underlying human disease and would greatly expand insights into highly specific processes controlling translation.

Altogether, the aim of my PhD study was to uncover the mechanistic details of translation REI in mammals and the yeast *S. cerevisiae*, under non-stress *versus* stress conditions. Using the model of mammalian *ATF4* and yeast *GCN4* mRNA, I focused on determining the molecular principles of REI, and, in addition, the role of eIFs (in particular, eIF4G) in this process.

2. Literature review

2.1 Translation in general with focus on initiation

2.1.1 Canonical cap-dependent eukaryotic translation initiation mechanism

Initiation of translation is an important step that starts with the acquisition of a ternary complex (TC) by the small 40S ribosomal subunit. TC consists of the methionyl initiator methionine tRNA ($\text{Met-tRNA}_i^{\text{Met}}$), a guanosine triphosphate (GTP) molecule and eIF2. Next, the 40S subunit with TC, together with incoming eIF1, eIF1A, eIF3 and eIF5 form the 43S preinitiation complex (43S PIC) (Figure 1) (Hinnebusch and Lorsch, 2012; Valášek, 2012; Sonenberg and Hinnebusch, 2009).

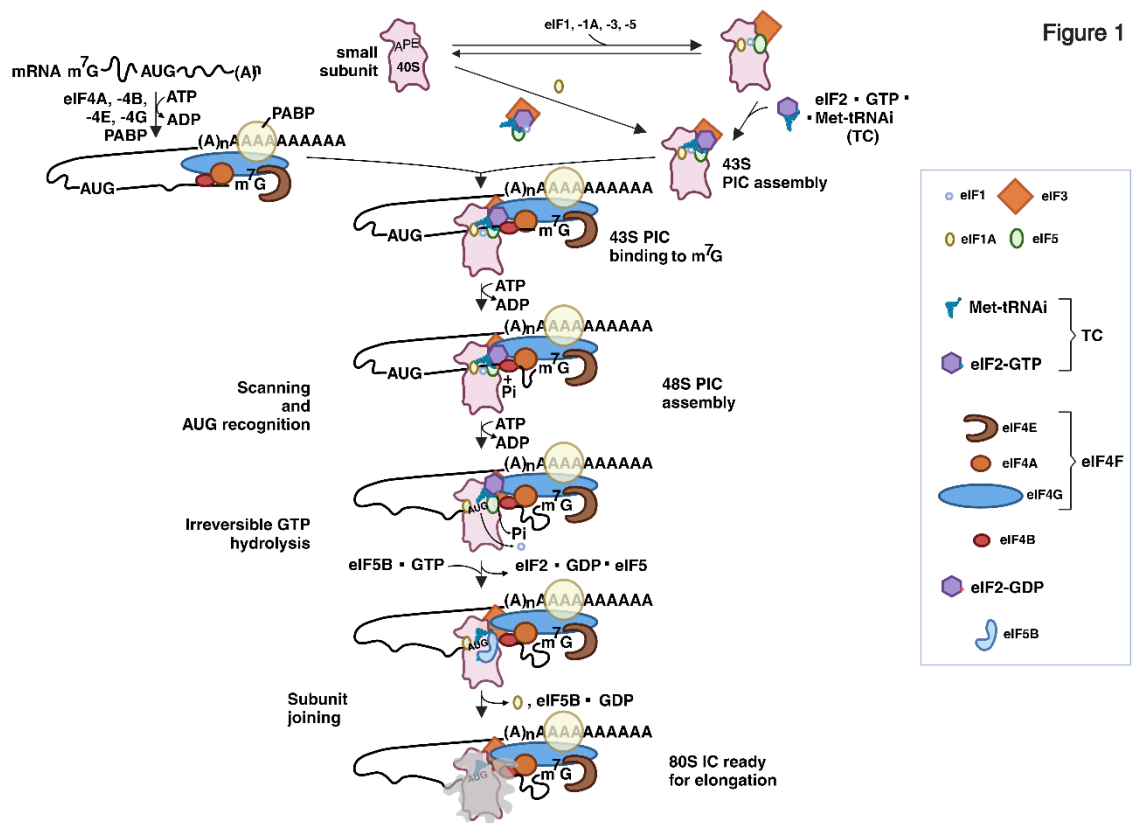


Figure 1. Schematics of the translation initiation process steps in eukaryotes. For details, please see the main text. Abbreviations: A, P, E are decoding sites in the 40S subunit; Met-tRNA_i , methionyl initiator tRNA; 43S PIC, 43S preinitiation complex; 48S PIC, 48S preinitiation complex; 80S IC, 80S initiation complex; eIFs, eukaryotic initiation factors; TC, ternary complex; m^7G – ^{7} -methyl guanosine cap; PABP, poly(A)-binding protein. Adapted from (Hinnebusch, 2014; Valášek et al., 2017). Created with BioRender.com

Further, the binding of 43S PIC to the 5' 7-methyl guanosine cap (m⁷G-cap) of mRNA happens, and it is conveyed by eIF4F factors, leading to the assembly of 48S PIC complex (Hinnebusch, 2014; Mitchell et al., 2010). The 48S PIC moves along the 5' untranslated region (5' UTR) of the mRNA in a process that runs from the 5' to 3', called scanning, and leads to the recognition of the AUG start codon (in the most cases) in a favorable Kozak consensus sequence, or Kozak context (Kozak 1989; Hinnebusch, 2017; Hinnebusch and Lorsch, 2012).

After base pairing between Met-tRNA_i^{Met} anticodon triplet CAU and the initiation codon AUG on the mRNA, an irreversible GTP hydrolysis on eIF2 occurs (Algire et al., 2005; Valášek et al., 2017). This eventually results in the ejection of most of the eIFs (Hinnebusch, 2017) but, intriguingly, not of eIF3 (Mohammad et al., 2017; Wagner et al., 2020).

After that, the subunits join (the process is facilitated by eIF5B) to create the 80S initiation complex (IC) and elongation begins. When the stop codon is recognized, translation is terminated, and the nascent peptide is released (Fringer et al., 2007; Valášek, 2012).

2.1.2 Translation initiation factors eIF4F and eIF3

To initiate the translation cycle accurately, a complex mechanism has been developed in eukaryotes, and this mechanism can be regulated by the action of certain initiation factors. Canonically, the recruitment of ribosome to mRNA starts within the 5'UTR: 43S ribosomal complex binds to the 5' cap of mRNA, and this is normally mediated by eIF4F complex (the components of this complex are eIF4E, eIF4A and eIF4G).

The first member of the complex, eIF4E, is a cap-binding protein, the second is eIF4A, an RNA helicase that participates (along with its stimulatory factors eIF4B and eIF4H) in unwinding the secondary structures of mRNA during the scanning process, and finally, eIF4G, a scaffold protein that mediates the binding of mRNA and its associated proteins to ribosome (Hinnebusch et al., 2016).

2.1.2.1 eIF4G domain structure, binding partners and interactions

As for the eIF4G, two initiation-promoting isoforms, eIF4G1 and eIF4G2, have been reported in the yeast *S. cerevisiae* (Clarkson et al., 2010), and three, eIF4G1 (referred to as

eIF4GI), eIF4G3 (referred to as eIF4GII) and eIF4G2 (referred to as DAP5, p97 or NAT1), in mammals (Liu et al., 2023; Pelletier and Sonenberg, 2019) (Figure 2). The formation of several eIF4G isoforms is likely due to alternative promoter choice, alternative splicing, and the use of the different start codons (Coldwell et al., 2012).

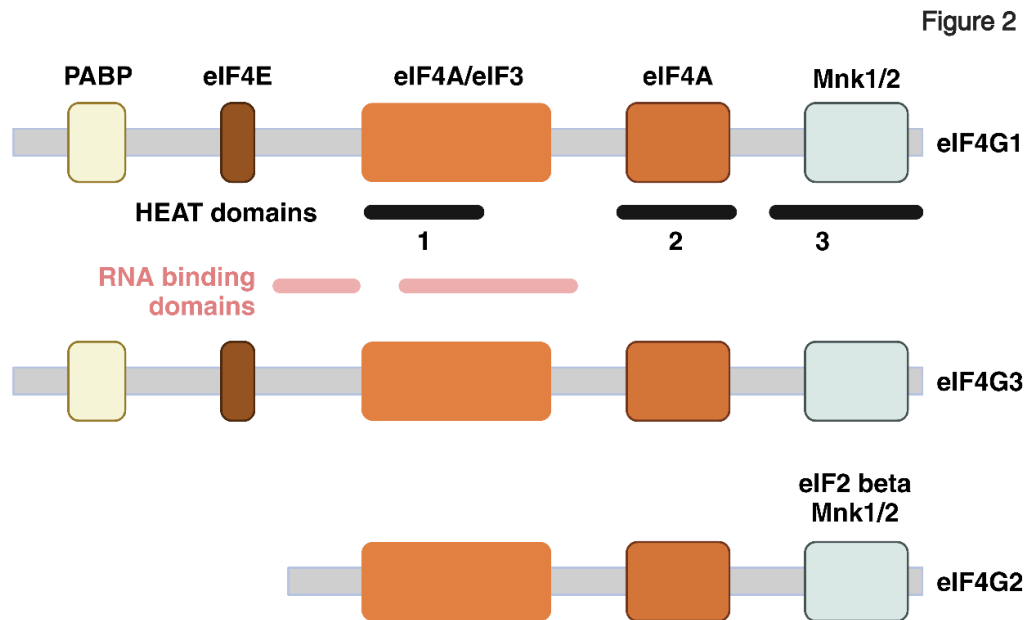


Figure 2. eIF4G1, eIF4G3 and eIF4G2 domain structure in mammals. Blocks of the same colour indicate homologous regions. The HEAT domains and RNA binding domains for eIF4G1 are depicted. Abbreviations: eIFs, eukaryotic initiation factors; HEAT domains, Huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1; Mnk, MAP kinase-interacting kinase; PABP, poly(A)-binding protein. Adapted from (Pelletier and Sonenberg, 2019). Created with BioRender.com

eIF4G domain structure differs among isoforms (Figure 2). eIF4G1 harbors RNA binding domains (RBDs), three HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1) domains, binding regions for poly(A) binding protein (PABP), eIF4E, eIF4A/eIF3, eIF4A, and Mnk1/2 (MAP kinase-interacting kinase) (Berset et al., 2003; Pelletier and Sonenberg, 2019). Although eIF4G2 (DAP5) is homologous to eIF4G1 at the C-terminal part and shares 39% sequence identity in RBDs and eIF4A/eIF3 binding region (hence has them as binding partners), the N-terminal part is different and lacks eIF4E and PABP binding regions, as it does not interact with them (Virgili et al., 2013). Lastly, eIF2 subunit beta (eIF2 β) has been shown to be a binding partner specifically for DAP5 but not eIF4G1 (Lee and McCormick, 2006). In humans, the eIF4A/eIF3 binding site is important for eIF4G function as it offers a platform for eIF4G-eIF3 interaction, which is essential for facilitating mRNA recruitment to the ribosome (Villa et al., 2013).

2.1.2.2 eIF4G and eIF3 involvement in non-canonical translation initiation

It is important to note that besides the canonical eIF4E-mediated initiation, non-canonical eIF4G- or eIF3-mediated translation initiation can also occur, often caused by the action of various stresses (Alard et al., 2015; Dennis et al., 2013; Haizel et al., 2020).

Regarding eIF3-mediated initiation, it can be cap-dependent or cap-independent (Kwan and Thompson, 2019). In the first cap-dependent scenario, the cap-binding activity of eIF3 subunit d (eIF3d) drives the alternative mechanism (Lee et al., 2015, 2016). It has also been shown that eIF3d is activated upon exposure to a number of stressors and is a major force underlying the ISR program (Lamper et al., 2020; Mukhopadhyay et al., 2023). Then, in eIF3-mediated and cap-independent case of initiation, a potential role of N⁶-methyladenosine (m⁶A) was described (Genuth and Barna, 2018). An interplay between m⁶A reader YTHDF1 and eIF3 with the subsequent m⁶A-directed initiation under stress has been previously proposed (Coots et al., 2017; Meyer et al., 2015; Mitchell et al., 2010; Peer et al., 2019; Zhou et al., 2018). It was further shown that eIF3d is a direct binding partner of DAP5 and their interplay ensures translation under conditions of inactivated eIF4E (de la Parra et al., 2018).

A different type of initiation mechanism is still possible even in uncapped mRNAs under conditions of cellular stress, where the presence of partially assembled initiation complexes containing eIF3 and eIF4G is also required. So-called cap-independent translation enhancers (CITEs) control translation initiation *via* binding the components of

the translation machinery within both 5' and 3' UTR, with efficiency similar to that of m⁷G-capped mRNAs (Lacerda et al., 2017; Shatsky et al., 2018; Terenin et al., 2013).

2.2 Translational control in general

In eukaryotes, the general control of the translation initiation step may be mediated by translation-related factors and regulated by modification of eIFs, in particular eIF2 or eIF4F. This type of regulation addresses the majority of mRNA molecules permanently present in the cell and has an impact on ribosome scanning-dependent initiation events (Hinnebusch, 2014; reviewed in Dever et al., 2023).

In the alternative, translation can be controlled through mRNA-specific regulation, which will be discussed in the following chapters.

There are two main mechanisms of general translational control. The first is the signaling pathway of eIF2 subunit α (eIF2 α) regulation and the other one is control of eIF4F formation by the activity of eIF4E-BPs (eIF4E-binding protein family of translational repressors).

2.2.1 Signaling pathway of eIF2 α regulation by eIF2 α phosphorylation on serine 51

Signaling pathway of eIF2 α regulation by eIF2 α phosphorylation (eIF2 α -P) on serine 51 represents a mechanism dependent on the formation and recruitment of eIF2•GTP•Met-tRNA_i^{Met} TC to the 40S ribosome, and as will be described in the next chapters, this pathway is a key factor determining cell fate during stress (Harding et al., 2003; Pathak et al., 1988).

A major point of eIF2 α -mediated regulation is that eIF2 can participate in the formation of new TCs only when it is in an active GTP-bound state.

It was mentioned previously (see 2.1.1) that during the initiation phase, the 43S PIC binds to m⁷G mRNA cap and the assembled 48S PIC scans from 5'- to 3' in search of the start codon. After the start codon is recognized, eIF2 (specifically the eIF2 subunit gamma (eIF2 γ)) hydrolyzes its GTP to yield eIF2•guanosine diphosphate (eIF2•GDP) and the ribosomal subunits join to form 80S (Wek, 2018). For the next round of initiation, eIF2•GDP has to be replaced by GTP (Figure 3). This process is facilitated by eIF2's guanine nucleotide exchange factor (GEF) eIF2B.

