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Characterization of the molecular mechanism of translation reinitiation in yeast.

Charakterizace molekulárních mechanismů reiniciace translace v kvasinkách.

Ph.D. Thesis

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INSTITUTE OF MICROBIOLOGY AS CR

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Declaration

With this, I declare that I have written this work on my own, appropriately acknowledged citations, and used no other than the listed resources and aids.
The thesis serves only and exclusively for my PhD graduation on Faculty of Science of Charles University in Prague.

Prague

Mgr. Vanda Pondělíčková

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List of abbreviations

3-AT	3-aminotriazole
bp	base pair
CTD	C-terminal domain
eIF	eukaryotic initiation factor
GEF	guanine-nucleotide exchange factor
GTI	general translation initiation
IRES	internal ribosomal entry site
MBU	minimal binding unit
MFC	multifactor complex (pre-formed complex of eIF1, 3, 5 and TC)
NMD	nonsense mediated decay
nt(s)	nucleotide(s)
NTD	N-terminal domain
PIC	pre-initiation complex
REI	reinitiation
RPE	reinitiation promoting element
RRM	RNA recognition motif
TC	ternary complex (comprises eIF2, GTP and Met-tRNA$_i^{Met}$)
uORF	upstream open reading frame
UTR	untranslated region

Abstract

Translation initiation is a multi-step process culminating in formation of the elongation-competent 80S ribosome. It requires accurate assembly of small and large ribosomal subunits, mRNA, initiation Met-tRNA$_i^{Met}$ and at least 12 eukaryotic initiation factors (eIFs). This phase of protein synthesis is also one of the key points of regulation of gene expression. One of the main aims of our laboratory is a complex characterization of the multiprotein eIF3 complex that has been implicated in most of the steps of translation initiation. For example, we revealed and described its novel role in translation reinitiation (REI), a gene-specific translational control mechanism that among others governs expression of an important yeast transcriptional activator *GCN4*.

Here I present a detailed characterization of the multi-functional N-terminal domain of Tif32 (subunit eIF3a). We demonstrated that the Tif32-NTD functionally interacts with the 5' sequences of short upstream ORF (uORF1) in the *GCN4* mRNA leader and thus allows efficient reinitiation downstream of this critical reinitiation-permissive uORF. Four REI-promoting elements (RPEs) were identified in the 5' sequences of uORF1, two of which were shown to work in the Tif32-NTD-dependent manner. The structure of the 5' sequences was determined computationally as well as experimentally and one structurally similar motif was observed also in the 5' sequences of yet another REI-permissive uORF in the mRNA leader of yeast transcriptional activator *YAP1*. Moreover, we experimentally verified that it also works in the Tif32-NTD-dependent manner. This is, to our knowledge, the first evidence of a conserved mRNA feature that markedly promotes reinitiation at least in yeast. In addition, we also identified two distal regions in the Tif32-NTD that carry the REI-promoting role of this eIF3 domain by interacting with two RPEs, as mentioned above.

In separate study we showed that the N-terminal domain of Tif32 also interacts with the small ribosomal protein Rps0A residing nearby the mRNA exit channel. A partial deletion of the Rps0A-binding domain in Tif32 (*tif32-Δ8* mutant) impaired translation initiation and reduced 40S-binding of eIF3 and its associated eIFs. We revealed that Rps0A interacts with amino acid residues 200-400 of Tif32 via its extreme C-terminal tail (CTT). We demonstrated that both Rps0A-depleted cells as well as cells deleted for *rps0A* but expressing the Rps0A protein lacking its CTT displayed the same 40S-binding defect by eIF3 and other MFC components as was observed earlier in the *tif32-Δ8* mutant cells. Together we concluded that the interaction between the Tif32-NTD and the Rps0A-CTT forms an important molecular bridge anchoring the MFC factors to the small ribosomal subunit *in vivo*.

Finally, we also functionally analyzed the N-terminal domain of Nip1 (subunit eIF3c). A semirandom mutagenesis of the Nip1-NTD was performed to distinguish the residues engaged in AUG selection from those promoting the assembly of the pre-initiation complexes. We successfully obtained several mutations that impaired either one of these initiation steps or both. We also mapped the binding sites for eIF5 and eIF1 in the Nip1-NTD and thus characterized their contribution to its dual role.

