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Translation reinitiation mechanism on mRNA of transcriptional activator *GCN4*
Mechanismus translační reiniciace na mRNA transkripčního aktivátoru *GCN4*

Bachelor thesis

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Author's Declaration:

I hereby declare that I have written this thesis independently with the use of literature listed. Neither the thesis nor any substantial part of it was submitted with the aim to obtain another or the same academic degree.

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Abstract

Translation reinitiation is a gene-specific translational control mechanism exploiting the ability of some short upstream open reading frames (uORFs) to retain post-termination 40S ribosomal subunit on the mRNA. Reinitiation efficiency depends on *cis*-acting sequences surrounding the uORF, translation elongation rates on the uORF, selected initiation factors, and the intercistronic distance of the short uORF from the main ORF. Although the precise mechanism of reinitiation is still not known, great progress in elucidating some of its details has been recently made with help of the *GCN4* translational control model system. Among them, involvement of eIF3 was shown to play a critical role for efficiency of this process. In particular, it was proposed that eIF3 specifically interacts with sequences located upstream of a reinitiation-permissive uORF upon termination, and that this step is instrumental in stabilizing the 40S ribosomal subunit on the mRNA to allow subsequent resumption of scanning for reinitiation downstream. In this thesis, the current knowledge of the translation reinitiation mechanism is summarized. As a typical example, the yeast transcriptional activator *GCN4* has been chosen, the mRNA of which is subjected to a tight translational control via the very reinitiation mechanism.

Key words: translation reinitiation, eIF3, 40S subunit, *GCN4*, uORF

Abstrakt

Translační reiniciace je genově specifický mechanismus kontroly translace, který využívá schopnosti některých krátkých otevřených čtecích rámců (uORFs) předcházejících v mRNA hlavní otevřený čtecí rámec zadržet 40S ribosomální podjednotku na mRNA poté, co ribosom na tomto uORF translaci ukončil. Efektivita tohoto procesu je ovlivněna tím, jaké nukleotidové sekvence uORF obklopují a jak rychle je ribosomem překládán, zdali jsou přítomny vybrané iniciační faktory, a také vzdáleností tohoto uORF od hlavního otevřeného čtecího rámce. Přestože přesný mechanismus reiniciace není dosud zcela znám, v posledních letech byly některé jeho dílčí kroky objasněny za pomoci studia mechanismu translační kontroly genu *GCN4*. Ukázalo se například, že naprosto nezbytná pro tento proces je účast eIF3. eIF3 specificky interaguje s mRNA sekvencí předcházející uORF, a tím významně napomáhá zadržení 40S podjednotky na mRNA a následné reiniciaci. Cílem této práce je shrnutí současných poznatků týkajících se mechanismu reiniciace translace. Jako příklad byl zvolen kvasinkový transkripční aktivátor *GCN4*, translace jehož mRNA je přísně řízena právě mechanismem reiniciace.

Klíčová slova: reiniciace translace, eIF3, 40S podjednotka, *GCN4*, krátký otevřený čtecí rámec

Contents

List of abbreviations

3' UTR	**3' u**n**t**ranslated **r**egion
40S subunit	eukaryotic small ribosomal subunit
43S PIC	**p**re**i**nitiation **c**omplex comprising 40S subunit, TC, eIF1, eIF1A, eIF3 and eIF5
48S PIC	**p**rein**i**tiation **c**omplex comprising a 43 PIC bound to mRNA
5' UTR	**5' u**n**t**ranslated **r**egion
60S subunit	eukaryotic large ribosomal subunit
80S ribosome	eukaryotic ribosome comrprising both small and large ribosomal subunit
a/TIF32	yeast ortholog of eIF3a
A-site	**a**minoacyl-**site** of ribosome
ATF4	**a**ctivating **t**ranscription **f**actor 4
ATP	**a**denosine **t**ri**p**hosphate
AUG	**a**denine-**u**racil-**g**uanine; start codon
b/PRT1	yeast ortholog of eIF3b
c/NIP1	yeast ortholog of eIF3c
CTD	**C**-**t**erminal **d**omain
eIF	**e**ukaryotic **i**nitiation **f**actor
eIF1	**e**ukaryotic **i**nitiation **f**actor **1**
eIF1A	**e**ukaryotic **i**nitiation **f**actor **1A**
eIF2	**e**ukaryotic **i**nitiation **f**actor **2**
eIF2B	**e**ukaryotic **i**nitiation **f**actor **2B**
eIF2α	**alpha** subunit of **e**ukaryotic **i**nitiation **f**actor **2**
eIF3	**e**ukaryotic **i**nitiation **f**actor **3**
eIF3a	**a** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF3b	**b** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF3c	**c** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF3g	**g** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF3h	**h** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF3i	**i** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF3j	**j** subunit of **e**ukaryotic **i**nitiation **f**actor **3**
eIF4A	**e**ukaryotic **i**nitiation **f**actor **4A**
eIF4B	**e**ukaryotic **i**nitiation **f**actor **4B**
eIF4E	**e**ukaryotic **i**nitiation **f**actor **4E**
eIF4F	**e**ukaryotic **i**nitiation **f**actor **4F**
eIF4G	**e**ukaryotic **i**nitiation **f**actor **4G**

eIF5	**e**ukaryotic **i**nitiation **f**actor **5**
eIF5B	**e**ukaryotic **i**nitiation **f**actor **5B**
g/TIF35	yeast ortholog of eIF3g
GAL1	yeast gene encoding the enzyme of **gal**actose catabolism pathway
GAL1-lacZ	fusion construct of ***GAL1*** gene and ***lacZ*** gene
Gcd$^-$	**g**eneral **c**ontrol **d**erepressed phenotype
Gcn$^-$	**g**eneral **c**ontrol **n**onderepressible phenotype
GCN2	**g**eneral **c**ontrol **n**onderepressible **2**; a protein kinase
GCN4	**g**eneral **c**ontrol **n**onderepressible **4**; an amino acid biosynthesis regulatory protein
GCN4	yeast gene encoding GCN4 protein
GCN4-lacZ	fusion construct of ***GCN4*** gene and ***lacZ*** gene
GDP	**g**uanosine **di****p**hosphate
GEF	**g**uanine nucleotide **e**xchange **f**actor
GTP	**g**uanosine **t**ri**p**hosphate
GTPase	enzyme that can bind and hydrolyze guanosine triphosphate (**GTP**)
HIS3	yeast gene encoding the enzyme of **his**tidine biosynthesis pathway
i/TIF34	yeast ortholog of eIF3i
IRES	**i**nternal **r**ibosome **e**ntry **s**ite
j/HCR1	yeast ortholog of eIF3j
lacZ	gene encoding β-galactosidase (**LacZ**), enzyme that cleaves lactose
m^{7}G-cap	**7**-**m**ethyl**g**uanosine **cap**
Met-tRNA$_i^{Met}$	**met**hionyl **i**nitiator **met**hionine **tRNA**; initiator tRNA
MFC	**m**ulti**f**actor **c**omplex
mRNA	**m**essenger **r**ibo**n**ucleic **a**cid
NMD	**n**onsense-**m**ediated **d**ecay
NTD	**N**-**t**erminal **d**omain
PABP	**p**oly(**A**)-**b**inding **p**rotein
PERK	**p**rotein kinase-like **e**ndoplasmic **r**eticulum **k**inase
P$_i$	**i**nogranic **p**hosphate
PIC	**p**re**i**nitiation **c**omplex
PK	**p**rotein **k**inase
P-site	**p**eptidyl-**site** of ribosome
Pub1	**p**oly(**U**)-**b**inding protein **1**
RNA	**r**ibo**n**ucleic **a**cid
RPS0A	40S **r**ibosomal **p**rotein **S0A**