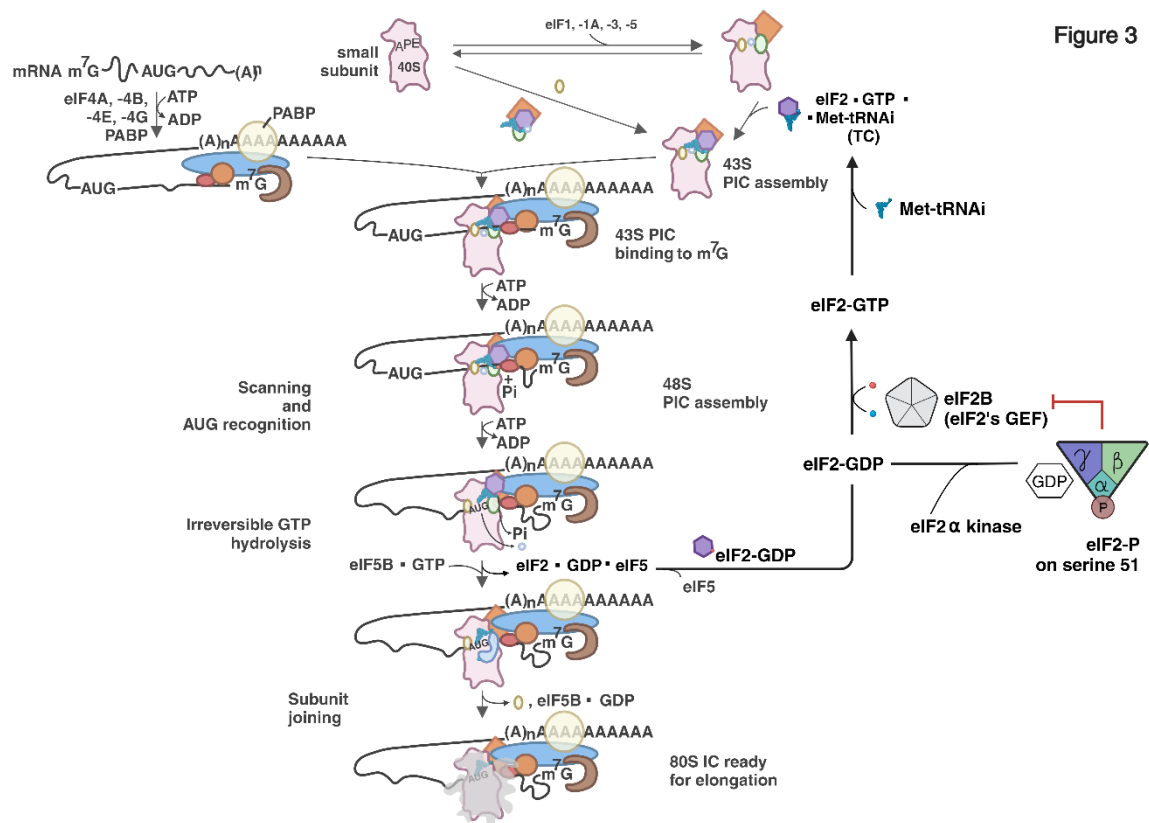


Figure 3. Signaling pathway of eIF2 α regulation by eIF2 α phosphorylation (eIF2 α -P) on serine 51. eIF2 α -P on serine 51 inhibits the eIF2's guanine nucleotide exchange factor (GEF) eIF2B from recycling eIF2 to eIF2-GTP-active form. For legend, see Figure 1. Adapted from (Hinnebusch, 2014; Ron and Walter, 2007). Created with BioRender.com

eIF2B is a decameric complex that consists of two copies each of subunit α , β , γ , δ and ϵ (Kashiwagi et al., 2016). In the dephosphorylated form, flexible trimers of eIF2 associate with the static catalytic centers of the eIF2B decamer to accelerate nucleotide exchange (eIF2B's subunits β - γ or δ are implicated). When phosphorylation is occurring, the α subunit of eIF2 is refolded, making contact with eIF2B at another interface (Gordiyenko et al., 2019; Kashiwagi et al., 2016; Krishnamoorthy et al., 2001) and this modification changes eIF2 from being a substrate to a high-affinity GEF inhibitor of eIF2B (Dever et al., 1995; Kenner et al., 2019).

The eventual outcome of phosphorylation of eIF2 α on serine 51 is that recycling of eIF2•GDP to its active GTP-bound form is impeded, thereby decreasing the delivery of TC to ribosomes, ultimately leading to a global attenuation of translation (Dever, 2002; Hinnebusch and Lorsch, 2012).

In yeast, phosphorylation of eIF2 α can only be governed by a single general control nonderepressible 2 (Gcn2) kinase *via* general amino acid control (GAAC) pathway (Gunišová et al., 2018), but in mammals there are four eIF2 α kinases that can be activated by several different stresses and act *via* a signaling pathway known as integrated stress response (ISR) (Pakos-Zebrucka et al., 2016; Ron, 2002; Taniuchi et al., 2016).

There are the following stress kinases in mammals: haem regulated inhibitor (HRI) (Chen, 2007; Chen et al., 1994; Han et al., 2001), double-stranded RNA-activated protein kinase (PKR) (Dey et al., 2005; García et al., 2007; Pindel and Sadler, 2011), GCN2 (Dong et al., 2000; Wek et al., 1989; Wek et al., 1995) and protein kinase-like endoplasmic reticulum kinase (PERK) (Harding et al., 1999; Harding, Zhang, et al., 2000; Shi et al., 1998), which can be activated by haem deficiency, viral infection, amino acid deficiency and ER stress, respectively (Chen and London, 1995; Donnelly et al., 2013; Wek, 2018) (Figure 4). The ISR pathway, including kinases and stimuli, will be described in more detail in the following chapters.

Figure 4

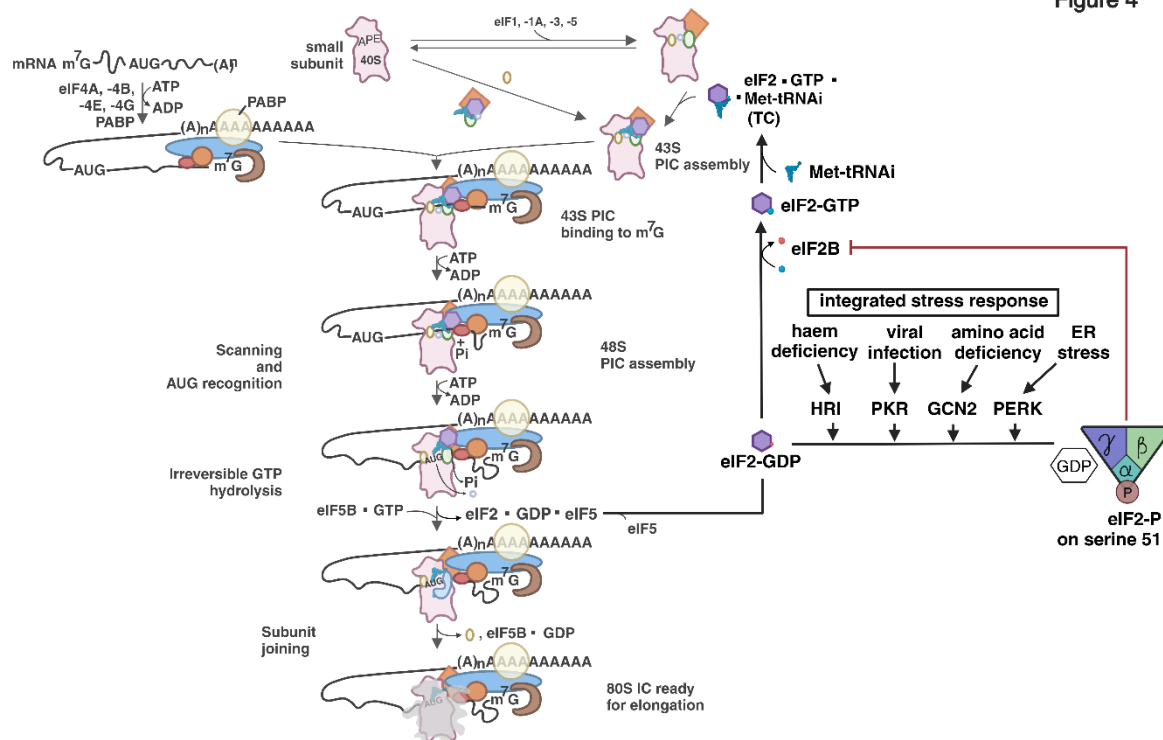


Figure 4. eIF2 α stress kinases (HRI, PKR, GCN2 and PERK) in mammals that are activated by different stresses (haem deficiency, viral infection, amino acid deficiency and ER stress, respectively) and converge into a core event of the integrated stress response, eIF2 α phosphorylation (eIF2 α -P). For legend, see Figure 1. Abbreviations: HRI, haem regulated inhibitor; PKR, double-stranded RNA-activated protein kinase; GCN2, general control nonderepressible 2 kinase; PERK, protein kinase-like endoplasmic reticulum kinase. Adapted from (Hinnebusch, 2014; Pakos-Zebrucka et al., 2016). Created with BioRender.com

When eIF2 α -P and activation of eIF2 kinases occur as a result of stress stimuli, translation of most mRNAs is repressed due to a general translational shut down (Hinnebusch, 2014; Sonenberg and Hinnebusch, 2009), whereas several key stress response factors, including yeast *GCN4* and mammalian *ATF4*, are paradoxically not inhibited but are even selectively upregulated and drive the adaptation program (Vattem and Wek, 2004; Wek, 2018; Harding, Novoa, et al., 2000).

2.2.2 Signaling pathway of eIF4F formation regulation by the activity of eIF4E-BPs

The second major mechanism of general translational control is regulated by the activity of eIF4E-BPs binding proteins (Sonnenberg and Hinnebusch, 2009).

eIF4E-BPs can compete with eIF4G for binding to eIF4E, blocking their association and assembly of the eIF4F complex, which is responsible for 43S recruitment to mRNA, and thereby repressing canonical translation initiation (reviewed in Valášek, 2012; Brito Querido et al., 2024).

eIF4E-BPs are rapidly phosphorylated by mammalian target of rapamycin (mTOR) kinase (as a component of the PI 3-K/AKT/FRAP/mTOR signaling pathway) under conditions when cell growth is stimulated, resulting in dissociation of eIF4E-BPs from eIF4E (Ma and Blenis, 2009; Mader et al., 1995). eIF4E is unbound and its binding to the 5' m⁷G-cap is accelerated by the specific eIF4E-eIF4G interaction, and eIF4F can be formed (Brito Querido et al., 2024; Rhoads, 2009).

As mTOR activity is inhibited under starvation or stress conditions, eIF4E-BPs are hypophosphorylated and may prevent formation of eIF4F leading to repression of canonical translation (Lin et al., 1994; Pause et al., 1994).

Nevertheless, translation of specific mRNAs (Gingras et al., 2003; Hernández, 2022; Meyuhas and Kahan, 2015), generally poorly translated, is dramatically induced under stress conditions, which besides growth factors may also include hormones, cytokines and mitogens (Sonnenberg and Gingras, 1998).

Both pathways of general translational control induce a reprogramming of translation, which allows the cell to conduct adaptive programs upon cellular stress (Hinnebusch et al., 2016; Roux and Topisirovic, 2018; Sonnenberg and Gingras, 1998).

2.3 Translational control by mRNA-specific elements

2.3.1 Translational control by upstream open reading frames (uORFs)

In addition to general translational control, there is mRNA-specific regulation that is activated in response to various stimuli. It affects only a certain group of mRNA molecules and can be promoted, for example, by RNA-binding proteins or micro-RNAs, modulating gene expression by silencing or degradation of mRNA (Dever et al., 2023; Sonenberg and Hinnebusch, 2009). This type of regulation also depends on the composition of the mRNA itself and involves gene-specific regulation by reinitiation (REI) or by leaky scanning on short uORFs (Mueller and Hinnebusch, 1986; Abastado et al., 1991; Calvo et al., 2009; Rodriguez et al., 2019).

2.3.1.1 Definition of uORFs, diversity across species and distribution in the genome

uORFs are defined this way since they occur in 5' UTR before, or “upstream”, of the main CDS and usually at least one sense codon should follow start codon of uORF, before stop codon in-frame. This stop codon lies prior to the authentic initiation codon of the main CDS or may be located within the CDS in the overlapping sequence; both variants where uORF is formed were found to be functional (Gray and Wickens, 1998; Johnstone et al., 2016; Kozak, 1987; Whiffin et al., 2020).

Although the process of uORF annotation is complex, it can be stated that about 15% of mRNAs in yeast and 49% in mammals carry at least one uORF, many of which are evolutionarily conserved across the species (Calvo et al., 2009; Iacono et al., 2005; May et al., 2023; Resch et al., 2009). One of the reasons why finding uORF-encoded genes is a challenging task is that uORFs may utilize alternative start sites or near-cognates for starting translation process (Dever et al., 2023; Ingolia et al., 2014; Peabody, 1989; Spealman et al., 2018). Fortunately, a number of modern robust tools such as ribosome profiling, or Ribo-Seq, large scale RNA sequencing, mass spectrometry-based proteomics, CRISPR tiling screens and guide RNA libraries are nowadays available to greatly assist in prediction, data mapping and identification of uORFs (Chen et al., 2020; Chothani et al., 2022; Fritsch et al., 2012). In fact, to date, more than 2.4 million uORFs for human transcripts have been predicted using bioinformatics-based approaches (Dever et al., 2020; Manske et al., 2023; McGillivray et al., 2018). Evidently, uORFs are distributed

throughout the genome, suggesting that they possess a critical function in the regulation of gene expression (Baird et al., 2014; Couso and Patraquim, 2017; Young and Wek, 2016).

2.3.1.2 The contribution of uORFs in processes and diseases

The mRNAs containing uORFs mainly encode genes that belong to functional categories such as transcription factors, growth factors and tumor-associated, or proto-oncogenes (Davuluri et al., 2000; Kozak, 1991a; Morris, 1995). Beyond that, uORFs are abundantly represented in receptor proteins, signal transduction components, and their translation contributes to the immune response (Child et al., 1999; Della Bella et al., 2022; Fujii et al., 2017; Marceca et al., 2015; Park et al., 2017; Starck et al., 2016). Translational control by uORFs was demonstrated for mRNAs that participate in cell differentiation, cell cycle regulation and stress response (Andreev et al., 2015, 2018; Araud et al., 2007; Brar et al., 2012; Clemm von Hohenberg et al., 2022; David et al., 2022; Makeeva et al., 2019; Young and Wek, 2016).

Despite the undeniable importance of the role of uORFs in cellular processes, genetic variants creating novel uORFs have been suggested to be subject to strong negative selection due to their ability to cause pathogenic loss of function of related proteins (Whiffin et al., 2020). Aberrant uORF-mediated translational control has been linked to human diseases among which are neurologic or metabolic disorders and inherited syndromes (Lee et al., 2021; Lee, 2002; Wen et al., 2009; Wiestner et al., 1998). uORF-associated genetic variability has been found to be implicated in several human cancers (Hofman et al., 2024; Jürgens et al., 2021; Nelde et al., 2022; Rodriguez et al., 2019; Roiuk et al., 2023; Schulz et al., 2018). It has been shown that uORF sequences can harbor single nucleotide variants that introduce new stop codons or strengthen already existing ones within the translated uORFs and hence underlie disease-associated processes (Lee et al., 2021). An interesting point is that the generation of new stop codons in uORF may result in ribosome stalling or collisions that drive suppression of the main CDS expression level (Fang et al., 2000).