Abstrakt

Iniciace translace je několika stupňový proces končící sestavením 80S ribozomu umožňujícího elongaci. Při tomto procesu se musí bezchybně složit velká a malá podjednotka ribozomu, mRNA, iniciační Met-tRNA$_i^{Met}$ a nejméně 12 eukaryotických iniciačních faktorů (eIF). Tato fáze výroby proteinů je také klíčovým místem pro regulaci genové exprese. Naše laboratoř se zaměřuje zejména na iniciační faktor eIF3, který je tvořen více proteiny a uplatňuje se ve většině fází iniciace translace. Odhalili jsme a popsali roli eIF3 v reiniciaci, genově specifickém mechanismu kontroly translace, uplatňujícím se i v regulaci exprese důležitého kvasinkového transkripčního faktoru *GCN4*.

V této práci přináším detailní studii N-terminální domény proteinu Tif32 (podjednotka eIF3a), která umožňuje několik funkcí. Předně ukazujeme, že Tif32-NTD funkčně interaguje s 5' sekvencí krátkého předcházejícího ORF (uORF1) v *GCN4* mRNA a umožňuje tak následnou účinnou reiniciaci. V 5' sekvenci uORF1 byly identifikovány čtyři elementy podporující reiniciaci (REI-promoting elements, RPEs) a dva z nich působí v součinnosti s Tif32-NTD. Struktura 5' oblasti byla počítačově modelována a následně experimentálně potvrzena. Společný strukturní motiv byl nalezen i v 5' sekvenci dalšího kvasinkového genu *YAP1*, který rovněž obsahuje uORF umožňující reiniciaci. Tento motiv navíc také pracuje v součinnosti s Tif32-NTD. Poprvé tak byl ukázán způsob fungování reiniciace, který je konzervovaný u více genů, zatím v rámci kvasinek. Na straně Tif32-NTD jsme popsali dva oddělené regiony podporující reiniciaci ve spolupráci se dvěma výše zmíněnými RPE.

Dále ukazujeme, že N-terminální doména Tif32 interaguje také s proteinem malé ribozomální podjednotky Rps0A, jenž se nachází v blízkosti výstupního kanálu pro mRNA. Částečná delece domény sloužící pro vazbu Rps0A v proteinu Tif32 (mutant *tif32-Δ8*) narušuje iniciaci translace a snižuje vazbu eIF3 a přidružených iniciačních faktorů na 40S ribozom. Zjistili jsme, že Rps0A (a to svým C-terminálním koncem, CTT) se specificky váže do úseku 200-400 aminokyselin proteinu Tif32. Zároveň jsme ukázali, že v buňkách depletovaných na protein Rps0A nebo s delecí *rps0A,* ale exprimující protein Rps0A bez CTT domény, se objevuje stejný defekt vazby iniciačních faktorů na 40S jako u buněk s mutací *tif32-Δ8*. Z toho plyne, že interakce Tif32-NTD a Rps0A-CTT je významná pro vazbu MFC na malou ribozomální podjednotku *in vivo*.

Také se věnujeme funkční analýze N-terminální domény proteinu Nip1 (podjednotka eIF3c). Použili jsme částečně nahodilou mutagenezi (semirandom mutagenesis) pro rozlišení aminokyselinových zbytků, které se podílí buď na výběru AUG start kodonu nebo na sestavování pre-iniciačních komplexů. Získali jsme několik mutací, jež narušují buď jeden, druhý nebo oba kroky iniciace. Zároveň jsme určili vazebná místa pro eIF5 a eIF1 a objasnili jejich příspěvek k dvojí roli Nip1-NTD.

1. Introduction

Translation is a fundamental biological process that, as the last step of gene expression, ensures production of all proteins necessary for living cells. Protein synthesis depends on the current availability of nutrients, the stage of cell development, the morphological specialization of the cell or its role in tissue arrangement. These factors determine the demand for different sets of proteins and their different amounts in each cell.

Compared to transcriptional regulation, translational control of existing mRNAs allows for more rapid changes in cellular concentrations of the encoded proteins and as such it can be used for maintaining homeostasis in addition to modulating more permanent changes in cell physiology or fate.