RRM	**R**NA **r**ecognition **m**otif
rRNA	**r**ibosomal **r**ibo**n**ucleic **a**cid
S. cerevisiae	*Saccharomyces cerevisiae*
Ser-51	**ser**in at amino acid position **51**
STE	**st**abilizer **e**lement
TAV	**T**rans**a**ctivator/**v**iroplasmin protein
TC	**t**ernary **c**omplex
tRNA	**t**ransfer **r**ibo**n**ucleic **a**cid
UAA	**u**racil-**a**denine-**a**denine; stop codon
UAG	**u**racil-**a**denine-**g**uanine; stop codon
uAUG	**u**pstream **AUG** codon
UGA	**u**racil-**g**uanine-**a**denine; stop codon
uORF	**u**pstream **o**pen **r**eading **f**rame
uORF1	the first **u**pstream **o**pen **r**eading **f**rame in *GCN4* mRNA 5' UTR
uORF2	the second **u**pstream **o**pen **r**eading **f**rame in *GCN4* mRNA 5' UTR
uORF3	the third **u**pstream **o**pen **r**eading **f**rame in *GCN4* mRNA 5' UTR
uORF4	the fourth **u**pstream **o**pen **r**eading **f**rame in *GCN4* mRNA 5' UTR
YAP1	**y**east **AP-1**-like transcription factor

1. Introduction

Protein synthesis is a fundamental process supporting life of every single cell. In order to cope with changing environmental conditions, cells have to be able to regulate their protein expression. This can be exerted at multiple levels and by numerous regulatory mechanisms. One of them is the gene-specific translational control mechanism called reinitiation. It exploits short coding sequences – uORFs (for upstream open reading frames) that precede sequences encoding a main gene product and can be found in numerous eukaryotic mRNAs. Ribosomes initiate in the normal way at the uORF's AUG codon; however, at its termination codon, the 40S subunit remains bound to the mRNA, resumes scanning, and initiates again at downstream start site. This process requires a specific interplay between mRNA sequences surrounding this uORF and selected initiation factors.

Translational control of the *S. cerevisiae GCN4* gene is one of the best characterized examples of reinitiation (Szamecz *et al*., 2008). GCN4 acts as a transcriptional activator of biosynthetic genes and its synthesis is stimulated by amino acid starvation conditions (Hinnebusch, 1984). *GCN4* mRNA contains in its 5' leader four uORFs whose concerted action results in a very sophisticated regulatory mechanism (Abastado *et al.*, 1991) ensuring that the *GCN4* mRNA translation is stimulated under amino acid starvation conditions, despite the fact that the general translation initiation is shut down (Dever *et al*., 1992).

As growing data indicate, translation reinitiation belongs to widely utilized regulatory tools employed in various eukaryotic organisms. However, the understanding of this process is still rudimentary. The aim of this thesis should be to summarize up-to-date knowledge of this topic and describe both *cis* and *trans*-acting players involved in this process primarily with the help of *GCN4* mRNA regulatory system.

2. Gene expression

In living cells, information encoded by a particular gene is used in the synthesis of functional gene product. In eukaryotes, this complex process called gene expression involves these consequential steps: transcription, mRNA splicing, mRNA export from the nucleus, mRNA stability mechanisms, translation, and post-translational modifications of a protein product.

Regulation of gene expression is important for many cellular processes. In comparison with other steps, translational control of existing mRNAs enables rapid changes in cellular concentrations of the encoded proteins (Sonenberg & Hinnebusch, 2009). Translation consists of four subsequent steps (initiation, elongation, termination and ribosome recycling) but the most regulation is exerted at its first, rate limiting stage – translation initiation.

2.1. Canonical translation initiation in eukaryotes

Initiation of translation is a complex process resulting in the assembly of the elongation-competent 80S ribosome loaded with methionyl–initiator tRNA (Met-tRNA$_i^{Met}$) whose anticodon is base-paired with the start codon (AUG) of mRNA (see Figure 1). The initiation process can be divided into several consequent steps that are masterminded by numerous proteins called eukaryotic initiation factors (eIFs).

A critical step early in the translation initiation pathway is formation of the ternary complex (TC), comprised of eIF2, GTP and Met-tRNA$_i^{Met}$, and its recruitment to the free 40S ribosomal subunit. In yeast, this step resulting in the 43S preinitiation complex (43S PIC) assembly was shown to be stimulated by eIF1, eIF1A, eIF3, and eIF5, both *in vitro* (Algire *et al.*, 2002; Asano *et al.*, 2001; Phan *et al.*, 1998) and *in vivo* (Fekete *et al.*, 2005; Jivotovskaya *et al.*, 2006; Olsen *et al.*, 2003). In the meantime, the mRNA 5' m^{7}G-cap is bound by eIF4F protein complex, which consists of the cap-binding protein eIF4E, an ATP-dependent RNA helicase eIF4A, and the protein serving as a scaffold for other factors, eIF4G (Gingras *et al.*, 1999). Subsequently, the 43S PIC contacts this eIF4F – m^{7}G-cap structure to bring about the mRNA producing the 48S PIC. The mRNA recruitment step is mediated by eIF3, eIF4F complex and poly(A)-binding protein (PABP).

The 48S PIC then scans 5' UTR (5' untranslated region) of the mRNA in the 5' to 3' direction until it encounters the first AUG (Kozak, 1989) which is recognized by base pairing with the anticodon in Met-tRNA$_i^{Met}$ (Cigan *et al.*, 1988). In mammalian reconstituted system, scanning process was shown to be stimulated by eIF1, eIF1A, and eIF4F complex, out of which eIF4A and its cofactor eIF4B utilize the energy of ATP to unwind secondary structure

present in the 5' UTR of a given mRNA (Pestova & Kolupaeva, 2002). Genetic data from yeast suggest that also eIF5 and eIF3 participate in scanning process *in vivo* (Nielsen *et al.*, 2004; Yamamoto *et al.*, 2005). The probability that a given AUG codon will be selected as the initiation codon by the scanning 48S PIC complex depends on the "strength" of nucleotide sequence flanking this AUG codon (so called the Kozak consensus sequence) (Kozak, 1986).

In the scanning 48S PIC, the GTP bound to eIF2 is partially hydrolyzed to GDP and inorganic phosphate (P_i) which is stimulated by eIF5 (Das *et al.*, 2001). Successful AUG recognition triggers dissociation of eIF1 from 40S subunit with subsequent irreversible P_i release from eIF2-GDP-P_i, driving GTP hydrolysis to completion (Algire *et al.*, 2005; Maag *et al.*, 2005). eIF1 displacement induce an irreversible transition to a closed, scanning-incompatible conformation of the 48S PIC (Cheung *et al.*, 2007), serving as the decisive step stalling the entire machinery at the AUG start codon. After eIF2-GDP ejection, leaving the Met-$tRNA_i^{Met}$ in the ribosomal P-site, eIF5B-GTP promotes joining of the 60S ribosomal subunit with the 40S-Met-$tRNA_i^{Met}$-mRNA complex (Pestova *et al.*, 2000). Subunit joining is thought to facilitate ejection of all other eIFs with the exception of eIF1A (Unbehaun *et al.*, 2004) and eIF3 (Szamecz *et al.*, 2008). Finally, after GTP hydrolysis and eIF5B dissociation, the 80S initiation complex is ready to accept the appropriate aminoacyl-tRNA into the ribosomal A-site and synthesize the first peptide bond.