2.3.1.3 Inhibitory effect of uORFs on translation of the main ORF

Even though there are a few exceptions where uORF and main CDS seem to be regulated in the same direction, it is widely accepted that uORFs act as negative barriers on the way to the main CDS and may inhibit its translation up to 80%, which is the basis of the effect

of uORFs on human phenotypes (Calvo et al., 2009; Hinnebusch, 1988; Johnstone et al., 2016; Reynolds et al., 1996; Somers et al., 2013). This inhibition may likely be present in an amino acid-specific manner (Dever et al., 2020). In consequence, removal of uORF by alternative splicing or alternative selection of the start site results in translation upregulation of the main CDS (Örd et al., 2009).

Factors that were proven to be important in determining the magnitude of the effect of a particular uORF on the expression of the main CDS are shown on the (Figure 5A) and include the distance from mRNA cap to uORF (increased one is inhibitory), the length of uORF, the number of uORFs incorporated in the 5' UTR (the presence of multiple uORFs is inhibitory because of cumulative effect), the strength of the Kozak consensus, the presence of overlapping uORF (Figure 5A and 5B, upper part), the stress status and a combination of factors (i.e., the Kozak context of the uORFs was found to be instrumental in controlling translation under stress) (Kozak, 1987; 2001; Park et al., 2006; Baird et al., 2014).

Crucially, with some, usually small, frequency, the scanning 48S PIC may bypass the uORF start site in a process referred to as 'programmed leaky scanning' (Figure 5B, middle part) (Gunišová et al., 2018).

2.3.1.4 Leaky ribosome scanning through the inhibitory uORFs

The leaky ribosome scanning through the inhibitory uORF start codon mitigates its negative effect and leads to elevated primary translation initiation and thus an increase in expression of the main CDS compared to non-leaky uORFs (Young and Wek, 2016).

Among other things, one of functional significance of the leaky scanning lies in the rapid response to stress conditions, and cellular stress can modulate its severity (Baird and Wek, 2012).

Further, initiation at the uORF's AUG could generate a prolonged isoform (if this AUG was in-frame) that could have a different function or be pathological compared to the canonical form of full-length protein (in the case of downstream AUGs, a truncated form could be generated) (Noderer et al., 2014), and hence the particular importance of the leaky scanning in regulating the ratio of isoforms present in the cell.

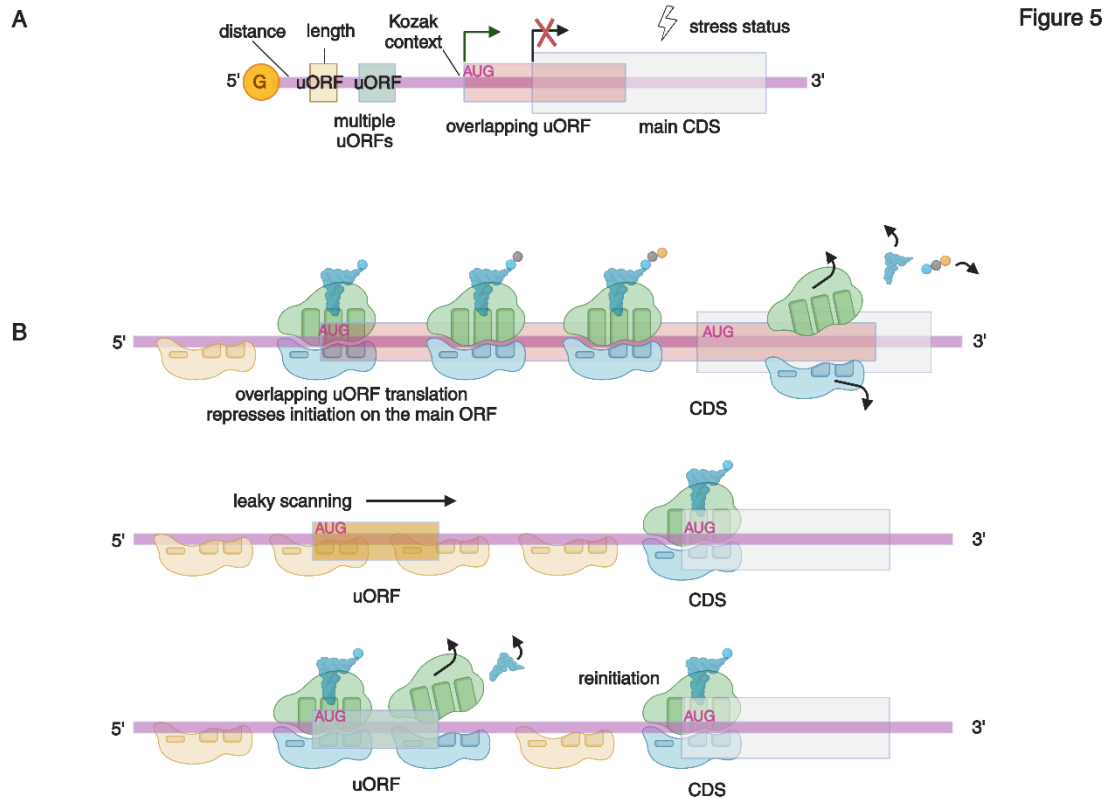


Figure 5. uORFs and overlapping uORFs regulate downstream main coding sequence (CDS) translation. **A.** The factors that are important in determining the magnitude of the effect of uORF on the expression of the main CDS include the distance from mRNA cap to uORF, the length of uORF, the presence of multiple uORFs, the Kozak context, the presence of overlapping uORF, the stress status and combination of several factors. For details, please see the main text. **B.** Translational control by uORFs. Overlapping uORF translation represses initiation on the main ORF (upper part). In detail, the 40S ribosome encounters the uORF, translates it, even past the CDS start codon, and undergoes a full recycling phase. In another case, when the ribosome skips uORF by leaky-scanning mechanism (middle part), the main CDS is being translated instead. In addition, the ribosome can translate the uORF, recycle only the 60S subunit, resume scanning, reacquire the TC and reinitiate downstream at the AUG of the main CDS (bottom part). Adapted from (Young and Wek, 2016). Created with BioRender.com

In fact, two proteoforms that differ by only a few amino acids at N-terminus can be functionally distinct, and interestingly, the ratio of proteoforms can be altered if the start codon recognition fidelity balance changes (Hopkins et al., 2013; Liang et al., 2014; Loughran et al., 2020; Shestakova et al., 2023; Zhang et al., 2015).

Initiation codon recognition fidelity, or how often a particular start codon in the optimal context is recognised, and the degree of leaky scanning are also impacted by changes in the levels of eIFs, concretely eIF1, eIF1A, eIF3 and eIF5 (Fijalkowska et al., 2017; Hinnebusch, 2014; Valášek et al., 2017). The interactions between eIF1 and eIF5, and changes of their levels that are controlled by uORFs in their mRNAs (and by the strength of Kozak consensus surrounding initiation sites) provide an excellent illustration of the stringency of start codon selection and how the degree of leaky scanning can be modulated (Ivanov et al., 2010; Loughran et al., 2012).

And finally, yet another group of uORFs include those that, following their translation, allow the 40S ribosome to continue scanning downstream to the AUG of the main CDS and reinitiate there (Figure 5B, bottom part) (Kozak, 2002; Gunišová et al., 2018).

2.3.2 Translational control by reinitiation (REI)

2.3.2.1 Translation REI mediated by short uORFs

Translation reinitiation (REI) is a widespread event in eukaryotes that represents a gene-specific regulatory mechanism in which only the large 60S subunit and deacylated tRNA are recycled upon translation and termination at a stop codon of REI-permissive short uORFs, whereas the 40S subunit escapes normal ribosome recycling, remains bound to the same mRNA molecule, resumes scanning, recognizes a different initiation site downstream, and re-initiates again there (Figure 5 B, bottom part). Essentially, the ribosome does not fully go through all the recycling steps and starts the translation process again, allowing one ribosome to make more than one protein from one mRNA (Gunišová et al., 2018; Munzarová et al., 2011; Sherlock et al., 2023; Valášek et al., 2017).

2.3.2.2 Factors affecting REI-permissiveness of uORFs

The ability of uORF to sustain 40S-mediated REI, or REI-permissiveness, is reliant on several characteristics. The first is the presence of *cis*-acting mRNA features in the vicinity of uORF, which are termed REI-promoting elements (RPEs), with REI being largely

reduced upon their removal or upon any extra insertion into RPEs sequences (Munzarová et al., 2011; Gunišová et al., 2016, 2018). The second is the ribosome capacity to retain critical eIFs that make a contribution to primary initiation at the uORF start codon. Functional interaction between eIF3 and RPEs has been demonstrated to be required for stabilization of 40S subunit on mRNA (Hinnubusch, 2005; Szamecz et al., 2008; Gunišová et al., 2018).

The length of the uORF is another factor affecting its permissiveness. The shorter the uORF, the more likely it is that certain eIFs will remain associated with the ribosome even throughout the first few rounds of elongation (Hronová et al., 2017; Mohammad et al., 2017; Valášek et al., 2017). With increasing uORF length, the ribosome transit time, and the chance that REI-promoting eIFs would fall off increases too (Kozak, 2001). The time required for uORF elongation may also be defined by the predisposition of its sequence to form stable secondary structures (Gunišová et al., 2018).

It is further worth taking into account that after resumption of scanning, the 40S subunit needs some time to re-acquire a new TC, making TC availability a rate-limiting factor for REI downstream, as well as the intercistronic distance from uORF to the main CDS. Introducing the artificial sequences after uORF and before main CDS and thus extending the distance between them brought experimental confirmation of the proportionality of distance to REI efficiency (Grant et al., 1994; Munzarová et al., 2011). In the light of the above considerations, the size and arrangement of the uORF is important. In mammals, REI-permissive uORFs are fewer than 30 codons and in yeast they are typically fewer than 10 codons. uORFs that are longer or overlap main CDS are defined to be REI non-permissive and may vary widely in their ability to capture scanning ribosomes (Kozak, 2001; Szamecz et al., 2008).

The best-studied examples of REI on short uORFs are yeast *GCN4* and mammalian *ATF4* transcriptional activators, they are functional homologues. *GCN4* has four uORFs (two REI-permissive and two REI-non-permissive), whereas *ATF4* has uORF1 and uORF2, which partially overlaps *ATF4* main CDS (Mueller and Hinnebusch, 1986; Lu et al., 2004; Vattam and Wek, 2004). Detailed information on the translational control of *GCN4* and *ATF4* via REI will be provided in the following chapters.

Aside from canonical eIFs, it has been described that recycling factors eIF2D/Ligatin, in human (Tma64 in yeast), and MCTS1 (Tma20) and DENR (Tma22) heterodimer are

essential on selected mRNAs for both REI and recycling (Bohlen, Harbrecht, et al., 2020; Young et al., 2018), and the lack of these factors alters REI rates in the cells. Recent studies have reported that a canonical initiation factor eIF3j (Hcr1) has this capacity too (Young and Guydosh, 2019). Defects in translation REI or ribosome recycling factors can be the cause of human disease (Bohlen et al., 2023; Castelo-Szekely et al., 2019; Young et al., 2018).

Regulation of translation by means of REI has been proposed to have many functions, in particular rapid response to environmental conditions (Liu and Qian, 2014). For example, oxidative stress provoked by arsenite treatment has been proven as a factor that significantly increases REI levels on uORF-containing mRNAs (Makeeva et al., 2023).

In addition to the presence of environmental stress and the activity of eIFs, the efficiency of REI can be affected by many other factors, including the strength of the Kozak initiation context, and the presence of mRNA secondary structures.

2.3.2.3 Other factors affecting the efficiency of REI

The selection of nucleotides adjacent to the start codon, where REI can be expected to take place, significantly influences its efficiency (Bohlen, Fenzl, et al., 2020; Hinnebusch, 2011). The optimal selection of the sequence of these nucleotides to maximize probability of REI involves 3 nt upstream and 1 nt immediately downstream of the potential start codon and is called the Kozak consensus (Kozak, 1984, 1986b; Pisarev et al., 2006). In vertebrates, for a ‘strong’ Kozak consensus GCCR(R = A or G)CCAUGG, both the purine base at the -3 position (where A of AUG codon is set to +1) and the purine at the +4 position should match (Kozak, 1987). Substitution of one of the purines at the -3 or +4 position downgrades the ‘strong’ initiation codon to ‘adequate’, and changing the purines at both positions turns the context to ‘weak’. In *S. cerevisiae*, the Kozak-like consensus is typically AAAAAAAAAUGUCU, where the most important positions are underlined (Hamilton et al., 1987).

Additional quantitative analysis of possible contexts covering positions -6 to +5 of the mammalian translation initiation sites was recently performed and the strongest context was found to be RYMRMVAUGGC (R=A or G, Y= C or U; M = A or C; V = G, C or A) (Noderer et al., 2018).

For the case when start codon recognition occurs at near cognate codons, it was noted that the optimal context is not necessarily the same as for the AUG codon, and that the +5 and +6 positions are relevant determinants too (Boeck and Kolakofsky, 1994; De Arce et al., 2018; Grünert and Jackson, 1994).

Even though the context is important for the translation initiation site recognition, the ribosome might initiate at start codon even in a weak Kozak context. In that case, translation control can be impacted by the presence of mRNA secondary structures. It has been shown that the stem loop located 14 nucleotides downstream of the beginning of the main CDS (the estimated distance between the leading edge of the ribosome and its P-site) can most facilitate recognition of the initiation codon. Moreover, it also increases translation from near cognate codons, even in suboptimal or weak contexts (Kozak, 1990; Kwon et al., 2001; Loughran et al., 2020; Wang et al., 2022). A possible reason is that a secondary structure in this position would slow down the scanning process, suppress leaky scanning and give the ribosome more time to identify the start codon (Kozak, 1994, 2001).

2.3.3 Alternative mechanisms of translation control by mRNA-specific elements

2.3.3.1 Programmed ribosomal frameshifting stimulated by mRNA secondary structures and mRNA-specific motifs

Apart from REI and leaky scanning, non-canonical mechanisms of translation in eukaryotes include programmed ribosomal frameshifting, in which a ribosome slips typically +1 nucleotide forward or -1 backward to translate a protein in a new alternative reading frame (Atkins et al., 2016; Ketteler, 2012). The utilization of -2 frameshifting has also been described in eukaryotes (Atkins et al., 2016; Fang et al., 2012).

As a result of the frameshifting, the sequence changes from the shift point, i.e. the frameshift site, and subsequently new stop codons may appear in the new reading frame leading to premature termination. In case the frameshifting product were synthesized, it would be the same as the annotated product from N-terminus, but the C-terminus would be different (Atkins et al., 2016).

Specific motifs on the mRNAs, e.g. slippery sequences, rare codons, which are present less frequently in particular organism and their cognate tRNAs are low-abundant, can effectively stimulate programmed ribosomal frameshifting (Elf et al., 2003; Lindsley et al., 2005). The presence of mRNA structures is also among the signals that stimulate

frameshifting (Atkins and Björk, 2009; Dinman, 2012). The contribution of mRNA secondary structure properties, such as rigidity or plasticity, to the efficiency of frameshifting can vary widely (Halma et al., 2019; Wu et al., 2018).