The translation is controlled throughout all four of its phases, namely initiation, elongation, termination and ribosome recycling, which seem to communicate with each other to achieve an optimal outcome. Among them the initiation phase is the main target of regulation, though. To elucidate the complexity of the whole process, it is necessary to characterize all features of the system and understand the molecular basis of its individual steps. Only the perfect knowledge of translation as a complex molecular mechanism will allows us to efficiently modulate the process of protein synthesis, influence the production of proteins according to predefined requirements and thus, for example, to prevent or cure various genetic diseases that are associated with malfunctioning translation.

In order to produce a protein of specific function, first its encoding mRNA has to be recognized by the translational apparatus. Subsequently, the correct start codon has to be identified by the initiator Methionyl tRNA and a functional ribosomal complex composed of small 40S and large 60S subunits has to assemble before the first step of elongation can occur successfully. This seemingly trivial process of translation initiation also requires many effector proteins called translation factors, proteins or protein complexes that execute or at least facilitate all of the aforementioned reactions.

Research projects of my post-graduate appointment were devoted to better understanding of the process of translational initiation and its regulation. The major goal of my Ph.D. thesis was to elucidate the phenomenon of the gene specific translation control called reinitiation, which is a special type of translation initiation occurring after translation of short upstream open reading frames (uORFs). This process has been extensively studied since the 80s especially with help of the yeast model *GCN4* mRNA but in spite of that there are still a lot of aspects of this mechanism, mainly at the molecular level, that remain to be characterized. The long-term goal of our laboratory is not only to contribute to the knowledge of regulation of *GCN4* translational control and to our understanding of the role of the translation initiation factor 3 (eIF3) in it, but also to figure out the basic principles of reinitiation in general across species.

2. Literature review

2.1. Translation initiation

Translation in eukaryotes starts usually by a canonical mechanism called the General Translation Initiation (GTI). The GTI consists of several steps that ultimately lead to formation of the 80S initiation complex (IC) with the initiator tRNA (Met-$tRNA_i^{Met}$) base-paired with the AUG start codon in the ribosomal P- (peptidyl) site. This complex is fully competent to proceed with the first elongation step and join the second aminoacyl-tRNA according to codon - anticodon base pairing. Preparing of the 80S IC comprises two major steps: formation of 48S preinitiation complex and subunits joining. The entire process is orchestrated by separated small (40S) and large (60S) ribosomal subunits and by numerous proteins and protein complexes commonly called eukaryotic initiation factors (eIFs). These factors "prepare" the mRNA for translation and facilitate all processes that lead to formation of the aforementioned elongation-competent 80S ribosome. All components are reused multiple times and hence have to be recycled to their "active forms" after the end of each translational cycle, which includes, for example, recharging some of them with high-energetic molecules like GTP etc.

The first initiation step is assembly of the so called ternary complex (TC). The ternary complex consists of the initiation factor eIF2, GTP and initiator Met-$tRNA_i^{Met}$. A crucial step of the TC formation is replacing GDP on eIF2 generated in the previous cycle with GTP. Only in this GTP bound form, eIF2 can stably anchor Met-$tRNA_i^{Met}$ to form a new ternary complex. This process is handled by guanine-nucleotide exchange factor (GEF) eIF2B. The TC production has to be tightly regulated because availability of the TC not only determines the overall rate of protein synthesis but it also importantly influences the degree of expression of mRNAs, the initiation rates of which are especially sensitive to the TC levels. As such, the TC formation is one of the two major targets of the general translational control (reviewed in Sonenberg and Hinnebusch 2009). I will describe this general regulatory mode in more detail using the specific example of the yeast *GCN4* mRNA translation later in text.

The ternary complex is believed to be loaded to the small ribosomal subunit with the help of eIF3, eIF1, eIF1A and eIF5, producing the 43S pre-initiation complex (PIC) (reviewed in Jackson, Hellen, and Pestova 2010; Hinnebusch and Lorsch 2012). At least in budding yeast, it was shown that a delicate network of interactions links eIF1, 3, 5 and the TC into a multifactor complex (MFC), which is believed to approach the 40S ribosome as a pre-organized unit (Asano et al., 2000, Hinnebusch and Dever 2007). Recent data suggest that the MFC exists also in human (Sokabe, Fraser, & Hershey, 2012) and plants (Dennis, Person, & Browning, 2009).