For a new round of initiation, the eIF2-GDP released from the 40S must be recycled to eIF2-GTP by its guanine nucleotide exchange factor (GEF) eIF2B to reform the TC (Webb & Proud, 1997). This exchange reaction can be inhibited under conditions of amino acid starvation or other types of stress by phosphorylation of the α-subunit of eIF2 by specialized protein kinases (PKs) (see chapter 4.), owing to the fact that the phosphorylated eIF2-GDP is a competitive inhibitor rather than a substrate for eIF2B (Rowlands *et al.*, 1988). Reducing the TC assembly rates leads to a general translational shutdown (Krishnamoorthy *et al.*, 2001).

In yeast, it was shown that eIF3, eIF5, eIF1 and TC physically associate with each other (Asano *et al.*, 1999; Valasek *et al.*, 2002; Valasek *et al.*, 2003; Yamamoto *et al.*, 2005) and together they form so called multifactor complex (MFC). This complex binds to the 40S subunit as a preformed unit, is able to exist free of ribosomes and thus represents an important intermediate in translation initiation in yeast (Asano *et al.*, 2000). Detailed functional studies of mutual interactions among MFC components revealed that the MFC promotes the TC and mRNA recruitment to the 40S ribosome as well as the subsequent steps such and scanning and AUG selection (Nielsen *et al.*, 2004; Valasek *et al.*, 2002; Valasek *et al.*, 2004).

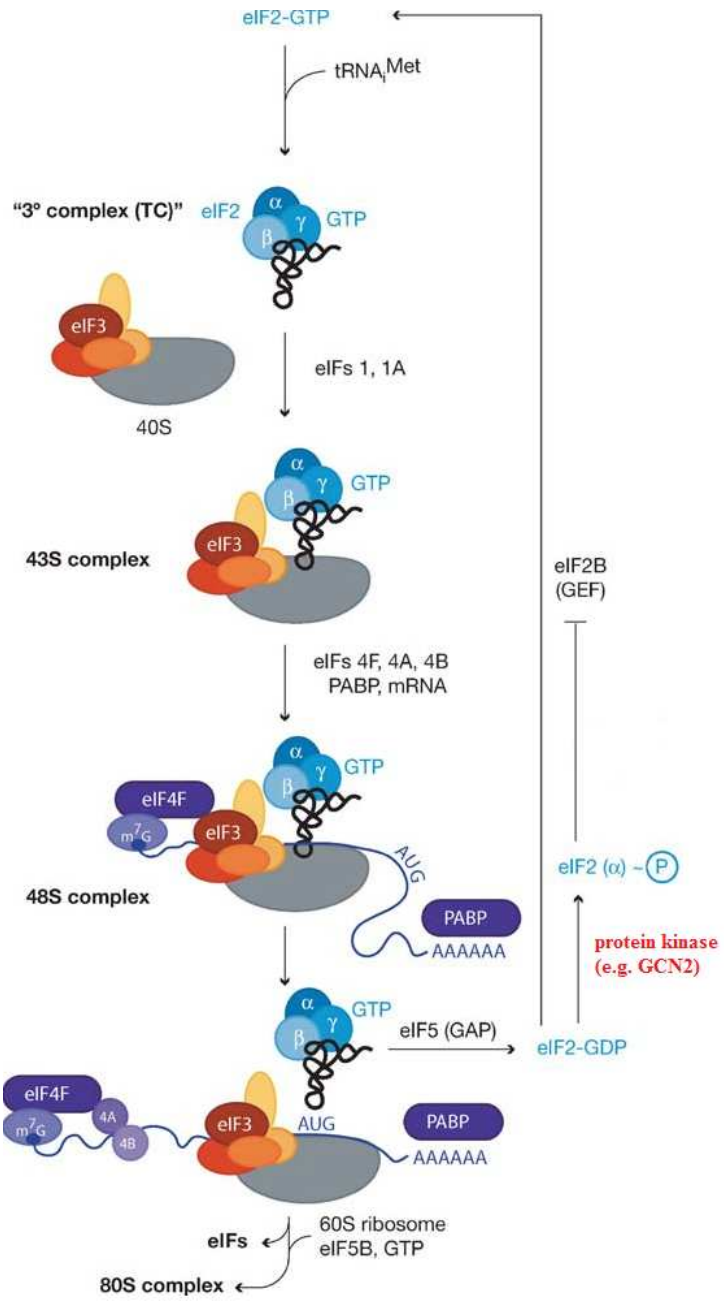

Figure 1. Model of the canonical pathway of eukaryotic translation initiation.
For details, see chapter 2.1. (adapted from Hinnebusch, 2005).

2.1.1. eIF3

Among all initiation factors, eIF3 is the largest and the most complex one. In the budding yeast, eIF3 is formed by five core subunits (a/TIF32, b/PRT1, c/NIP1, i/TIF34 and g/TIF35) and one loosely associated, nonessential subunit, namely j/HCR1 (see Table 1). All these six subunits have the corresponding orthologs in the 13-subunit mammalian eIF3 (Phan *et al.*, 1998; Valasek *et al.*, 1999). eIF3 was shown to stimulate nearly all steps of translation initiation pathway (see chapter 2.1.). Recently, it was also revealed that eIF3 plays the crucial

role in special reinitiation events (Szamecz *et al.*, 2008) (see chapter 6.3.). Importantly, eIF3 is thought to reside on the solvent side of the 40S subunit (Siridechadilok *et al*., 2005; Valasek *et al.*, 2003). This location seems to be an ideal site with the respect to regulatory functions proposed for eIF3.

Table 1. eIF3 subunits in *Saccharomyces cerevisiae.*

unified nomenclature	*S. cerevisiae* nomenclature
eIF3a	a/TIF32*
eIF3b	b/PRT1*
eIF3c	c/NIP1*
eIF3g	g/TIF35*
eIF3i	i/TIF34*
eIF3j	j/HCR1

*core subunits

2.2. Alternative translation initiation pathways

As the majority of eukaryotic mRNAs are almost exclusively monocistronic, their translation is initiated by the canonical mechanism employing the scanning mechanism (Kozak, 1989) described above. However, there are several examples in eukaryotic traslation initiation that deviate from this general mechanism and start protein synthesis either without scanning or at internal sites of an mRNA. These involve translation initiated in a cap-independent manner at internal ribosome entry sites (IRESes) (Gilbert *et al.*, 2007; Chen & Sarnow, 1995), ribosomal shunting (Rogers et al., 2004) and reinitiation after translation of short upstream open reading frame (uORF) (Rajkowitsch *et al.*, 2004). Many of these mechanisms are utilized by viruses (Herbreteau *et al.*, 2005; Kolupaeva *et al.*, 2000; Pelletier & Sonenberg, 1988) or serve as regulatory tools for gene-specific translational control of transcription factors (Abastado *et al.*, 1991; Vattem & Wek, 2004).

3. uORFs as regulators of translation initiation

uORFs represent mRNA elements located in 5' UTR which contain a start codon (AUG) followed by at least one additional coding triplet and an in-frame termination codon (UAA, UAG, UGA) (see Figure 2).