In eukaryotes, the frameshifting event within antizyme mRNA, the ornithine decarboxylase inhibitor, responsible for the concentration of polyamines in the cell, is well described. Remarkably, high levels of polyamines increase the efficiency of +1 programmed translational frameshifting by up to 30%. In addition to the frameshifting site, the antizyme mRNA contains a 3' signal in the form of a pseudoknot that slows down the ribosome, which then should decide to terminate or continue translation, but in a +1 frame (Howard et al., 2001; Ivanov and Atkins, 2007).

There are many other examples of frameshifting events identified in human mRNAs (Belew et al., 2014; Clark et al., 2007; Wills et al., 2006).

2.3.3.2 N⁶-methyladenosine (m⁶A)-dependent translation initiation

In addition to the REI and leaky scanning mechanisms underlying the selective translation of mRNAs that are upregulated under stress and all the other factors mentioned above, an important role of N⁶-methyladenosine (m⁶A) methylation has been reported as well (Zhou et al., 2018).

The m⁶A is the most abundant reversible mRNA modification in eukaryotes, and it is known to recognize the particular motifs in the sequence: RRACH (R = G or A; H = A, C or U) and DRACH (R at position -2 to m⁶A can be, in addition to G or A, the nucleotide U) (Dominissini et al., 2012; Linder et al., 2015; Meyer et al., 2012).

Potential involvement of m⁶A writers, erasers and readers in the regulation of m⁶A levels by interaction with eIF3 and modifications in UTRs of mRNA has been described (Brito Querido et al., 2024; Zhou et al., 2018).

Moreover, the m⁶A potential role in an eIF3-mediated cap-independent initiation has been reported, but the mechanism remains unclear (Genuth and Barna, 2018; Meyer et al., 2012; Zhou et al., 2015). One possibility which has been hypothesized is that m⁶A modification may be involved in a 5' end-dependent scanning mechanism (see 2.1.2.2) by slowing down ribosomes *via* direct recognition of m⁶A sites by eIF3 subunit a, or alternatively, not only

owing to motifs in the sequence, but also through alteration of nearby mRNA secondary structures (Meyer et al., 2015).

Taking everything into account, the most of eukaryotic mRNAs have features that maintain the regulation at the level of translation, including initiation and REI fidelity control (Kozak, 1991b, 2005).

2.4 Translational control *via* REI on yeast *S. cerevisiae* *GCN4* mRNA

2.4.1 *GCN4* mRNA as a model of translational control *via* REI

The *GCN4* mRNA is one of the best characterized examples to date of translational control *via* REI on four short uORFs in yeast *S. cerevisiae* (reviewed in Hinnebusch, 2005). *GCN4* encodes the 281 amino acid long Gcn4 protein, which contains a basic leucine zipper (bZIP) domain and which functions as a global transcriptional factor of the GAAC pathway. Tight regulation of *GCN4* mRNA expression in response to amino acids starvation is facilitated by Gcn2 kinase, which phosphorylates eIF2 α (Dever et al., 1992; Hinnebusch, 1993) and upregulates *GCN4* expression, which further triggers activation of amino acid biosynthetic genes (Hronová and Valášek, 2017; Jennings et al., 2017).

In non-starved cells, four uORFs inhibit *GCN4* translation by constraining the progression of scanning ribosomes across the 5'UTR to AUG of the *GCN4* main CDS (Hinnebusch, 1997). These uORFs include REI-permissive uORF1 and uORF2 (~90% efficiency, if compared to uORF1) and REI-non-permissive uORF3 (~18%) and uORF4 (~4%), and all of these uORFs 1-4 are required for preventing of Gcn4 translation under normal conditions but mainly uORF1 translation is needed for efficient expression of Gcn4 upon starvation (Figure 6) (Mueller and Hinnebusch, 1986; Munzarová et al., 2011).

2.4.2 Fail-safe mechanism of *GCN4* translational control

After translation of uORF1, 5' proximal positive element of *GCN4* (due to RPEs i.-iv. in its 5' sequence), about 50% of ribosomes stay bound to mRNA and REI downstream. Under normal, or non-starvation conditions, the major proportion of 40S subunits translate uORF1, then terminate and, because of the high level of eIF2 and TC availability in the cell, have time enough to recover and still have the opportunity to REI at uORF2, uORF3 or uORF4. The latter one is the most 5' distal negative element, which represses Gcn4 expression. After translation of uORFs, the terminating ribosomes undergo full recycling, and, in this case, Gcn4 protein is not produced (Figure 6A, upper part) (Grant et al., 1995; reviewed in Gunišová et al., 2018).

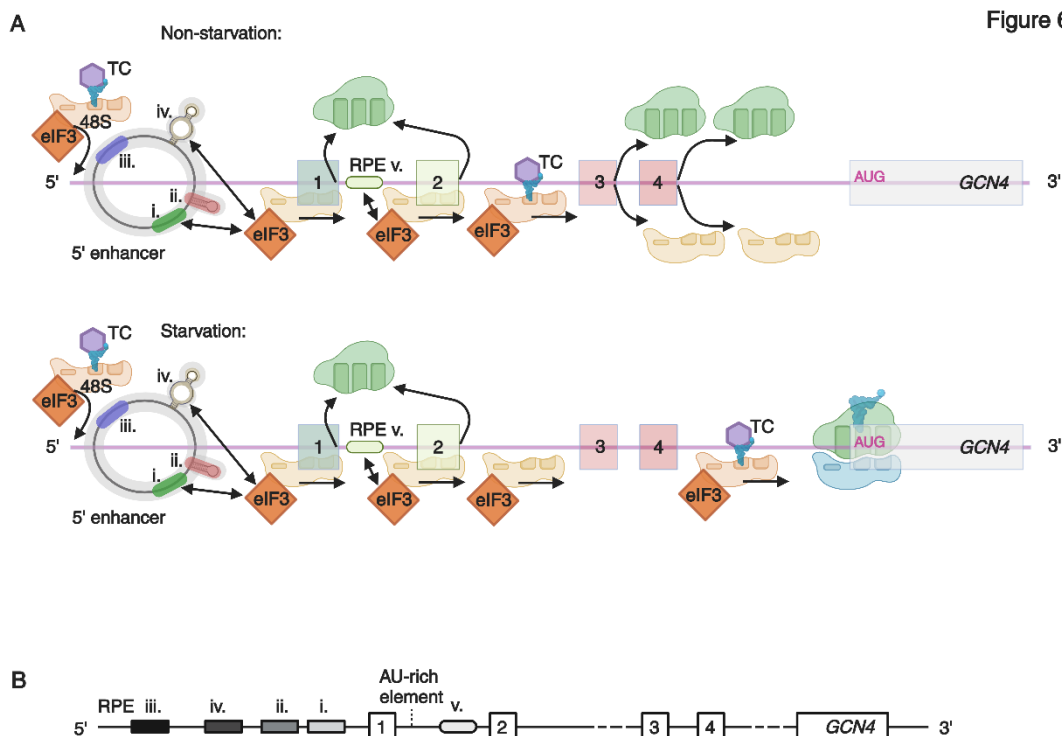


Figure 6. A. Model for fail-safe mechanism of *GCN4* translation control mediated *via* REI under non-starvation (upper part) *versus* starvation conditions (bottom part). Functional interactions between eIF3-dependent RPEs and NTD of eIF3a/TIF32 are indicated by arrows. For details, please see the main text. Abbreviations: TC, ternary complex; eIF3, eukaryotic initiation factor 3; 48S, 48S preinitiation complex; RPE, reinitiation-promoting element; 1-4, four short uORFs 1-4. **B.** Schematic of the *GCN4* leader containing four short uORFs (1-4) and RPEs. Summary of the features of uORF1 and uORF2 that affect the molecular mechanism of *GCN4* and ensure high REI efficiency. Adapted from (Gunišová et al., 2018; Gunišová and Valášek, 2014). Created with BioRender.com

Conversely, under amino acid starvation conditions, due to eIF2 α -P and reduced cellular levels of TC, the small subunit of the ribosome must scan for a much longer time after uORF1 translation. It traverses through the AUG of uORF2-4 and becomes REI-competent later, after scanning extra sequences on its way to AUG of the main CDS of *GCN4*. Then, the delayed acquisition of eIF2-TC occurs, the ribosome recognizes *GCN4* start site and REI there (Figure 6A, bottom part) (Abastado et al., 1991; Hinnebusch, 1997).

This mechanism of *GCN4* translational control is referred to as ‘fail-safe’. In more detail, uORF2, like uORF1, is a REI-permissive and serves as a functional back up for uORF1

(i.e., as a ‘fail-safe’ for scanning PICs that leaky scanned uORF1 during the primary initiation). uORF3 does the same for uORF4. This makes the entire *GCN4* system more stringent, more effectively preventing Gcn4 from being expressed under non-starvation conditions and enables greater derepression of Gcn4 under amino acid starvation conditions (Gunišová and Valášek, 2014).

2.4.3 Alternative ways to amino acid starvation to induce *GCN4*

It has been suggested that alternative ways to amino acids starvation are also possible involving the same short uORF-dependent mechanism in the 5' leader of *GCN4* and increased translation of *GCN4* mRNA. Among them are purine deprivation, where eIF2 α -P is also present and the control is also Gcn2 dependent (Hinnebusch, 1997; Rolfes and Hinnebusch, 1993), and glucose limitation, which leads to elevated translation of *GCN4* and can induce activation of Gcn2 protein kinase too (Yang et al., 2000). Then, alkylation damage with methyl methanesulfonate leads to activation of protein kinase Gcn2, with subsequent derepression of Gcn4 function and its synthesis (Natarajan et al., 2001). The mTOR cascade inhibitor rapamycin can also modulate Gcn4 activity by regulating *GCN4* mRNA translation *via* causing an increase in eIF2 α -P (Kubota et al., 2003; Valenzuela et al., 2001).

Summarizing, the main mode of activation of Gcn4 expression operates at the level of *GCN4* mRNA translation.

2.4.4 REI-promoting elements (RPEs) upstream of *GCN4* uORF1 and uORF2

It was previously found that there is a functional interplay between multiple RPEs (i.-iv.) in 5' sequence of uORF1 of *GCN4* and N-terminal domain (NTD) of eIF3 subunit α /TIF32, which underlies uORF1 REI-permissiveness (Munzarová et al., 2011).

RPEs i. and iv. of uORF1, two elements out of four (i.e., RPEs i., ii., iii. and iv.) (Figure 6B), were discovered to interact with Box 6 and Box 17 regions in eIF3 subunit α /TIF32-NTD, and this regulatory interaction (and likely physical) is critically required to enhance REI. Accordingly, the mutations of Box 6 and Box 17 strongly decrease uORF1 REI-permissiveness (Szamecz et al., 2008). Notably, the NTD of α /TIF32 subunit of eIF3 is situated on the 40S subunit close to the mRNA exit channel and can directly contact these RPEs (emerging from the mRNA exit channel) to stabilize the post-TC (Kouba et al., 2012).

The reported secondary structure of RPEs showed the presence of 9 base pair-long stem representing RPE ii., and a double-circle hairpin shaping RPE iv. RPE i. and iii. remain unstructured (Figure 6A and 6B). RPEs ii. and iii. act in the eIF3-independent manner (Gunišová and Valášek, 2014). Taken together, uORF1 makes use of a total of four RPEs (i., ii., iii. and iv.).

Another RPE is in uORF2, RPE v., which resembles the sequence of RPE i. uORF1. RPE v. of uORF2 interacts with Box 6 and Box 17 regions in eIF3 subunit α /TIF32-NTD too. RPE ii. is common for uORF1 and uORF2.

Downstream of uORF1 termination codon is an AU-rich element specific for uORF1 (Figure 6B), which has a profound effect on the REI-permissiveness of uORF1, because it is largely compromised upon AU-rich sequence substitution (Gunišová et al., 2016).

Concerning eIF3, it stays bound to the small subunit scanning upstream of uORF1 or 2. Upon their translation, RPEs emerge from the ribosome mRNA exit channel and successively fold into a secondary structure. Afterwards, eIF3 makes a contact with RPEs in order to stabilize the 40S on uORF, thus preventing full ribosome recycling and enabling efficient REI. uORF3 and 4 do not have RPEs and consequently are unable to use the assistance of eIF3 to stabilize the 40S (Aitken et al., 2016; Mohammad et al., 2017).

2.5 Translational control *via* REI on mammalian *ATF4* mRNA

2.5.1 Activating transcription factor 4 (*ATF4*) mRNA as a model for studying translational control

Translational control *via* REI has also been shown to govern expression of the mammalian functional homologue of yeast *GCN4* (Gunišová et al., 2018; Gunišová and Valášek, 2014), *ATF4*, a master transcription factor (Lu et al., 2004; Vattam and Wek, 2004) that is upregulated by various stress stimuli (Liu and Qian, 2014).

The human ATF4 is a 351 amino acid long protein that belongs to the cAMP response element binding-2 transcription factor (CREB-2) family that under conditions of cellular stress undergo homodimerization *via* a bZIP motif. Then, in the nucleus, ATF4 provokes the induction of the downstream target genes, which have cAMP-responsive elements (CRE) (Han and Kaufman, 2017; Wek, 2018) and can trigger the expression of chaperons in the ER, buffering folding reactions under the ER stress (Han et al., 2013).

2.5.2 *ATF4* is a key player in the integrated stress response (ISR)

In eukaryotes, cell intrinsic stresses such as ER stress can also activate the integrated stress response (ISR), an important signaling pathway that enables the cells to adapt and survive when exposed to different stresses (Pakos-Zebrucka et al., 2016).

The stress response is “integrated” as it is the outcome of different inputs (stresses) action, through converging paths of eIF2 α kinases (see 2.2.1 and Figure 4) (Costa-Mattioli and Walter, 2020).

One of the activated kinases phosphorylates eIF2 α on serine 51 and thereby influences the level of TC required for every new round of translation initiation (Baird and Wek, 2012).

Despite the fact that this causes protein synthesis to be shut down, the cell may remain capable of activating pathways in response to stress conditions by selectively upregulating the translation of specific mRNAs, among them mammalian *ATF4* (Ron, 2002; Pakos-Zebrucka et al., 2016).

2.5.2.1 Activation of PERK kinase by ER stress

Although the induction of *ATF4*, the primary effector during ISR (Wek et al., 2023), can be achieved by activation of one of the four members of the eIF2 α kinase family, PERK is the only kinase that can be induced by accumulation of proteins (Figure 7) that cannot properly fold in the ER lumen (Walter and Ron, 2011).