The pre-assembled 43S PIC is then recruited to the capped 5' end of mRNA, which is facilitated by the eIF4F complex, to form the 48S PIC. The eIF4F comprises the cap-binding protein eIF4E, RNA-helicase eIF4A and eIF4G. eIF4G is a scaffold protein harboring binding domains for eIF4E and poly(A)-binding protein (PABP) in its N-

terminus and for eIF4A and (in mammals) also for eIF3 in its central and C-terminal regions (Imataka, Gradi, & Sonenberg, 1998; Imataka & Sonenberg, 1997; Morino et al., 2000; Villa et al., 2013). All these interactions enable eIF4G to connect the cap (bound by eIF4E) and the poly(A) tail of mRNA (bound by PABP) to form the so called 'closed-loop' mRNA structure (Wells et al., 1998). This structure probably helps to re-use the translational machinery after termination for new initiation on the same messenger RNA. Whereas in mammals it is eIF3 that seems to mediate the major connection between the eIF4F-mRNA and the 43S PIC, in budding yeast, a direct eIF3-eIF4G interaction has not been detected and the eIF3-binding domain is not evident in yeast eIF4G (LeFebvre et al., 2006). Instead, it was proposed that eIF5 might bridge the contact between eIF4G and eIF3 in the 48S PIC, as it was shown to be capable of simultaneous binding to both factors *in vitro* (Asano et al., 2001). Taking into account that yeast eIF3 is also considered to be more critical factor for mRNA recruitment than eIF4G (Jivotovskaya et al., 2006; Mitchell et al., 2010), it could be that the molecular mechanism of this particular initiation step differs in certain aspects between lower and higher eukaryotes (Valášek, 2012).

Translation initiation in eukaryotes differs from other kingdoms in several aspects, one of which – perhaps the most obvious one - is the identification of the AUG start codon. Whereas prokaryotic mRNAs possess a Shine-Dalgarno sequence that places the start codon directly to the P-site on the 30S ribosome and viruses use the IRES (internal ribosomal entry site) for the same purpose, the eukaryotic ribosome has nothing guaranteed and has to conduct a systematic search for the initiation codon in the process called scanning. The assembled 48S PIC moves along the 5' untranslated region (UTR) of an mRNA in the 5' to 3' direction inspecting successive triplets as they enter the P-site for complementarity to the anticodon of Met-$tRNA_i^{Met}$ (Kozak, 1978). The first AUG in 5' UTR is usually but not always recognized as the start codon because the AUG selection is also influenced by the AUG sequence context (Kozak sequence), which enhances or represses its usage as the start codon. Optimal Kozak sequence is gcc(A/G)cc<u>AUG</u>G (the A of the AUG codon is designated +1) in mammalian cells and deviations, especially at -3 and +4 positions (written in capital), lead to diminishment of translation efficiency by at least 10-fold (Kozak, 1986). In budding yeast the optimal context is typically aAaAaA<u>AUG</u>TCt (the most important positions are in capital) (Hamilton, Watanabe, & de Boer, 1987).

Even though the basic principles of scanning were proposed ~35 years ago offering the most coherent explanation for ribosomal start-site selection (Kozak, 1978), our knowledge of the mechanistic details of the scanning process remains fragmentary. It is known that unstructured 5' UTRs can be scanned by the 43S ribosomal complex without ATP consumption, i.e. in the absence of factors with helicase activity such an eIF4A, eIF4B or eIF4H. Thus the presence of the eIF1, 1A, 3 and the ternary complex in the 48S PIC is sufficient for reaching the AUG start site in the mammalian reconstituted system (Pestova & Kolupaeva, 2002). But even a weak internal secondary structure in the 5' leader requires both of the aforementioned factors – ATP and helicase(s) (Jackson 1991; Svitkin et al. 2001). Nevertheless, the exact role of helicases

and other factors in scanning is still not properly understood and the molecular principle of the actual movement along the mRNAs is absolutely unclear.