The latest reports indicate that uORFs are wide-spread among various organisms. A study of *S. cerevisiae* transcriptome revealed that 6% of yeast mRNAs contain short uORFs (Nagalakshmi *et al.*, 2008). Surprisingly, it was predicted that in human and mouse cells the number of mRNAs containing short uORFs is even higher and reaches nearly 50% (Calvo

et al., 2009). It does not mean that all of them have regulatory functions, nevertheless, many examples of genes utilizing short uORFs to govern their own expression have already been published either in fungi (Luo & Sachs, 1996; Mueller & Hinnebusch, 1986) or in mammals (Vattem & Wek, 2004) including humans (Harigai *et al.*, 1996) (see also below).

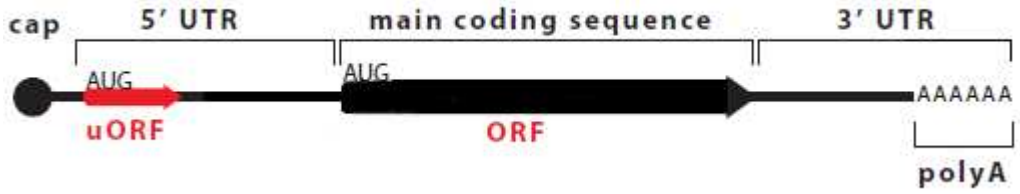

Figure 2. A schematic picture of mRNA transcript containing an upstream open reading frame (uORF). For details, see chapter 3. (adapted from Calvo *et al.*, 2009)

3.1. uORFs-linked pathologies

It is noteworthy that mutations altering the surrounding sequences of some uORFs of human mRNAs disrupt their translational control and can cause various human diseases (Liu *et al.*, 1999; Wen *et al.*, 2009; Wiestner *et al.*, 1998). For example, it was also shown that translational control mediated by uORFs influences the emergence of atherosclerosis in diabetics (Griffin *et al.*, 2001). Hence it is obvious that better understanding of the molecular details of the uORF-mediated regulation can significantly contribute to improvement of human health in the future.

3.2. Types of mechanisms by which uORFs affect mRNA translation

Regarding the fact that the start codon is in eukaryotes selected by the scanning mechanism and that these short reading frames are situated upstream of a main coding sequence, uORFs are primarily considered to serve as a barrier severely reducing the expression of the main gene (Kozak, 1984; Kozak, 1999). Indeed, a global concurrent analysis of mammalian transcriptomes and proteomes revealed that the occurrence of uORFs in mRNAs closely correlates with significantly reduced protein expression of downstream ORFs carried by these mRNAs (Calvo *et al.*, 2009). Importantly, not all uORFs impose such a negative effect on expression of a main ORF. In fact, the degree of reduction of a major ORF expression is determined by the "Kozak strength" of AUG start codon of a particular uORF (Kozak, 1986). Intuitively, uORFs with poor initiation context can be skipped by numerous 48S PICs via "leaky scanning", which mitigates their negative effect on general translation initiation (Vivier *et al.*, 1999).

On the other hand, several uORFs affect downstream translation through their ability to mediate ribosome stalling at coding or termination regions (Wang *et al.*, 1999). This is usually a result of the action of the nascent peptide encoded by this uORF (Gaba *et al.*, 2001). Such a stall at uORF termination codon prevents the scanning ribosomes from reaching another downstream start site.

In addition to these “downregulation effects”, some uORFs influence expression of a main gene by affecting stability of its mRNA through so called nonsense-mediated mRNA decay (NMD), which is triggered by increased ribosome occupancy of a premature termination codon (Gaba *et al.*, 2005). Conversely, many other short-uORF-containing mRNAs (like *GCN4* and *YAP1*) are fully resistant to this destabilization pathway thanks to specific stabilizer elements (STE) contained in the 5' UTR (Ruiz-Echevarria *et al.*, 1998; Vilela *et al.*, 1998). These STEs were shown to interact with the RNA binding protein Pub1 and this interaction is instrumental in mRNA stabilization as it prevents rapid NMD (Ruiz-Echevarria & Peltz, 2000).

The last but not least class of uORFs are those that permit 40S subunit to stay bound to the same molecule of mRNA after the elongating 80S terminated their translation (Abastado *et al.*, 1991). The rest of this thesis will be focused on this last class of regulatory uORFs that allow efficient resumption of scanning followed by reinitiation at a downstream start site.

3.2.1. Reinitiation after an uORF translation termination

Historically, in an early study by M. Kozak (1987b) she showed that out of 699 vertebrate mRNAs encoding proto-oncogenes, nearly two thirds contained one or more uORFs preceding the start site of the main ORF. This led to the idea that such a regulation exerted at the translational level and mediated by uORFs might be a tool for limiting the expression of potentially harmful proteins if overproduced (Kozak, 1991).

Reinitiation is exploited as a regulatory mechanism in many various eukaryotic organisms starting from yeasts (Abastado *et al.*, 1991; Vilela *et al.*, 1998), over plants (Futterer & Hohn, 1992; Wang & Wessler, 1998) up to mammals (Vattem & Wek, 2004). This mechanism of translation regulation is also greatly employed by invading viruses (Park *et al.*, 2001; Powell *et al.*, 2011). And, according to the newest bioinformatical data, the number of mRNAs proven to utilize the reinitiation mechanism for their translational control is expected to rise (Cvijovic *et al.*, 2007; Selpi *et al.*, 2009).

4. General requirements for reinitiation of translation

The ability of an uORF to retain the 40S subunit on the mRNA after it has terminated translation at the uORF stop codon generally depends on: i) the time required for the uORF translation, which is determined by the length of the uORF and the translation elongation rates, ii) various initiation factors, and iii) special *cis*-acting mRNA features. Finally, reinitiation efficiency is also determined by iv) the intercistronic distance.

The first two requirements are united in the idea that eIFs important for promoting efficient reinitiation remain at least transiently associated with the elongating ribosome, and that increasing the uORF length or the ribosome transit time increases the likelihood that these factors are dropped off (Kozak, 2001). Accordingly, reinitiation is most efficient after short uORFs translation and its efficiency declines as the uORF is lengthened (Kozak, 2001; Luukkonen *et al.*, 1995). There is now an evidence for this hypothesis showing that in yeast *S. cerevisiae* eIF3 remains bound to elongating 80S for the first few elongating cycles and upon termination critically enhances reinitiation capacity of post-termination 40S subunits (Szamecz *et al.*, 2008). Among other eIFs implicated in promoting efficient reinitiation in mammalian cells are eIF4A and eIF4G (Poyry *et al.*, 2004), but the mechanism of their operation is not known.

As for *cis*-acting mRNA features, with the exception of the uORF-mediated translational control of the *S. cerevisiae GCN4* discussed below, there is nearly nothing known about what reinitiation-promoting mRNA features are required.

Importantly, the reinitiation efficiency also directly depends on the distance between uORF termination codon and a downstream initiation codon (Grant *et al.*, 1994; Kozak, 1987a). This reflects the fact that the rescanning 40S subunit require a certain time for *de novo* recruitment of the TC necessary for another AUG recognition (Abastado *et al.*, 1991). Owing to this, reinitiation event can be delicately regulated by manipulating eIF2-GTP availability in a cell via eIF2α phosphorylation by specific protein kinases s such as GCN2, the only PK in yeasts (Dever *et al*., 1992), or mammalian PERK (Harding *et al.*, 2000). Importantly, reduction in the TC assembly decreases the rate of general translation but at the same time can paradoxically stimulate the translation of specialized mRNA, such as *GCN4* or *ATF4*, which both encode stress-induced transcriptional activators of biosynthetic genes (Dever *et al.*, 1992; Vattem & Wek, 2004).