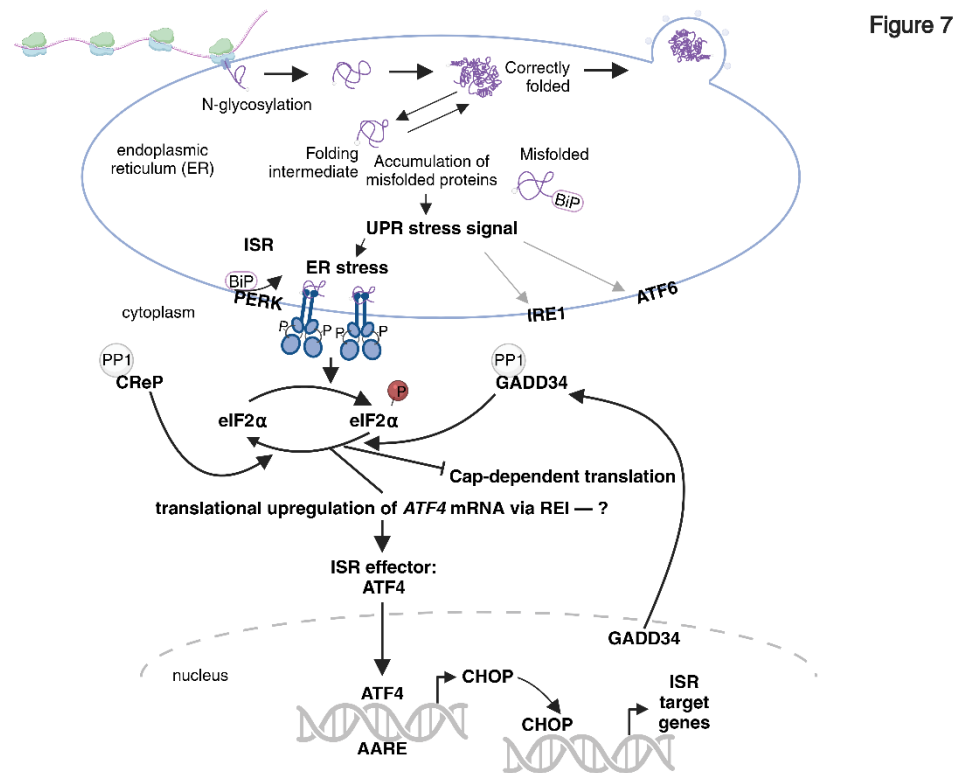


Figure 7. *ATF4* is a key player in the integrated stress response (ISR). UPR, ER stress, ISR, eIF2 α kinase PERK and ATF4 with its downstream targets are indicated. For details, please, see the main text. Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; ISR, integrated stress response; BiP, binding immunoglobulin protein; PERK, protein kinase-like endoplasmic reticulum kinase; IRE1, inositol-requiring enzyme-1; ATF6, activating transcription factor 6; CReP, constitutive repressor of eIF2 α phosphorylation; GADD34, growth arrest and DNA damage-inducible protein; PP1, protein phosphatase 1; ATF4, activating transcription factor 4; AARE, amino acid response element; CHOP, C/EBP homologous protein. Adapted from (Pakos-Zebrucka et al., 2016; Ron and Walter, 2007). Created with BioRender.com and kegg genome pathway database www.kegg.jp

PERK is known to be present in metazoans (Hollien, 2013) and located in the ER membrane. The *PERK* gene encodes a type I transmembrane ER resident protein (Harding, Zhang, et al., 2000). Its domain architecture comprises a transmembrane domain bridging the IRE1-like luminal domain with the cytoplasmic domain, which itself has serine/threonine protein kinase activity and is composed of a conserved kinase domain (Cui et al., 2011).

As for the mechanistic details of PERK activation, it is mediated by interaction with binding immunoglobulin protein (BiP) or alternatively named 78-kDa glucose-regulated protein (GRP78) (Ma et al., 2002). BiP is a molecular chaperone with high affinity for short hydrophobic peptides on the surface of unfolded proteins (Blond-Elguindi et al., 1993; Flynn et al., 1991).

The luminal domain of PERK is normally bound by BiP, preventing its oligomerization (Bertolotti et al., 2000).

When ER stress is triggered by the accumulation of unfolded proteins, BiP dissociates, leading to oligomerization of the luminal domain, autophosphorylation of the kinase domain and further activation of PERK. Another possible model suggests that PERK may be activated by direct recruitment of unfolded proteins to the luminal domain (Urano et al., 2000; Wiseman et al., 2010).

Upon activation, the affinity of PERK for the substrate, i.e. eIF2, is increased (Marciniak et al., 2006), and PERK directly phosphorylates the α -subunit of eIF2 on serine 51. Phosphorylation of eIF2 α inhibits global protein translation (see 2.2.1) and the load of proteins entering the ER is lowered, resulting in ER stress relief (Dever, 2002; Lavoie et al., 2014).

2.5.2.2 Dephosphorylation of eIF2 α as a signal to termination of ISR

After ER stress has been resolved, an important event in the later phase of the stress response is the timely termination of ISR, which can be accomplished by facilitating the dephosphorylation of eIF2 α with the help of growth arrest and DNA damage-inducible protein (GADD34) and allowing the resumption of the synthesis of essential pro-survival proteins (Lee et al., 2009).

Upregulation of *GADD34* expression may be mediated by *ATF4* or also by CAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and activating transcription factor 3 (ATF3), ISR target genes to be described later (Jiang et al., 2004; Marciniak et al., 2004).

When *GADD34* is upregulated during the ISR program, it makes a complex with the catalytic subunit of protein phosphatase 1 (PP1) (Figure 7) and acts to promote dephosphorylation of eIF2 α -P (Neill and Masson, 2023).

Alternatively, PP1 phosphatase activity may be modulated by constitutive repressor of eIF2 α phosphorylation (CReP), which under non-stress conditions directs PP1 to eIF2 α (Figure 7) to keep eIF2 α -P levels low in the cell (Brush et al., 2003; Kojima et al., 2003; Novoa et al., 2003).

2.5.2.3 The role of the ISR program in transition from acute to chronic ER stress

Adaptation of the cell to stress, stress alleviation and eIF2 α dephosphorylation are signs of the end of the ISR program and usually occur when the stress does not last very long (Novoa et al., 2003).

The subsequent normalization of protein synthesis is accompanied by a decrease in the expression of accumulated ISR-mRNAs carrying short uORFs, such as *ATF4*, *CHOP*, *GADD34* and activating transcription factor 5 (*ATF5*) mRNAs (Pakos-Zebrucka et al., 2016).

Initially, ISR is assumed to be an adaptation-promoting pathway, but the cellular response elicited by stress responsive genes and dependent on the cellular context, origin and severity of the stress may influence the outcome of the program (Harding et al., 2003). Exposure to intense or prolonged stress can turn an acute adaptive ISR program into a

chronic one, leading to programmed cell death (Novoa et al., 2003; Pakos-Zebrucka et al., 2016).

The chronic ISR program maintains permanently increased translation of mRNAs containing inhibitory uORFs as a major hallmark. However, in this program, PERK-dependent transcription, translation and epitranscriptome-based reprogramming of gene expression involves a transition from eIF4F/eIF2B-dependent initiation mechanism to eIF3d-dependent (Guan et al., 2014, 2017).

The chronic ISR program facilitates both stress-sensing and adaptive response, leading to partial recovery of protein synthesis that specifically upregulates the expression of proteins with a particular role in ER, and when this program is compromised, it can lead to ER dysfunction, pathological state and “foamy cell” phenotype (Guan et al., 2017; Mukhopadhyay et al., 2023).

The study of the signaling pathways underlying acute and chronic ISR gene programs is important for understanding the dual roles of ISR in cell survival and cell death (Lin et al., 2008; Walter and Ron, 2011).

2.5.2.4 The pro-survival and pro-death signaling by the ISR and unfolded protein response (UPR)

The ISR, together with another cellular pathway of which it is a part, termed the unfolded protein response (UPR), regulate the decision whether to protect the cell by an adaptive response or to promote cell death (for example, by apoptosis or proteotoxicity) by regulating the signaling of the respective genes (Dever et al., 1992; Scheuner et al., 2001; Walter and Ron, 2011).

In addition to PERK-eIF2 α pathway involved in ISR, there are two other branches of the UPR (Figure 7), represented by the inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6) ER-transmembrane proteins (Walter and Ron, 2011). The cooperation between these three distinct branches of the UPR determines cell fate (Marciniak et al., 2004).

IRE1 has two isoforms in mammals, IRE1 α and IRE1 β , the first of which carries an ER-luminal domain that has high sequence homology to the PERK domain and can sense unfolded proteins. Then, BiP dissociates and IRE1 α oligomerization occurs with following

trans-autophosphorylation. The other domain of IRE1 α , the RNase domain, then activates mRNA of the mammalian transcription factor *XBPI* (*HAC1*, in yeast), by cleavage and removal of the intron. Activated *XBPI* upregulates UPR stress genes to restore homeostasis (Bertolotti et al., 2000; Calton et al., 2002; Cox et al., 1993; Sidrauski and Walter, 1997).

In yeast *S. pombe*, the model has been suggested, where Ire1-catalysed cleavage in ER targeted mRNAs results in ribosome stalling at the 3' end of truncated mRNAs further causing the degradation by the mRNA no-go decay machinery (Gyrdosh et al., 2017).

The ATF6 sensor of the UPR plays an important role in the induction of chaperone-encoding genes and, after BiP dissociation, is delivered from the ER to the Golgi body where it is activated by protease-mediated cleavage. Then, after subsequent intra-membrane protease cleavage, the cytosolic portion of ATF6 is released, which can eventually be imported into the nucleus, to bind promoter elements and activate the expression of UPR target genes (Ron and Walter, 2007; Tabas and Ron, 2011).

Long-term activation of IRE1 α is known to lead to cell survival, whereas persistent activation of PERK promotes apoptosis (Lin et al., 2007).

Although downstream signaling mechanisms between UPR branches are not directly interconnected, their functional effects overlap considerably (Ron and Walter, 2007).

2.5.2.5 Reagents inducing ER stress and targeting components of ISR and UPR signaling pathways

For a comprehensive understanding of how ISR and UPR signaling pathways cause distinct cellular outcomes, pharmacological modulation by reagents, including ER-stress inducing ones, represents a promising strategy.

The ER stress agent Thapsigargin (Tg) is known to block sarco/ ER Ca²⁺-ATPase (SERCA) and deplete Ca²⁺ in ER and is therefore widely used to study ISR and UPR (Guan et al., 2014; Jackson et al., 1988; Lu et al., 2004; Vatter and Wek, 2004).

Another agent, tunicamycin, provokes ER stress and induces the accumulation of misfolded proteins by a mechanism of inhibition of N-linked glycosylation (Noda et al., 1999; Pincus et al., 2014; Rendleman et al., 2018).

Treatment with sodium arsenite (NaAsO_2) induces stress, which also results in rapid phosphorylation of eIF2, inhibition of global translation, and activation of stress signaling pathways (Andreev et al., 2015, 2018; McEwen et al., 2005).

There is evidence of UPR signaling associated with ER stress caused by saturated free fatty acids and induction of apoptosis upon their exposure (Cunha et al., 2008; Han and Kaufman, 2016).

The activation of ISR signaling pathway can be antagonized by integrated stress response inhibitor (ISRIB), a small compound that can recover translation downstream of eIF2 α (Halliday et al., 2015; Zyryanova et al., 2021).

An interesting concept has recently been proposed suggesting that certain conditions may more readily trigger one UPR signaling mechanism at the expense of another, and in some tissues, there is a special membrane bilayer environment facilitating this (Mitra and Ryoo, 2019). In yeast *S. cerevisiae*, for example, the molecular details of *IRE1* activation by lipid bilayer stress have been described, and lipid bilayer stress has been posited as the cause that results in the *IRE1*-mediated branch of UPR (Covino et al., 2018).

2.5.2.6 ISR target genes activated by *ATF4* upregulation during stress

The balance of appropriate gene expression during the stress response is essential for the cell pro-survival or pro-death decisions (Chen et al., 1996; Han et al., 2013; Teske et al., 2011). Activation of part of the protective branch of UPR, represented by *IRE1* and *ATF6*, ultimately leads to increased transcription of genes that promote degradation of the unfolded proteins (Pakos-Zebrucka et al., 2016).

The consequences of stress response activation also involve the translation of *ATF4* mRNA and its downstream targets. Remarkably, the pathways leading to the induction of apoptosis can be regulated through the transcriptional activity of *CHOP* (Han et al., 2013; Palam et al., 2011). *CHOP* forming a heterodimer with *ATF4* can enhance the expression of *ATF5* (Watatani et al., 2008; Zhou et al., 2008), ultimately reducing cell survival and enhancing cell death signaling by modulating the expression of apoptotic genes (Teske et al., 2013).

ATF4 mRNA upregulation provoked by stress activates expression of downstream ISR target genes *via* binding to C/EBP-ATF response element (CARE) enhancers. These

CARE enhancer sites can act as an amino acid response element (AARE) (Figure 7) during stress caused by amino acid limitation (Fawcett et al., 1999; Kilberg et al., 2009). As previously mentioned, ATF4 can form heterodimer with CHOP and in this way regulate AARE-dependent transcription (B'Chir et al., 2013).

The target genes that are upregulated in response to increased translation of *ATF4* mRNA are mainly represented by transcription factors containing the bZIP domain and are commonly implicated in apoptosis, autophagy, biogenesis of protein components, amino acid biosynthesis and a number of other processes that are discussed in the later parts of this thesis (Han et al., 2013; Neill and Masson, 2023).

2.5.2.7 Regulatory mechanisms, contributing to ATF4 upregulation in stress

ATF4 levels increase in response to a variety of stress signals other than ER stress, including anoxia or hypoxia, amino acids deprivation and oxidative stress (Ameri and Harris, 2008; Blais et al., 2004; Harding, Novoa, et al., 2000; Shin et al., 2015; Siu et al., 2002). Another classical stimulus is glucose deprivation, which potentiates mitochondrial bioenergetics and the formation of respiratory supercomplexes through modulation of PERK activity. In turn, PERK activity facilitates upregulation of oxidative phosphorylation *via* *ATF4*-promoted expression of supercomplex assembly factor 1, which improves mitochondrial respiration (Marciniak et al., 2022). In addition, it is generally recognized that *ATF4* levels respond to different types of stresses affecting mitochondria (Mitra and Ryoo, 2019).

Among other examples, upregulation of *ATF4* and other ER stress regulators involved in the PERK-eIF2 α pathway was observed in human cells exposed to ultraviolet A radiation (Chen et al., 2019).

Next, *ATF4* mRNA was reported to be a direct target of the nonsense-mediated mRNA decay (NMD) surveillance pathway. NMD was originally presented as a pathway helping the cell get rid of mRNAs with premature termination codons appearing during transcription or splicing errors, but it was later discovered that NMD also targets mRNAs encoding full-length proteins and thus controls their expression level. Inhibition of NMD *via* UPF1 depletion was found to increase the stability of ATF4 protein, along with CHOP, ATF3 and other ISR components (Bohlen et al., 2020; Gardner, 2008; Karousis et al., 2021; Usuki et al., 2017).

Then, post-translational modifications (PTMs) and epigenetic mechanisms were observed to influence ATF4 protein stability and the balance for pro-survival *versus* pro-apoptotic effects during ATF4 overexpression (Köditz et al., 2007; Wortel et al., 2017).