Originally, it was suggested that the small ribosomal subunit carrying a number of initiation factors moves along the messenger RNA in the specific conformational state called "open" – scanning conducive. This arrangement is promoted by binding of eIF1 and eIF1A to 40S subunit that triggers adoption of a specific position of the 40S head relative to its body, and as a result, opens the mRNA binding channel for mRNA loading and presumably also for subsequent scanning (Passmore et al., 2007). Importantly, during scanning the anticodon of Met-$tRNA_i^{Met}$ is not fully accommodated in the ribosomal P-site in order to prevent premature engagement with putative start codons. GTP on eIF2 is partially hydrolyzed with the help of the GTPase accelerating factor (GAP) eIF5. The resultant phosphate ion is not released from the complex and its retention, most probably by eIF1, blocks the ribosome to change its conformation from open to close – scanning arrested till the AUG start codon in the optimal Kozak context is recognized (Algire, Maag, & Lorsch, 2005). Then the exact codon-anticodon base pairing enables Met-$tRNA_i^{Met}$ to fully accommodate in the P-site, eIF1 is either ejected or moved to a different site on the 40S ribosome, free phosphate is released and the 48S PIC thus irreversibly switches to the closed scanning arrested form. This conformational switch is further stabilized for example by a functional interaction between eIF1A and eIF5 (Cheung et al., 2007). Besides the aforementioned factors, our laboratory and others provided additional evidence that also the eIF3 complex is involved in the process of AUG recognition mainly via the N-terminal domain of its Nip1 subunit (Valášek et al. 2004; Nielsen et al. 2006; Elantak et al. 2010; Chiu et al. 2010, Karásková et al. 2012, reviewed in Valášek 2012).

Recently, this mechanism was investigated in two structural studies (Lomakin and Steitz 2013; Weisser et al. 2013). Both groups proposed a slightly different model of action. The open conformation is, according to their structures, necessary only for mRNA loading and for the actual onset of scanning rather than for its arrest upon AUG recognition. A proper start codon selection thus supposedly does not require any large conformational changes even though it is still accompanied by dissociation or displacement of eIF1.

To finalize the initiation phase of translation, the large ribosomal subunit (60S) joins the scanning arrested 48S PIC with the help of the second GTP-binding protein in the entire pathway, eIF5B (Pestova et al., 2000). This factor, in accordance with the observed position of its prokaryotic homolog IF2 (Allen et al., 2005), occupies the region in the intersubunit cleft and was proposed to promote subunit joining by burying large solvent-accessible surfaces on both subunits (Unbehaun et al., 2007). As a result, all initiation factors occupying the intersubunit interface have to be ejected not to impede the process of 60S joining. The only two exceptions are eIF3 and eIF4F, which are localized at the opposite, solvent-exposed side of the 40S subunit, were proposed to stay 80S-bound for at least a few elongation cycles (Unbehaun et al. 2004; Fringer et al. 2007, this PhD thesis and its publications). In addition to eIF5B, eIF1A also contributes to subunit joining by interacting with the C-terminal domain of eIF5B via its C-terminal tail (Acker et al., 2006; Marintchev et al., 2003; Olsen et al., 2003). This interaction is

most probably possible only after the displacement of eIF1A's C-terminal tail from the P-site upon correct AUG recognition. Upon forming of all intersubunit bridges between small and large subunits, eIF5B-bound GTP is hydrolyzed by the GTPase-activating center (GAC) on the large subunit and this triggers coupled dissociation of eIF5B and eIF1A from the assembled 80S ribosome. The elongation phase of translation can now proceed.