5. The mechanism of *GCN4* translational control

The mechanism of *GCN4* translational control represents the most studied example of translation reinitiation (Szamecz, 2008), thus, here it will be described in more details.

5.1. *GCN4* - a gene encoding specific transcriptional activator

Yeast cells, as well as other cells, govern their gene expression in response to various environmental stimuli. One of such important regulatory means in yeasts is known as General amino acid control which help the cells to cope with amino acid imbalancy (Niederberger *et al*., 1981). When a yeast cell suffers from amino acid starvation, it responses by mobilizing transcriptional activators in order to induce genes important for its survival (Delforge et al., 1975; Mirande & Waller, 1988; Zhou *et al*., 1987).

One of the most important positive regulators of General amino acid control of lower eukaryote *Saccharomyces cerevisiae* is that encoded by *GCN4* gene (Hinnebusch, 1984). GCN4 specifically binds TGACTC sequence common in coregulated genes such as *HIS3* gene in *S. cerevisiae* encoding a histidine biosynthetic enzyme (Hope & Struhl, 1985). In amino acid starvation conditions, GCN4 activates at least 35 genes encoding amino acid biosynthetic enzymes (Hinnebusch, 1992). Genome-wide expression profiling analyses revealed that besides that, additional genes involved in cofactor biosynthesis, organelle biogenesis, mitochondrial transport, autophagy and others are induced by this yeast transcriptional activator during stress conditions (Natarajan *et al.*, 2001).

5.2. The regulation of *GCN4* mRNA translation is exerted by four short uORFs

GCN4 mRNA contains in its 5' UTR four short uORFs of two to three codons in length none of which is in frame with the long ORF of *GCN4* downstream (see Figure 3). The regulation of *GCN4* expression occurs at the translational level (Hinnebusch, 1984) and relies on sophisticated interplay between these short uORFs (Abastado *et al.*, 1991).

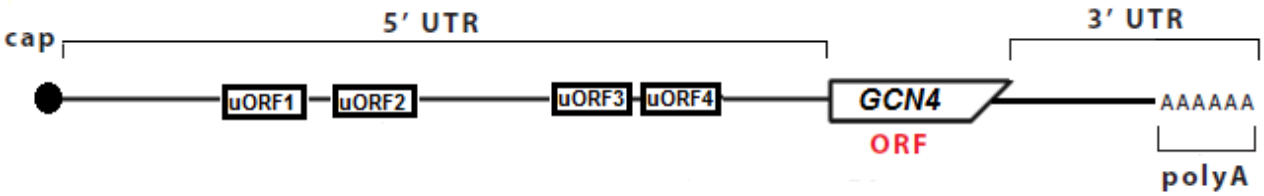

Figure 3. A schematic picture of *GCN4* mRNA. It contains four upstream reading frames all of which precede the main open reading frame encoding the GCN4 transcriptional activator. For details, see chapter 5.2. (based on Calvo *et al.*, 2009).

5.2.1. Regulatory functions of uORFs in *GCN4* mRNA – the historical overview

Many experiments were performed to elucidate precise functions of particular uORFs in *GCN4* mRNA 5' leader (5' UTR). The theory that uORFs function as translational barriers (Kozak, 1984) was confirmed also in the case of *GCN4* mRNA by removing of all four uORFs, resulting in a constitutive derepression of GCN4 synthesis (Hinnebusch, 1984). However, the first uORF (uORF1) later turned out to be a weak translational barrier. Instead, it performed a stimulatory role in GCN4 synthesis because it enabled the ribosomes to overcome the inhibitory effects of the remaining downstream uORFs (Mueller & Hinnebusch, 1986). Another deletion mutagenesis in *GCN4* mRNA 5' leader revealed that the presence of uORFs 1 and 4 is sufficient for significant degree of *GCN4* expression regulation, comparable to the wild-type mRNA containing all four uORFs intact (Williams *et al.*, 1988). This helped to simplify the subsequent analyses of uORFs functioning in *GCN4* translational control.

But the features of uORF1 underlying its intriguing properties still remained veiled. Start codons of uORFs 1 and 4 were shown to function as similarly efficient translational start sites *in vivo*, but uORF4 did not exhibit a positive effect on GCN4 synthesis when located upstream of uORF1 (Williams *et al.*, 1988). Efficient initiation was therefore unlikely to be a sole determinant of the positive regulatory role of uORF1. Thus, it was proposed that a very important property of uORFs mediating *GCN4* translational control is the ability to permit reinitiation following termination of translation and that the uORF1 is optimized for this function (Williams *et al.*, 1988).

Additional extensive genetic analyses of the *GCN4* mRNA 5' UTR finally helped to provide a detailed model for *GCN4* translational control (Abastado *et al.*, 1991). An important support for the *GCN4* translational control model came from the observation that increasing the distance between uORFs 1 and 4 to the wild-type spacing that separates uORF1 from *GCN4* start site impaired the ability of uORF1 to derepress *GCN4* mRNA translation. This

indicated that the time when ribosome reacquires certain factors needed for efficient reinitiation plays the crucial role in *GCN4* translational control mechanism (Abastado *et al.*, 1991). Thus, it was suggested that after a ribosome translates uORF1, 40S ribosomal subunit stay bound to the mRNA, resumes scanning and then reinitiates either at uORF4 start codon or *GCN4* start codon, according to amino acid availability.

5.2.2. The role of eIF2 phosphorylation in *GCN4* translational control

Although the model for *GCN4* translational control suggested that in amino acid starvation conditions the reassembly of factors required for efficient reinitiation is much slower in comparison to nonstartavion conditions, the underlying mechanism of this phenomenon remained veiled.

It was known that eukaryotic protein synthesis is regulated by common mechanism involving the phosphorylation of the alpha subunit of eIF2 (eIF2α) (Ranu, 1980). As phosphorylation of mammalian eIF2α occurs on serine residue at position 51 and amino acid sequence around this phosphorylation site turned out to be conserved among eukaryotes including the yeast *S. cerevisiae* (Cigan *et al.*, 1989), it was suggested that phosphorylation of Ser-51 of eIF2α could be exploited as a tool for translation regulation also in yeasts. Additionally, the catalytic domain of protein kinase GCN2, which has been earlier identified as an activator of GCN4 synthesis (Hinnebusch, 1984; Tzamarias & Thireos, 1988), was shown to be related to catalytic domain of another eukaryotic protein kinase that phosphorylates eIF2α (Chen *et al*., 1991). This implicated GCN2 protein kinase to be an eIF2α kinase in yeast.

Indeed, it proved true. In 1992 (Dever *et al.*), it was discovered that GCN2 phosphorylates the α-subunit of eIF2 and mediates translational control of the yeast transcriptional activator *GCN4* .

5.3. The final generally accepted model of *GCN4* translational control

Independent of amino acid availability, most ribosomes translate the first uORF supporting the retention of 40S subunit on the mRNA and, after termination, about a half of 40S subunits resumes scanning downstream (Abastado *et al.*, 1991).