Polyubiquitination has been shown to have a significant function in regulating ATF4 stability, and proteasomal degradation of ATF4 is dependent on its phosphorylation at a serine residue and is caused by the ubiquitin ligase SCF^{BT_{RC}P} (Lassot et al., 2001).

Increased ATF4 expression is associated with a mechanism of drug resistance to proteasome inhibitors such as Bortezomib (Rzymiski et al., 2009). Another proteasome inhibitor, MG132, triggers the expression of ER stress and UPR markers, including ATF4, as a result of cells coping with stress through the ISR pathway (Jiang and Wek, 2005; Nakajima et al., 2011).

Aside from that, the role of the ATF4 in the mTOR Complex 1 (mTORC1) during stress was determined. mTORC1 has been shown to direct the ATF4-driven translation program by means of eIF4E-BPs, but not by changes in the phosphorylation status of eIF2 α (Gingras et al., 1999; Park et al., 2017; Sonenberg and Hinnebusch, 2009; Yamaguchi et al., 2008).

Furthermore, a link between Gcn2 kinase and mTORC1 was established. Under conditions of amino acid deprivation, activated Gcn2 upregulates ATF4, which in turn triggers the expression of Sestrin2, which is required to maintain mTORC1 repression (Ye et al., 2015).

In summary, the increase in ATF4 expression during stress may be regulated by a variety of mechanisms, the major one is attributed to translational control on short uORFs by the delayed REI model.

2.5.3 Translational control of *ATF4* mRNA via delayed REI: old model

The mechanism of translational control of *Atf4* mRNA under stress has been originally demonstrated in a mouse model (Figure 8A) and it is essentially the same as that of *GCN4* mRNA (see 2.4.2 and Figure 6): it is mediated by eIF2 kinases and regulated via delayed REI (Vattem and Wek, 2004). Under non-stress (normal) conditions ribosomes translate uORF1 and then terminate. The 40S subunit stays bound to the mRNA and resumes the scanning process, relatively quickly re-binding TC (since the TC levels are normal), and reinitiates at the AUG of uORF2. As uORF2 overlaps main CDS of *Atf4*, the expression of *Atf4* is inhibited and ATF4 protein is not made (Figure 8A, upper part).

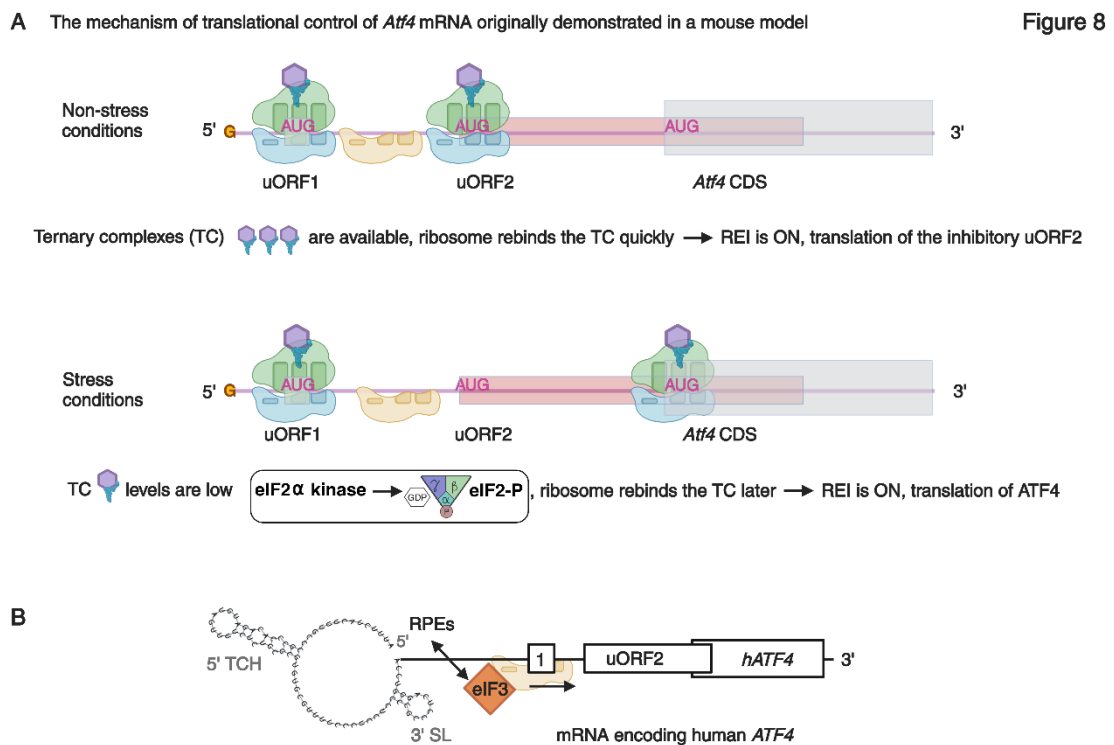


Figure 8. A. Schematics of the molecular mechanism of translational control of *Atf4* mRNA via delayed REI, originally demonstrated in a mouse model, under non-stress (upper part) versus stress (bottom part) conditions. uORF1, uORF2 and the main *Atf4* coding sequence (CDS) are indicated. B. Model of human *ATF4* (*hATF4*) mRNA leader containing short uORF1, uORF2 overlapping main CDS, and REI-promoting elements (RPEs). Functional interactions between RPEs and eIF3 (eukaryotic initiation factor 3) are indicated by arrows. For further details, please see the main text. Adapted from (Gunišová and Valášek, 2014; Lu et al., 2004; Vattem and Wek, 2004). Created with BioRender.com

Under stress conditions, ribosomes translate uORF1, then terminate. The 40S resumes scanning, but TC levels are reduced overall in the cell (eIF2 α is phosphorylated by PERK and the bound GDP cannot be exchanged for GTP (for details see 2.2.1)). The acquisition of TC is therefore delayed, ribosomes are not able to recognize AUG of uORF2 as a next initiation codon and bypass it, and only after that they re-bind new TC and reinitiate later, at the AUG of *Atf4*. In this way, the mechanism of translation control on *Atf4* mRNA is called delayed translation REI mechanism. As a final outcome, the *Atf4* mRNA in this case is being translated, and ATF4 protein is made (Figure 8A, bottom part) (Lu et al., 2004; Vatter and Wek, 2004).

2.5.4 RPEs-like features of ATF4 and their interaction with the eIF3 subunit h

In humans, *ATF4* mRNA possesses two uORFs in its 5' UTR (Figure 8B). The first one is uORF1, which is twelve nucleotides long and, similar to uORF1 of *GCN4*, is highly REI-permissive and hence a positive feature of *ATF4*. Most ribosomes translate uORF1, in a stress-independent manner, as observed for *GCN4*. The second uORF is uORF2, which is 180 nucleotides long and partially overlaps the main CDS of *ATF4* in a -1 frame, thus preventing ATF4 synthesis and serving as an inhibitory, negative element (Vatter and Wek, 2004).

Notably, uORF1 of human *ATF4*, like uORF1 of the yeast *GCN4*, is a positive REI-permissive element with the same length. 5'UTR of *ATF4* uORF1 carries RPEs-like *cis*-acting structural features that follow one after another in the sequence. The extreme 5' segment of *ATF4* folds into a stable 5' triple-circle hairpin (5' TCH), and the rest of the sequence is a 3' stem-loop (3' SL) (Figure 8B), by analogy to the successively shaped structure of 5' enhancer's RPEs ii. and iv. preceding uORF1 of *GCN4* (Figure 6). It was discovered that 5' TCH in *ATF4* comes into contact with eIF3 subunit h (this resembles the action of eIF3 subunit a/TIF32 in yeast) so that it can stabilize the ribosomes after translation and termination on uORF1 to enable REI (Munzarová et al., 2011; Hronová et al., 2017).

The significant effect of downregulation of eIF3 subunit h (*versus* to no effect of eIF3 subunit a) was empirically confirmed using siRNA system, in human cells. The decrease in ATF4 expression by more than 30% was detected in luciferase reporter assays. In this way, eIF3h belongs to factors that enhance REI on human *ATF4* mRNA (Hronová et al., 2017). According to cryo-EM data, human eIF3h appears to occupy position on the ribosome

immediately adjacent to mRNA channel, as it was observed in yeast for eIF3 subunit a/TIF32-NTD (Des Georges et al., 2015). As a matter of fact, human eIF3 comprises twelve subunits, which is seven more subunits than *S. cerevisiae* eIF3, which may explain their different involvement in securing efficient REI (Valášek et al., 2017).

2.5.5 Translational control of *ATF4* mRNA: new discrepancies.

Despite the depth of the original theory, there are a number of inconsistencies in the delayed REI model proposed by Vatter and Wek. The constructs used in the study did not contain the full-length *Atf4* CDS, which includes uORF2/*Atf4* overlap and may represent an important region for the control of *Atf4* mRNA translation by REI and overall upregulation of ATF4 protein during ER stress. Also, normalization to the co-transfected *Renilla* luciferase plasmid, which is by definition altered in expression under stress conditions due to global translation shutdown, was used. Consequently, the relationship between transcriptional and translational upregulation of ATF4 was not properly investigated. Furthermore, all analyses were performed using mouse *Atf4* mRNA, and therefore the contribution of a conserved start-stop element in the 5' UTR of e.g. human *ATF4* mRNA localized upstream of uORF1 to the overall control was ignored (Lu et al., 2004; Vatter and Wek, 2004; Wagner et al., 2020).

Further discrepancies have emerged over time suggesting that delayed REI mechanism may represent only one layer of the whole regulatory system. In direct contradiction with the postulated model, robust translation of uORF2 under stress has been reported in several studies (Andreev et al., 2015; Sidrauski et al., 2015; Starck et al., 2016). Later, by applying quantitative translation initiation sequencing (QTI-seq), the unexpected discordance between occupancy of ribosomes in CDS and translation initiation sites density was observed, and it was found that the annotated initiation site of the *ATF4* CDS was unclear. In the same study, an interesting hypothesis has emerged as to whether the *ATF4* CDS is translated by shifting the +1 frame from the uORF2 reading frame. Finally, m⁶A modification was reported to be involved in *Atf4* translational control in mouse model, and its role is still to be determined (Zhou et al., 2018).

These contradictions raised the question of what the actual molecular mechanism of translational control of human *ATF4* is and prompted us to revisit the *ATF4* delayed REI model.

Towards this end, we designed an entirely new *ATF4*-HA Tag reporter system, under control of CMV-promoter that faithfully recapitulates the regulation of the native mRNA. We used JessTM quantitative Western Blotting assay to compare the reporter constructs in non-stress *versus* ER stress, provoked by Tg treatment. The results of this ATF4 project are recently published and the part of this thesis.

In summary, we fully reproduced the proposed delayed REI mechanism but on top of it we found new aspects of *ATF4* translational control depending on newly identified *cis*-acting features in the 5' UTR and in the main CDS of *ATF4*, and the details of it would be described in the next chapters.

3. Aims

The general aim of this thesis was to decipher the molecular principles of translation REI in yeast and mammals and involvement of eIFs, specifically the components of eIF4F, in this process using the model yeast *S. cerevisiae* *GCN4* mRNA and human *ATF4*, under both non-stress and acute ER stress conditions.

List of particular tasks:

- to utilize the previously established in our laboratory *in vivo* Rap-Nip assay to study the persistence of the eIF4F complex components, concretely eIF4E, eIF4A and eIF4G, on elongating ribosomes on short uORFs and understanding their role in REI mechanism in yeast *S. cerevisiae*
- to revisit the original mechanism of translation control of human *ATF4* mRNA by delayed REI on short uORFs and, being inspired by new findings pointing to various discrepancies, to uncover additional modes of *ATF4* regulation
- to develop and employ JessTM Protein Simple assay to specifically detect and quantify protein expression levels of human *ATF4*-HA tag reporter constructs carrying mutations in regulatory features of *ATF4* mRNA, in order to explore the mechanistic details of *ATF4* translational control under non-stress and Tg-induced ER stress conditions

4. Materials and methods

The experiments to investigate the details of translation REI mechanism in yeast and mammals were performed in yeast *S. cerevisiae* and HEK293T and HeLa human cell lines.

List of methods:

- cultivation of bacterial and yeast cells
- cultivation of HEK293T and HeLa human cell lines
- preparation of whole cell extracts from yeast and human cells
- isolation of DNA and RNA, PCR, molecular cloning, including restriction digestion and RNase H cleavage
- RT-qPCR analysis
- DNA transformation into bacteria and yeast
- isolation of plasmid DNA and transfection of human cells with plasmid DNA
- RNA-protein Ni²⁺-pull down (RaP-NiP) assay
- ribosome protection assay
- T3 ligation assay
- conventional western blotting and automated Jess™ Simple western (SW) Protein Simple assay
- β galactosidase reporter assay in yeast

5. List of publications

Publication I

eIF4G is retained on ribosomes elongating and terminating on short upstream ORFs to control reinitiation in yeast

Mahabub Pasha Mohammad, Anna Smirnova, Stanislava Gunišová, Leoš Shivaya Valášek

Nucleic Acids Research. 2021 Sep 7;49(15):8743-8756.

DOI: 10.1093/nar/gkab652

IF₂₀₂₁ 19.160

IF₂₀₂₃ 16.7

Contribution of the author: 20%

I was involved in the preparation of manuscript and performed several *in vivo* RaP-NiP assay experiments in yeast.

Publication II

Stem-loop-induced ribosome queuing in the uORF2/*ATF4* overlap fine-tunes stress-induced human *ATF4* translational control

Anna M Smirnova, Vladislava Hronová, Mahabub Pasha Mohammad, Anna Herrmannová, Stanislava Gunišová, Denisa Petráčková, Petr Halada, Štěpán Coufal, Michał Świrski, Justin Rendleman, Kristína Jendruchová, Maria Hatzoglou, Petra Beznosková, Christine Vogel, Leoš Shivaya Valášek

Cell Reports. 2024 Apr 23;43(4):113976.

DOI: 10.1016/j.celrep.2024.113976

IF₂₀₂₃ 7.5

Contribution of the author: 40%

I was critically involved in the design, preparation and performance of the majority of experiments. I contributed significantly to data analysis, interpretation of results and preparation of the paper.

Publication III

Protocol for detecting and quantifying human *ATF4*-HA in non-stress *versus* stress conditions using automated and quantitative western blotting (Jess)

Anna M Smirnova, Anna Herrmannová, Leoš Shivaya Valášek

STAR Protocols. November 20, 2024.

DOI: <https://doi.org/10.1016/j.xpro.2024.103454>

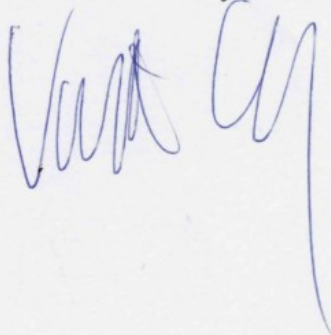
IF₂₀₂₄ 1.3

Contribution of the author: 80%

I contributed to the preparation and design of the experiments in the Protocol, including the key, quantitative western blotting JessTM assay. I carried out all the experiments, analyzed the data, prepared the figures and wrote the first draft of the manuscript.