Under nonstarvation conditions, eIF2-GDP is fast recycled to eIF2-GTP by eIF2B resulting in high levels of TC formation. Thus, rescanning 40S subunits readily reassamble TC and preferentially reinitiate at uORF4, blocking another reinitiation downstream. However, under starvation conditions, uncharged tRNAs accumulating in amino acid-starved yeast cells stimulate eIF2α kinase GCN2 (Dong *et al.*, 2000). As a result, the TC levels drop

and the rescanning 40S subunits have to travel for a longer period till they have rebound the TC. This significatnly increases the likelihood that they bypass uORF4 and reinitiate translation at *GCN4* start site instead (Dever *et al.*, 1992) (see Figure 4).

The critical biochemical evidence for the *GCN4* translational control mechanism, which was initially deduced from genetic data, was provided later on by mapping the positions of ribosomes translating *GCN4* mRNA *in vitro* using toeprinting (Gaba *et al*., 2001). The ultimate proof was presented recently by a novel technique based on the deep sequencing of ribosome-protected mRNA fragments (Ingolia *et al.*, 2009). Also this method detected a decrease in ribosome occupancy of the reinitiation-nonpermissive uORFs as well as an increase in translation of *GCN4* coding region during starvation.

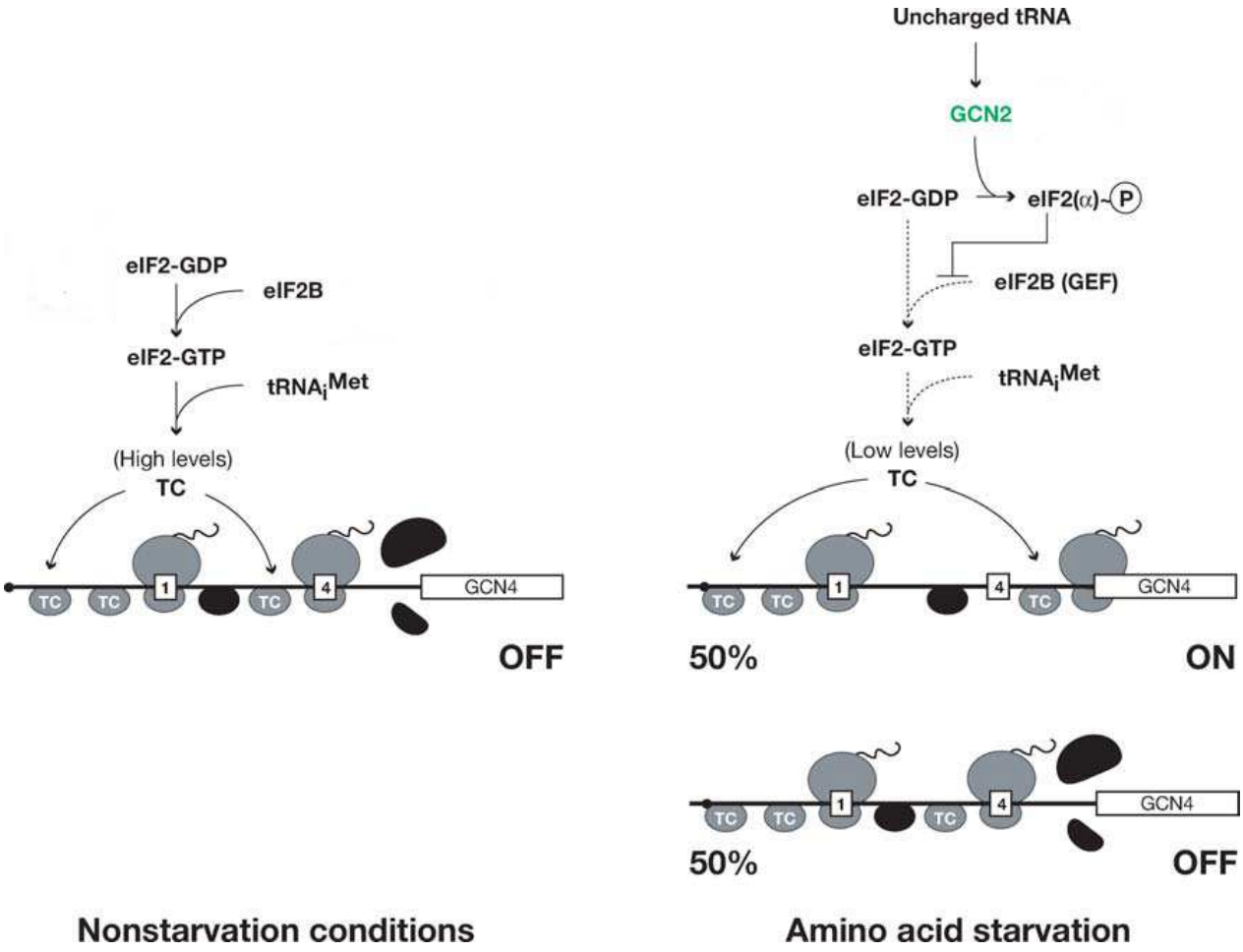

Figure 4. Model of translational control of GCN4 in yeast.

For details, see chapter 5.3. (adapted from Hinnebusch, 2005).

5.4 Phenotypes linked to *GCN4* expression

It is noteworthy that the impairment of translational control of *GCN4* expression can be monitored by specific phenotypes. These phenotypes then serve as a valuable genetic tool in defining various contributions of individual eIFs to translation initiation (Cuchalova *et al.*, 2010; Nielsen *et al.*, 2004; Szamecz *et al.*, 2008). Mutant cells defective in TC formation and/or its recruitment to 40S subunit constitutively derepress *GCN4* expression and thus produce so called Gcd$^-$ (General control derepressed) phenotype. By contrast, mutants that fail to derepress *GCN4* under starvation conditions embody a Gcn^{--} (General control noninducible) phenotype implicating a defect in one or more of the steps following assembly of 48S PIC such as scanning processivity, start codon recognition, or subunit joining (Cuchalova *et al*., 2010).

6. Reinitiation mechanism after translation termination on uORF1 of *GCN4* mRNA

6.1. The influence of the sequences downstream of uORF1 in the historical perspective

The probable importance of sequences surrounding the stop codons of uORFs in *GCN4* mRNA 5' leader in determining their distinct functions in translational control was shown already in 1989 (Miller & Hinnebusch). The replacement of uORF1 stop codon plus 10 nucleotides immediately following it with the corresponding downstream sequences from uORF4 was sufficient to convert uORF1 into a much stronger translational barrier in comparison with its former positive properties. The presence of thus altered uORF1 upstream of uORF4 in the 5' leader also reduced *GCN4* mRNA translation rate suggesting that the 3' sequences flanking uORF1 is required for ribosome ability to resume scanning afer uORF1 translation.

More extensive analysis of the last codon of uORF1 and 10 nucleotides following it revealed that a high content of A+U bases in this uORF1 termination region might cause a higher propensity of ribosome to reinitiate at *GCN4* start site (Grant & Hinnebusch, 1994). This led to a presumption that the presence of G+C-rich sequences in uORF1 termination region would prevent a fast resumption of scanning as a result of higher stacking energies of C+G pairs in comparison to A+U base pairs. Accordingly, the replacement of C+G-rich sequences flaking the uORF4 termination region with A+U-rich sequences restored a high reinitiation rate at the *GCN4* start site (Grant & Hinnebusch, 1994). Based on that it was

postulated that stable interactions between the termination region of an uORF and sites either on the rRNA, tRNA or elsewhere in the *GCN4* mRNA prevent fast exit of the ribosome from the termination region, increasing the probability that ribosome dissociates from the mRNA (Grant & Hinnebusch, 1994).

6.2. The influence of the sequences upstream of uORF1 in the historical perspective

For a while it seemed like the sequences surrounding the stop codons of uORF1 and uORF4 could be the only determinants of their different abilities to permit reinitiation at *GCN4* start site. However, in 1995 (Grant *et al.*), it turned out that uORF1 loses it ability to support reinitiation when inserted in the mRNA leader in place of uORF4, even when transferred part of uORF1 included the critical 13 nucleotides downstream previously assigned to be important for reinitiation (Grant & Hinnebusch, 1994). Furthermore, deletion of sequences located more than 20 nucleotides upstream of the uORF1 start site resulted in its conversion into an inhibitory element and blocked efficient reinitiation downstream (Grant *et al.*, 1995). Interestingly, the 5' leader of uORF1 of the *GCN4* mRNA is also unusually long (~200 nucleotides) when compared to other yeast mRNA leaders with an average length of 52 nucleotides (Cigan & Donahue, 1987). Therefore, it was anticipated that in contrast to sequences downstream of uORFs 1 and 4, sequences upstream of uORF1 might enhance reinitiation by a more active process, such as by the facilitation of rebinding of certain factors which could be necessary to resume scanning for efficient reinitiation downstream (Grant *et al.*, 1995). It took over ten years to resolve this puzzle (see below).

6.3. eIF3 as the key reinitiation-supporting factor

The key finding concerning the role of 5' sequences of uORF1 in translation reinitiation came when the largest eIF3 subunit, a/TIF32, was shown to play a critical role for the efficiency of this process. Previously, the N-terminal domain (NTD) of a/TIF32 was demonstrated to interact with the C-terminal domain (CTD) of the 40S ribosomal protein RPS0A situated near the mRNA exit channel (Valasek *et al.*, 2003). Strikingly, a partial deletion of the RPS0A-binding domain of a/TIF32 not only decreased binding of eIF3 and associated eIFs to native preinitiation complex *in vivo* but also greatly impaired the induction of *GCN4* mRNA translation. Detailed analysis of this defect revealed that it resulted from an inability of 40S subunits to resume scanning after uORF1 translation and, most importantly, implicated the a/TIF32-NTD in establishment of a post-termination interaction with the sequences 5' of

uORF1. It is important to note that the NTD of a/TIF32 is ideally positioned (near the exit channel) to contact mRNA sequences emerging from the mRNA exit pore (Valasek *et al.*, 2003). Indeed, this interaction proved to critically contribute to efficient reinitiation (Szamecz *et al.*, 2008).

6.4. The generally accepted model of the molecular events preceding efficient reinitiation after a short uORF translation

GCN4 mRNA provides the most detailed eukaryotic translation reinitiation model, whose current overview is described in Figure 5. (A, B) eIF3 associates with scanning 48S PIC and their connection is stabilized by eIF1, eIF1A, eIF5 and TC. (C) After uAUG recognition and 60S subunit joining, eIF3 (and possibly also eIF4F, based on work in mammalian *in vitro* systems (see chapter 6.5.)) remains bound to the 40S subunit. Since eIF3 resides on the solvent-exposed side of the 40S subunit, it does not prevent the 60S subunit to join. Post-initiation 40S-binding of eIF3 is mediated by several contacts between the head ribosomal proteins and some eIF3 sub-domains such as that between the a/TIF32-NTD and RPS0A. (D) In the course of elongation, weakly bound eIF3 stays 80S-bound for several elongation cycles; however, the more time it takes to translate a particular uORF, the higher is the probability of eIF3 dissociation. (E, F) After translation of an ideally short uORF, some of 80S ribosomes terminating at its stop codon still carry eIF3. (E, G) The specific sequences upstream of uORF1 contact the a/TIF32-NTD to stabilize the post-termination 40S on the mRNA after 60S dissociation. Thus stabilized 40S subunit probably recruits scanning-promoting factors such as eIF1 and eIF1A and resumes scanning for reinitiation downstream. (F, H) Lack of these specific sequences upstream of uORF4 and thus an absence of the a/TIF32-NTD – mRNA stimulatory interaction results in completion of ribosomal recycling by 80S ribosome and 40S release from the mRNA at uORF4.