I hereby confirm that the author of this thesis, Anna Smirnova, have significantly contributed to the publications listed above. In the case of her first-author publication, she has performed a major part of experimental work and contributed to the manuscript preparation.

Res. Prof. Leoš Shivaya Valášek, PhD



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6. Summary of presented publications

Publication I investigated the mechanism of REI and the role of the scaffold protein eIF4G in the yeast *S. cerevisiae* model of *GCN4* mRNA using a previously established Rap-Nip assay for *in vivo* detection of eIFs bound to mRNA fragments. We found that eIF4G and eIF4A, but not eIF4E (all are part of the eIF4F complex), are retained on ribosomes elongating and terminating on short REI-permissive uORFs and thus regulate REI in yeast.

Publication II presents new insights to and extensions of the conventional mechanism of translational control of human *ATF4* mRNA, the functional homolog of yeast *GCN4*, by REI. Importantly, the original studies (Lu et al., 2004; Vattam and Wek, 2004) ignored a few facts and thus produced several important discrepancies. For example, the constructs did not contain the full-length CDS of *ATF4*, but only a few amino acids after the start codon. One study was conducted using mouse *Atf4*, which lacks the so-called “start-stop” element upstream of uORFs, and therefore its translation barrier role was not examined and included in the overall model of *ATF4* translation control. Thus, it is no surprise that in the following publications, another layer of regulation of *ATF4* translation besides the delayed REI mechanism has been proposed. For example, that involving two DRACH methylation motifs in the uORF2 sequence, which have been suggested to serve as possible targets for m⁶A modification. It was also reported that uORF2 can be robustly translated even under stress and that the actual translation initiation start site of *ATF4* was obscure.

In our study, we first recapitulated the original theory on the translational control of *ATF4* by delayed REI and, beyond that, we uncovered new molecular insights into it. We demonstrated that a previously unknown stem-loop in the uORF2/*ATF4* overlap acts as a road block to the scanning ribosomes and provokes ribosome queuing, thereby introducing an additional layer of translational control of *ATF4*.

To elucidate this, we designed a comprehensive set of *ATF4*-HA tag reporter constructs that carry mutations in both rationally and computationally predicted features of *ATF4* mRNA (methylation motifs, near-cognates and stem-loops) and examined their effects using automated and quantitative western blotting JessTM assay.

Apart from the translational barrier effect of the newly discovered stem-loop in overlapping the region of uORF2 and *ATF4* on the ribosome processivity, we have shown that this stem-loop allows the recognition and exploitation of a CUG near-cognate in its close proximity, thanks to the fact that an authentic translational start site is substantially leaky scanned. We also suggested a role for two conserved methylated adenines in the *ATF4* sequence in regulating of *ATF4* translation.

In **Publication III**, we took advantage of the JessTM Protein Simple assay, which we optimized for the detection and quantification of the *ATF4*-HA tag constructs in human cells and which we used to obtain the main results for Publication II. Due to general translational shutdown, studying the translational control of *ATF4* mRNA under non-stress *versus* stress conditions is complicated because the protein expression level of any reference gene declines rapidly upon stress-induced translation shutdown. Therefore, finding an appropriate normalization control is a challenging task. To circumvent this obstacle, we have developed a thorough protocol describing the steps for high-throughput comparison of human *ATF4*-HA tag expression levels under non-stress and thapsigargin (Tg)-induced ER stress, while completely eliminating a need for any normalization.

7. Discussion:

The current thesis focuses on the molecular details of translational control by REI on short uORFs using human *ATF4* and yeast *GCN4* mRNAs, as well as understanding the role of eIFs, particularly eIF4G, in this process.

7.1 Human *ATF4* mRNA translational control by delayed REI and by additional regulatory modes

Translational control by REI has long been thought to be the main regulatory mechanism for *ATF4* mRNA upregulation during stress, but later studies have found that it may be mediated by other mechanisms, as discussed above.

To re-visit delayed REI model of *ATF4* regulation and to address new evidence for other potential factors affecting its translation, we designed a set of human *ATF4*-HA tag WT and mutant constructs under control of CMV-promoter and assessed their effects in ER stress *versus* non-stress.

We have developed a stepwise protocol that avoids normalization to any control gene and ultimately can accurately estimate the effects of mutations in the JessTM assay (Smirnova et al., 2024). The new protocol is important for this type of study because the expression of housekeeping genes is commonly altered under stress conditions due to overall protein synthesis shutdown, and finding a proper control becomes a challenging task.

In our study, we not only re-examined and reproduced the canonical model of *ATF4* mRNA translational control but also presented evidence that there is an additional layer of *ATF4* regulation, surprisingly provided by a stem-loop in the uORF2/*ATF4* overlapping region. This stem-loop functions as a road block to ribosomes and induces ribosome queuing (Smirnova, et al., 2024).

Of relevance, it has also been previously demonstrated by other investigators that the presence of a stable hairpin in the 5'UTR of mRNAs can modulate scanning dynamics by slightly reducing speed of the scanning ribosomes, compared with that without secondary structures (Babendure et al., 2006; Kozak, 1986, 1989; Wang et al., 2022).

As for mechanistic details, we have shown that the ribosome queuing on *ATF4* mRNA, triggered by the stem-loop, can lead to a particular proportion of the 48S PICs that leaky

scan the annotated *ATF4* start site (which we have found to be substantially leaky) to reinitiate at a near-cognate CUG located in close proximity to the stem loop.

This is consistent with the notion that initiation at near-cognate start codons, which usually occurs at lower levels than at canonical start sites, can become more efficient when a stable secondary structure would form downstream (Kearse and Wilusz, 2017; Loughran et al., 2020). And initiation at near-cognate codons such as, for instance, CUG and GUG, is likely to have widespread biological significance (Ingolia et al., 2011).

Although we attempted to examine the role of the stem-loop in the uORF2/*ATF4* overlapping region, the results from HA-tag reporters have not been supported by the insertion of mutations directly into the endogenous *ATF4* locus yet. Therefore, possible future experiments using a system such as CRISPR/Cas may enable us to create a stable cell line carrying the stem-loop mutation(s) and further support our findings.

Our proposed ribosome queuing model explains the robust translation of uORF2 upon ISR induction as well, which was not part of the original *ATF4* theory but has been previously noticed in several other studies employing ribosome profiling (Andreev et al., 2015; Sidrauski et al., 2015). The observation that the REI-non-permissive and inhibitory uORF2 of *ATF4* is strongly expressed even during ER stress may suggest that translation of uORF2 during ISR has an additional role in regulating the main ORF. This phenomenon could be a more wide-spread for mammalian uORFs, as high ribosome occupancy in the 5' UTR of mRNAs preferentially translated under stress has been noticed as well (Andreev et al. 2015).

Despite the fact that ribosome profiling and translational complex profiling can be instrumental in predicting peptides translated from uORFs under stress conditions (Andreev et al., 2024), a much more difficult task is to determine which peptide products formed from uORFs are functional, and their identification, for example, by mass spectrometry (Somers et al., 2013) could be a challenging (due to their small size) but optimal solution.

In another earlier published study focused on the detection of translation events from uORF in the 5' UTRs, a tracing translation by T cells, which was developed to accurately monitor uORF translation products, revealed pervasive peptide expression from both uORF1 and uORF2 of *ATF4* fused to luciferase even during induced ISR conditions

(Starck et al., 2016). However, by inserting the tracer peptide into the uORF2 sequence, the authors replaced part of the uORF2 sequence and unintentionally removed the highly conserved DRACH1 and RRACH1 motifs (for potential m⁶A modification sites), occurring in the non-overlapping part of the uORF2 sequence, the effect of one of which (RRACH1) we have examined and suggested that it may have a role in regulation in humans. Thus, even though this set-up was not technically sound, our data did support major conclusions of this study.

7.1.1 m⁶A modification site in the 5' UTR of human *ATF4* shows inhibitory effect and m⁶A site in the main ORF antagonizes stem-loop function

The m⁶A modification site in the mRNA at a similar position to that we identified has been previously described in the 5' UTR of mouse *Atf4* mRNA, but never in humans (Zhou et al., 2018). In mice, the possible involvement of ALKBH5 m⁶A demethylase in this dynamic m⁶A methylation was observed, as well as that of FTO demethylase. m⁶A methyltransferases knockdown, in turn, was shown to induce *Atf4* expression under amino acid starvation conditions.

As mentioned in previous chapters (see 2.1.2.2), the importance of m⁶A residues in the 5' UTR for mRNA translation under conditions of cellular stress has been suggested by earlier studies to promote a cap-independent mode of translation initiation (Meyer et al., 2015; Zhou et al., 2015).

Recently, the effect of m⁶A on translation initiation has been seriously challenged by (Guca et al., 2024). In this study, using a variety of experimental techniques, they demonstrated that a single m⁶A at the most frequently modified site in the 5' UTRs does not influence translation yield.

Nevertheless, the m⁶A modification can still have an impact on the mRNA, especially if it is located within secondary structures (Guca et al., 2024). m⁶A can destabilize them because the modified adenosine does not favor base pairing with uridines, and its position in the structure (involved in base pairing in the middle or unpaired on the top of the secondary structure) is also important (Kierzek and Kierzek, 2003; Roost et al., 2015).

It is noteworthy that m⁶A modification within the RNA secondary structure (like the location of the A326 site) may function as the “m⁶A-switch”, and in this case m⁶A functions as an RNA structure remodeler that interferes with mRNA maturation. It is also possible that such m⁶A may also recruit other factors, which directly recognize m⁶A, to destabilize the RNA structure and facilitate binding of other proteins (Liu et al., 2015).

Indeed, in our *ATF4*-HA tag system, we documented an interplay between a previously unrecognized m⁶A modification site and the stem loop. We detected a specific effect of a mutation in A326 (located within the RRACH2 motif, in the uORF2/*ATF4* overlapping region) that promoted ATF4 translation while antagonizing the stem-loop function.

In addition, in unstructured region of uORF2, in the sequence motif RRACH1, A235 site exerted an inhibitory effect on the translation of ATF4, which is consistent with a publication on the corresponding methylation site in mouse *Atf4* mRNA (Zhou et al., 2018).

Taken together, we identified two m⁶A modification sites (within RRACH motifs) with distinct roles in *ATF4* mRNA translational control, one of them antagonizing the role of the stem-loop.

7.1.2 Stem-loop-induced ribosome collisions on *ATF4* mRNA lead to ribosome queuing but not to + 1 frameshifting

As a matter of fact, the stem-loop in the uORF2/*ATF4* overlap represents an obstacle that impedes ribosome processivity along the *ATF4* mRNA leader. This event is widely recognized as a potential cause of ribosome collisions (Simms et al., 2017) that could be detrimental to cellular homeostasis if left unresolved.

To facilitate the degradation of such unprocessive mRNAs, an mRNA surveillance process termed no-go decay has evolved (Doma and Parker, 2006). Importantly, severely collided ribosomes should be rescued from transcripts, and this process can be promoted by sensors such as the E3 ubiquitin ligase, Hel2 (yeast)/ZNF598 (mammals) (Meydan and Gydosh, 2021), and several others that will be described below.

However, if no-go decay is compromised and endonucleolytic cleavage of the mRNA is inhibited, ribosome collision events may remain unresolved and lead to +1 frameshifting (Simms et al., 2019; Wang et al., 2018).

Since the stem-loop could induce collisions that could lead to uORF2-to-*ATF4* frameshift and we found typical ribosome frameshift signals including the “slippery” sequences (Dinman, 2012) in the uORF2-*ATF4* overlap, we decided to test it.

Our initial intention was to detect the uORF2-ATF4 fusion product, which we did not even after several tries. Next, we prolonged the uORF2 sequence by inserting several copies of the c-Myc tag in various arrangements with the *ATF4* main sequence and thanks to these experiments we could finally rule out the possibility of a uORF2-to-*ATF4* frameshifting event because no fusion product was detected under any conditions tested.

The second functional consequence of ribosome collisions imposed by the stem-loop (collided ribosomes can be made up of disomes or even trisomes) could be the so-called ribosome queuing phenomenon (Ivanov et al., 2018; Meydan and Guydosh, 2020; Young and Guydosh, 2022).

Indeed, we confirmed ribosome queuing with help of our JessTM assay and specifically designed reporter constructs. Moreover, we provided additional evidence strengthening the resulting ribosome queuing model.

Using the Ribo-Crypt tool and publicly available disome sequencing data (or so-called disome profiling (Guydosh and Green, 2014; Ingolia et al., 2009) from a study where eIF5A was knocked down (Han et al., 2020), we were able to uncover and visualize disome formations between the AUG of uORF2 and the stem-loop in overlapping region but virtually nowhere else.

Furthermore, ribosome protection assays designed in our lab demonstrated protection of the specific fragment covering almost the entire putative queuing region in front of stem-loop from the RNase cleavage. This region was no longer protected where the stem loop was removed by mutation.

Finally, we used open-access ribosome sequencing libraries from previously published studies, where the authors treated human cells with arsenite or tunicamycin to study the effects of the ER stress (Andreev et al., 2015; Ichihara et al., 2021), and compared the total number of footprints in the *ATF4* mRNA, as well as the distribution of footprints of ribosomes aligned according to the position of their P-site. We found clustering of footprints primarily upstream of the stem-loop with a marked preference for the near-

cognate CUG (discussed above) positioned at the ribosomal P site; hence most probably selected as an alternative initiation start site.

Overall, our observations are consistent with a model of a stem-loop induced queue formed by 80S-80S ribosome couples in non-stress and a mixed queue of 48S PICs and 80Ses in stress conditions, beginning with the start codon of uORF2 all the way up to the leading edge of the stem-loop.

Regarding the factors sensing collisions and ribosome queuing and resolving stalled translation events, to further support our queuing theory in a near future, we consider knocking down the following key players: ZNF598, ASCC3, Gcn1, ZAK, and 4EHP (Hickey et al., 2020; Juskiewicz et al., 2020; Pochopien et al., 2021; Wu et al., 2020).

In fact, we propose that the model of ribosome queuing on *ATF4* mRNA resembles, at least in part, a well-established mechanism of ribosome queuing and collisions on the *AZIN1* mRNA encoding the antizyme inhibitor 1 (Ivanov et al., 2018). The *AZIN1* mRNA expression is regulated in response to changing polyamine levels (Ivanov et al., 2008). High polyamine induces pausing of 80S elongating ribosomes in the upstream conserved coding region on the proline-proline-tryptophan motif, which causes ribosome stalling and queuing of scanning 40S PICs with 80Ses. This inhibits translation of the main ORF of *AZIN1*, because ribosomes initiate on the AUU near-cognate start codon of the upstream conserved uORF instead (reviewed in Dever et al., 2023).

The presence of collisions, specifically the accumulation of prominent disome footprints in the upstream conserved coding region of the *AZIN1* mRNA was also observed in (P. Han et al., 2020).

Other examples of a metabolite-triggered ribosome pausing can be found in translational control of *N. crassa arg-2* mRNA and mammalian *PTP4A2* mRNAs (Dever et al., 2020; Ivanov et al., 2009). *N. crassa arg-2* (and its homologue in *S. cerevisiae CPA1*) mRNA contains uORF, which inhibits translation of the main ORF under high arginine conditions (Fang et al., 2004; Wang and Sachs, 1997). On mammalian *PTP4A2* mRNA, ribosome stalling on the uORF is triggered by elevated Mg^{2+} levels, thereby repressing translation of the main ORF (Hardy et al., 2019).