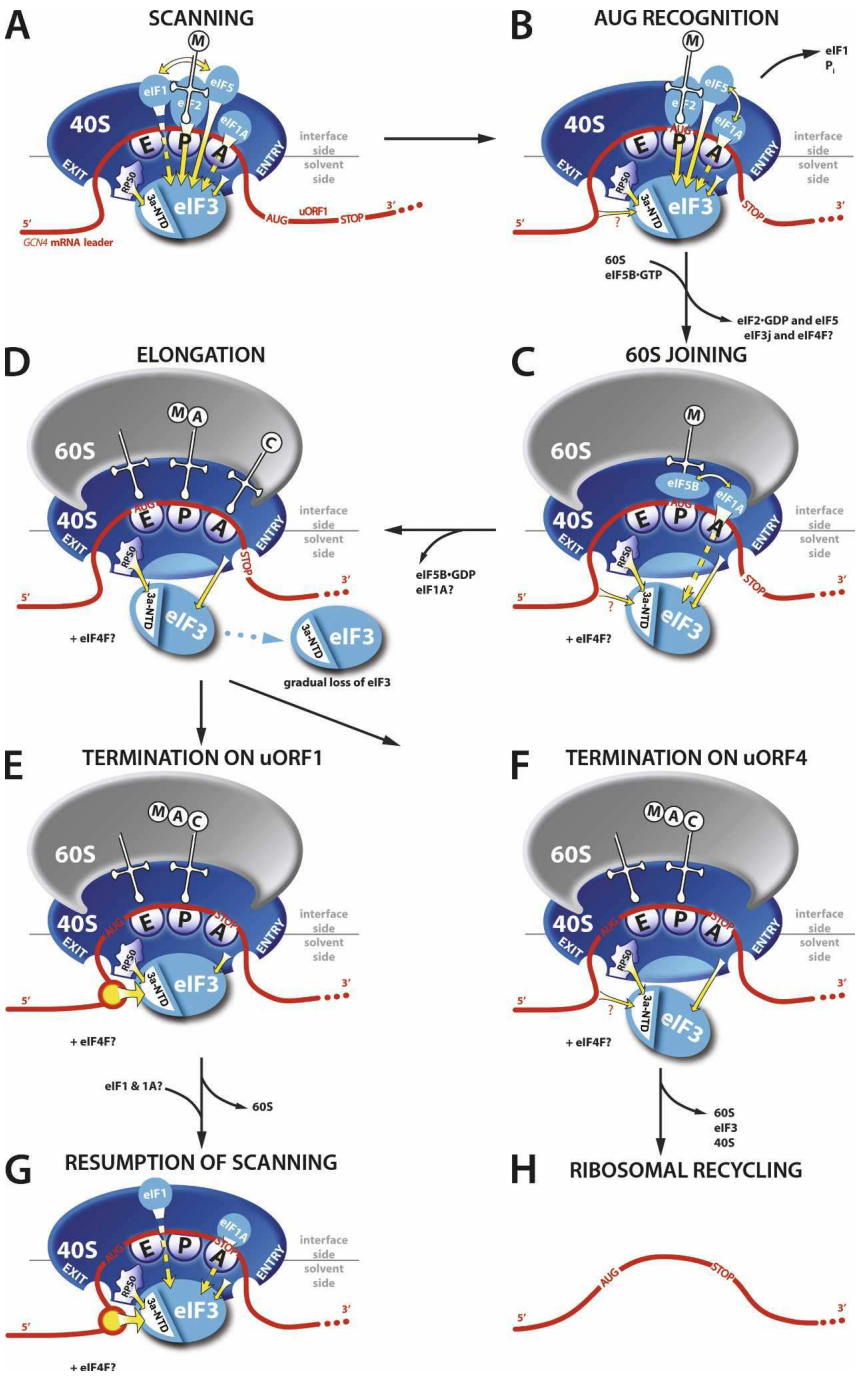

Figure 5. The generally accepted model of the molecular events preceding efficient reinitiation after a short uORF translation. For details, see chapter 6.4. (adapted from Szamecz *et al.*, 2008).

6.5. Other eukaryotic initiation factors implicated in translation reinitiation mechanism

Till today, another one essential eIF3 subunit, g/TIF35 was shown to directly promote the reinitiation mechanism after uORF1 translation. The *g/tif35-KLF* mutation in the RRM of the g/TIF35 subunit induced a strong Gcn$^-$ phenotype also owing to an inability of

the post-termination 40S subunits to resume scanning after the first uORF translation (Cuchalova *et al.*, 2010). However, detailed analysis revealed that the g/TIF35-RRM probably ensures efficient resumption of scanning by a different mechanism than by that of the a/TIF32-NTD described previously. It was shown that the g/TIF35-RRM does not cooperate with the 5' sequences of uORF1 and instead it was hypothesized that the g/TIF35-RRM may interact with *GCN4* 3' UTR (Cuchalova *et al.* 2010). In any case, a precise mechanism of g/TIF35 function in reinitiation mechanism remains to be elucidated.

Interestingly, the reinitiation-supporting role of eIF3 was also demonstrated in other organisms besides the yeast *S. cerevisiae* . For example, , the conserved h subunit of eIF3 was shown to ensure efficient reinitiation after uORF translation in *Arabidopsis thaliana* (Roy *et al.*, 2010). Also, it was proposed that the recruitment of eIF3, and in particular its g subunit, by the cauliflower mosaic virus transactivator TAV enables translation of polycistronic viral mRNAs by reinitiation (Park *et a*l., 2001). Although budding yeast lacks eIF3h, these findings show again that eIF3 plays a pivotal role in reinitiation events.

Besides eIF3, other initiation factors were also implicated in promoting efficient reinitiation in mammalian cells, namely eIF4A and eIF4G. It was proposed that an efficient reinitiation occurs only if the eIF4 family of initiation factors (either eIF4F or just the central domain of eIF4G plus eIF4A) have participated in the primary initiation event at the uORF start codon (Poyry *et al.*, 2004). However, the precise molecular mechanism is unknown.

7. Future perspectives

7.1. The secondary structure of mRNA preceding uORF1 supports efficient translation reinitiation

The most recent computational modeling data indicate that the a/TIF32-NTD-interacting mRNA sequences located 5' of uORF1 most probably progressively fold into a specific secondary structure as they leave the mRNA exit pore of the elongating ribosome (Munzarova *et al.*, 2011). It is assumed that establishment of a such specific fold of these *cis*-acting sequences is critically required for making the interaction with a/TIF32 to support retention of 40S subunit on the mRNA. Importantly, a similar secondary structure was predicted to form also upstream of yet another reinitiation-permissive uORF occuring in the 5' leader of the yeast *YAP1*, the gene encoding a transcription factor important in cell response to stress (Vilela *et al.*, 1998). The fact both these uORFs operate in the a/TIF32-dependent manner

(Munzarova *et al.*, 2011) may suggest that the underlying mechanism of reinitiation after short uORFs translation is conserved, at least in yeasts.

7.2. Does the 3' UTR of *GCN4* contribute to the intrigue regulation of its expression?

In 1980s it was shown that insertion of the *GCN4* mRNA 5' leader containing all four uORFs into the *GAL1-lacZ* fusion construct conferred the *GCN4*-like translational control upon the *GAL1-lacZ* transcript (Mueller *et al.*, 1987). It is surprising that whereas the absence of the sequences upstream of uORF1 led to a substantial reduction in the *GCN4-lacZ* expression (by approximately 8-fold) (Grant *et al.*, 1995), deletion of the very similar region in the recombinant *GAL1-lacZ* construct containing *GCN4* mRNA 5' leader resulted in less than 2-fold reduction (Mueller *et al.*, 1987). This discrepancy has never been resolved. Since besides the coding region, the recombinant *GAL1-lacZ* construct differs from the original *GCN4-lacZ* construct also in the 3' UTR following the coding region, it is conceivable that 3' UTR also contributes to the overall translational control of *GCN4* expression. Its inhibitory effect, however, is negligible when sequences upstream of uORF1 are present and interact with the a/TIF32-NTD. Thus, the mechanism by which the 3' UTR of *GCN4* mRNA contributes to the overall regulation of *GCN4* translational control is still unknown and should be subjected to future investigations.

8. Conclusion

Undoubtedly, there has been huge progress in unveiling several details of the reinitiation mechanism in the recent years. Above all, the indispensable role of eIF3 in translational control mechanism of *GCN4* expression, and perhaps some other genes as well, has confirmed the complexity and multifunctionality of this initiation factor, whose roles thus extend beyond canonical translation initiation. Particularly intriguing observations are those indicating that eIF3 remains transiently bound to the elongating 80S ribosome in some sort of a metastable state as it gradually drops off during successive elongation cycles. If this hypothesis that is mostly based on genetic data receives further biochemical support by future experiments, the currently favored textbook model declaring that all initiation factors dissociate from the 40S ribosomal subunit upon subunit joining will require a substantial revision to take this fact into account.

This special ability of eIF3 proved to play a critical role for the reinitiation mechanism by the virtue of its contact with the 5' sequences of REI-permissive uORFs that stabilizes

the 40S subunit on mRNA and allows it to resume scanning. But here again, most of the supporting evidence is based on genetic epistasis experiments obtained in yeasts and thus a clear biochemical proof of the direct contact between the NTD of a/TIF32 and uORF's 5' sequences is needed to strengthen the current model.

Even though several hypotheses picturing the molecular contribution of the 3' sequences of uORF1 of *GCN4* to efficient reinitiation exist, the true mechanism is unknown and will require other analyses. Similarly, the actual role of the 3' UTR of the *GCN4* mRNA is also a completely unexplored field that should be studied in the future.

Finally, as it appears now, the most of the features concerning reinitiation mechanism are highly conserved from lower to higher eukaryotes. The outstanding example supporting this claim is mammalian *ATF4*, a transcription factor representing a functional homologue of yeast *GCN4*. *ATF4* expression involves participation of two differently acting uORFs located in 5' leader of *ATF4* mRNA, and its regulation strongly resembles *GCN4* translational control mechanism (Vattem & Wek, 2004).

Thus, a very exciting theme to explore in the future will be the focus on biochemical and genetic analysis of the molecular mechanism of reinitiation in higher eukaryotes.

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