Taken together, our model of the stem-loop-imposed queuing on the *ATF4* transcript provides another essential piece into the jigsaw puzzle of *ATF4* regulation, without which a complete picture of how stress response is controlled cannot be properly assembled.

7.2 The role of *ATF4* in cellular processes and disease

Given the complex nature of the mechanism of *ATF4* translational control, studies aimed at understanding its molecular aspects may represent a promising therapeutic strategy.

However, it should be considered that in addition to ISR signaling, *ATF4* has been shown to play a role in a wide range of cellular and physiological processes. Among others, these include amino acid metabolism and glutathione biosynthesis, autophagy, metabolic adaptation, cell cycle regulation, differentiation (Chaveroux et al., 2016; Harding et al., 2003; Sarcinelli et al., 2020; Torrence et al., 2021). *ATF4* is implicated in hematopoiesis, osteogenesis, modulation of synaptic plasticity, learning and memory formation (Almeida, 2011; Ameri and Harris, 2008; Costa-Mattioli et al., 2005; Noyes et al., 2021; Rached et al., 2010).

Aberrant *ATF4* expression is linked to various pathological conditions and has been shown to reduce the effectiveness of chemotherapy through mechanisms involving dysregulated expression of genes associated with oxidase stress resistance and redox homeostasis. Overexpression of *ATF4* in tumor cells allows them to cope with chronic nutrient deficiencies and the need to maintain rapid growth in the tumor microenvironment (Verginadis et al., 2022; Wortel et al., 2017). Apart from *ATF4*, overactivation of PERK expression in hypoxic regions of solid tumors has also been previously described (Bi et al., 2005; Ma and Hendershot, 2004). Consequently, the idea of *ATF4* and PERK activity inhibition as an optimal approach for anticancer drug development seems plausible (Rzymiski et al., 2009).

ATF4 malfunction has been shown to be associated with other common progressive and multifactorial diseases such as neurodegenerative diseases. These include Parkinson's disease, Alzheimer's disease and Huntington's disease caused by polyglutamine expansion, amyotrophic lateral sclerosis and prion diseases (Baleriola et al., 2014; Bugallo et al., 2020; Devi and Ohno, 2014; Hoozemans et al., 2007; Matus et al., 2013; Scheckel et al., 2020; Sun et al., 2013).

ATF4 has been described to bind a site in the promoter of an E3 ubiquitin ligase parkin, a mutation in the encoding gene of which is the most common cause of the Parkinson's disease. Increased parkin expression has been reported to protect cells from mitochondrial damage and apoptosis induced with tunicamycin and thapsigargin, providing the link between the ER stress and the Parkinson's disease pathogenesis (Bouman et al., 2011).

A high-throughput screen of genes related to neurodegenerative diseases identified ATF4 as an interaction partner of Caspase 6, a protease known to promote programmed cell death pathways (Haenig et al., 2020). Consistent with this, earlier studies have suggested that *ATF4* plays a key role in mediating the apoptosis program (Han et al., 2013). Notably, reduced *ATF4* expression during PKR inhibition has been documented as a contributing factor of neuroblastoma cell resistance to ER stress-induced apoptosis (Vaughn et al., 2014). However, the effects of *ATF4* may differ between neuronal cell types, and its role in apoptosis appears to depend on the cellular context and stress conditions (Neill and Masson, 2023).

The development of pathologies leading to *Diabetes mellitus* is also associated with the ER stress (Ron and Walter, 2007). During the course of the disease, PERK kinase propagates diverse genetic defects that lead to a reduction in pancreatic β -cell-numbers in humans and to inadequate β -cell mass development in mice (Delépine et al., 2000; Scheuner and Kaufman, 2008). Conditional mutations affecting the PERK/eIF2 α pathway result in insulin resistance, glucose intolerance and hyperglycemia (Back et al., 2009).

The role of *ATF4*-mediated transcriptional control in pancreatic β -cells has been demonstrated in studies showing an interplay between ATF4 and the ISR enhancer Sephin1 (Kitakaze et al., 2021).

In research on the progression of diabetes in patients with Wolfram syndrome, it was found that deletion of wolframin in β -cells induced the expression of *ATF4*, *CHOP*, BiP, and sXBP1. β -cells deficient in wolframin showed decreased ER Ca²⁺ levels, causing activation of cell death (Cnop et al., 2017).

Therefore, in β -cells, proper control of ISR-mediated translation is essential for managing ER stress effectively, and modulating ISR activity could have therapeutic potential depending on the disease profile (Costa-Mattioli and Walter, 2020).

A comprehensive understanding of the mechanism of *ATF4* translational control, together with the cross-talk between the ISR and the other two branches of the UPR signaling network, is of considerable physiological importance and may represent a promising strategy for medical applications.

7.3 The yeast *S. cerevisiae* *GCN4* mRNA model for studying the role of eIF4G and eIF4A in translational control by REI on short uORFs

In addition to the *ATF4* mRNA model in human cells, we explored its functional homologue, the yeast *S. cerevisiae* *GCN4* mRNA model. To identify all critical players in REI, we performed the RNA-protein Ni²⁺-pull down (RaP-NiP) *in vivo* assay, which has been established in our laboratory, and which allows detection of specific eIFs bound to 80S during short uORFs translation (Mohammad et al., 2017).

Previous research has revealed that eIF3 is an REI-promoting eIF that is not only required to participate in primary initiation for efficient REI at the downstream start codon (Hronová et al., 2017; Munzarová et al., 2011; Szamecz et al., 2008), but also remains transiently retained on ribosomes even during elongation in yeast (Mohammad et al., 2017) and mammals (Bohlen, et al., 2020; Wagner et al., 2020). Recently, it was found that the eIF3 retention on the ribosome can be modulated *via* modification of its subunit a, which may alter the efficiency of REI (Shu et al., 2022).

It has previously been suggested by genetic data in yeast (Mohammad et al., 2017; Watanabe et al., 2010), experiments with mammalian *in vitro* reconstituted translation systems (Skabkin et al., 2013) and selective ribosome footprinting in human cells (Wagner et al., 2020) that perhaps some components of eIF4F complex could facilitate REI efficiency as well, in a similar manner as the eIF3 multi-subunit complex does. Although ribosome profiling in human cells has indicated that upon subunit joining, eIF4G1 and eIF4E do not immediately dissociate from the 80S ribosome and may stay attached throughout the first few elongation cycles with a decay half-length of ~ 12 codons, the mechanistic details were poorly understood (Bohlen et al., 2020).

Altogether, several studies have made progress in elucidating the role of the eIF4F complex in REI, but the overall picture was still unclear and, particularly in yeast (where the situation could be different from human cells), direct *in vivo* evidence that it stays bound to the elongating 80S ribosome was still lacking.

Therefore, to investigate whether yeast eIF4A, eIF4G and eIF4E might be eIFs that, together with eIF3, facilitate REI, we used an *in vivo* RaP-NiP assay. Following our previous findings on eIF3 (Mohammad et al., 2017), we compared the occupancy of the eIF4F components-bound ribosomes elongating and terminating at the REI-permissive *versus* REI-non-permissive uORFs of the *GCN4* mRNA leader fragments.

We found that co-purification of RNA segments containing REI-permissive uORF1 and uORF2, leads to higher yields compared to co-purification of the RNA segments containing REI-non-permissive uORF3 and uORF4. This was observed for both eIF4G and eIF4A, suggesting their involvement in the REI.

However, no difference was observed in co-purification of RNA segments containing REI-permissive *versus* non-permissive uORFs for eIF4E factor. In agreement with this notion, further analysis of mutations, impairing the specific contact between eIF4G-eIF4E, revealed only general effects that were independent of the REI-permissiveness of the tested uORFs. Therefore, we concluded that eIF4G and eIF4A, but not eIF4E, are preferentially retained on ribosomes involved in translation of short REI-permissive uORFs, and this retention is required for efficient REI downstream.

An important limitation to consider is that uORFs may use other determinants besides eIFs that promote REI, as mentioned in previous chapters (see 2.3.2.2). These include the length of the uORF because as it increases, the REI-permissiveness of the uORF decreases (Kozak, 2001; Luukkonen et al., 1995). The underlying explanation is that the probability that the eIFs in question gradually fall off 80S increases (Kozak, 1987). Indeed, the time required to translate a particular uORF could also be influenced by the complexity of the sequence region of the uORF and whether the uORF sequence has a tendency to form stable secondary structures (Gunišová et al., 2018).

Another critical factor is the presence of *cis*-acting mRNA features, i.e. RPEs (i., ii., iii. and iv.), which are located in the 5' sequence of uORF1. The interaction between some of these (i. and iv) and the NTD of a/TIF32 subunit of eIF3 has been reported to stabilize the 40S-mRNA-eIF3 post-termination complex to enable REI (Mohammad et al., 2017). Likewise, uORF2 exploits RPE v. Then, uORF2 also uses RPE ii., which is shared with uORF1 (Gunišová et al., 2018; Munzarová et al., 2011).

Remarkably, using the RaP-NiP assay, we tested the mutations which impair the interaction of eIF4G with eIF4A and revealed the specifically increased efficiency of REI

at downstream AUGs (due to increased retention of these eIFs on ribosome), after translation of REI-permissive uORF1 over REI-non-permissive uORF4.

The further combining loss of eIF4G-eIF4A binding with a mutation that alters the intactness of the eIF4G1-RNA2 binding domain abolished the specificity for REI-permissive (i.e. uORF1 and 2) *versus* non-permissive uORF (uORF4). This combination also nullified the negative effect of the mutation of RPE i. (uORF1-specific) on the recovered uORF1 mRNA fragment levels and the REI efficiency. The latter confirmed the presence of a functional interaction between eIF4G1 and eIF3 involving RPE i. (as this RPE has been shown to promote REI by binding to the α /TIF32 subunit of eIF3 (Munzarová et al., 2011)).

We assume that this specific contact of the eIF4G1-RNA2 binding domain with the RPE can stabilize the 40S subunits on the stop codons of the REI-permissive (and carrying RPEs) uORFs after termination, allowing only the 60S subunit to get recycled and the remaining 40S to efficiently continue scanning for the start codon downstream.

To summarize, we have provided the first *in vivo* evidence that yeast *S. cerevisiae* eIF4A and eIF4G, but not eIF4E stay persistently bound to the ribosome for several elongation cycles to promote REI downstream (Mohammad et al., 2021).

7.4 Mechanism of translational control by REI in yeast *versus* mammalian model

The mechanism of translational control by REI in yeast and mammals is generally conserved. In both organisms, stabilization of the post-termination 40S subunit on the short uORF on mRNA by eIFs, including eIF3 and eIF4G, is required for efficient REI (Bohlen, Fenzl, et al., 2020; Mohammad et al., 2017, 2021; Wagner et al., 2020). There are, however, several aspects that appear to be different.

Emerging examples of uORF translation suggest that uORFs are inhibitory elements that repress translation of the main ORF. In fact, uORFs in yeast are by definition REI-non-permissive (Jackson et al., 2012) and must be flanked by *cis*-acting RPEs to become REI-permissive (Gunišová et al., 2016; Munzarová et al., 2011). The vast majority of uORFs that carry mammalian mRNAs, by comparison, are less inhibitory than those in yeast and may allow at least some REI (Calvo et al., 2009).

The other difference between human and yeast REI lies in the effect of RPEs and RPE-like elements. Elimination of the REI-promoting sequences surrounding the uORF1 of *GCN4* has a strong effect on *GCN4* REI potential (Gunišová et al., 2016), whereas mutations in the sequences corresponding to the RPE-like elements in the 5'UTR of *ATF4* mRNA have been found to have more moderate effect (Hronová et al., 2017; Smirnova, et al., 2024).

Regarding factors contributing to the promotion of REI, efficient REI on *GCN4* mRNA in yeast requires a/TIF32 subunit of eIF3 (Munzarová et al., 2011; Szamecz et al., 2008), but the mechanism of REI on human *ATF4* mRNA is facilitated by the eIF3 subunit h (Hronová et al., 2017), which is also true for plants (Kim et al., 2007; Roy et al., 2010).

A possible explanation for the different subunit involvement may be in the composition of the eIF3 complex and the number of subunits. In humans and plants, the eIF3 complex is composed of almost twice the number of subunits as in yeast, and some of them are thought to have a specific role in promoting REI (Valášek et al., 2017).

One important note on the involvement of eIF3 subunits in REI in humans is that attempting to distinguish the pure contribution of eIF3a to this process, for example by simple downregulation using siRNA, would be problematic because the eIF3 factor is very complex and downregulating the subunit a affects the stability and integrity of the entire eIF3 complex (Herrmannová et al., 2020; Wagner et al., 2016).

A further major distinction between yeast and human control of translation by REI is the manner in which the components of the eIF4F complex interact with eIF3 (Brito Querido et al., 2024; Pöyry et al., 2004). In yeast, eIF4G is much shorter than in humans and lacks eIF3 binding domain, so it does not contact eIF3 directly, but their contact is mediated by eIF1 and eIF5 (He et al., 2003; reviewed in Brito Querido et al., 2024). In humans, the eIF3-eIF4G interaction is direct, and is required for mRNA recruitment (Korneeva et al., 2000; Morino et al., 2000; Villa et al., 2013).

In conclusion, elucidating the mechanistic details of translation control by REI on short uORFs in yeast and mammals, together with the critical role of eIFs, would help to uncover the intricate mechanisms of how the cell maintains tight regulation under normal and cellular stress conditions, providing valuable insights into the fundamental process of protein synthesis.

Conclusions

My PhD thesis has made significant contribution to understanding the molecular principles of translational control by REI in yeast *S. cerevisiae* and mammals and has revealed previously undescribed involvement of eIFs and other aspects of this process under normal *versus* ER stress conditions.

- we employed previously developed *in vivo* Rap-Nip assay and revealed that eIF4G and eIF4A but not eIF4E are transiently retained on the ribosome elongating and terminating on short uORFs of the *GCN4* mRNA in yeast *S. cerevisiae*
- using our newly designed human *ATF4*-HA tag system we have successfully recapitulated the original model of *ATF4* mRNA regulation by delayed REI and, on the top of that, we have identified new aspects of *ATF4* translational control
- we have developed a stepwise JessTM Protein Simple assay protocol for the measurement and quantification of human *ATF4*-HA tag under non-stress and Tg stress conditions, and took advantage of it to investigate the effects of *cis*-acting *ATF4*-mRNA-specific features
- we examined the effects of mutations in both the 5'UTR and the uORF2/*ATF4* overlapping region, and we have proposed roles for new players involved in *ATF4* regulation
- we discovered an entirely new layer of translational control in the uORF2/*ATF4* region facilitated by ribosome queuing in front of the stem-loop
- collectively, we have demonstrated that the translational control of *ATF4* is not controlled solely by the delayed REI mechanism but is the result of the combined action of specific factors and *trans*-acting elements

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