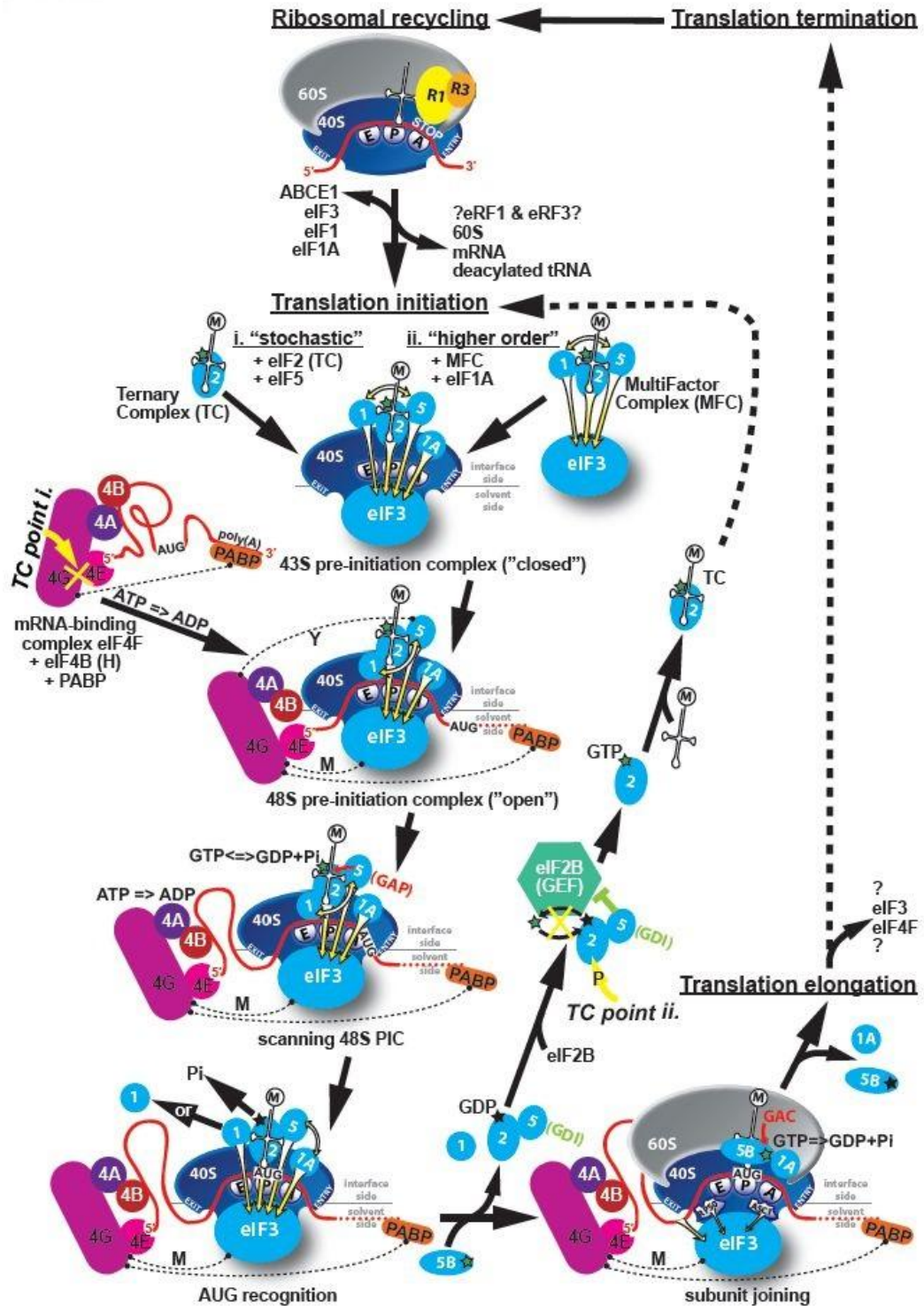


Figure 1. Schematic of the canonical translation pathway in eukaryotes with the ribosomal recycling and initiation phases shown in detail (adapted from Valášek, 2012). This figure combines findings from both yeast and mammals and indicates potential differences. The terminating 80S ribosome is split into

individual subunits with help of ABCE1/RLI1 and eIFs 1, 1A and 3. How eRFs 1 and 3 are recycled is not properly understood. The former eIFs either remain bound to the 40S subunit or dissociate prior to the initiation phase. In the former case, the ternary complex (TC) and eIF5 join the existing 40S-eIF1-eIF1A-eIF3 post-recycling complex in a "stochastic" way (i) to form the 43S pre-initiation complex (PIC). In the latter case, the 43S PIC is formed in the "higher order" manner via simultaneous binding of all components of the Multifactor complex (eIFs 1, 3, 5 and the TC) and eIF1A. Upon binding, eIFs1 and 1A induce conformational change that opens the mRNA binding channel of the 40S ribosome for mRNA loading. As a part of this major rearrangement eIFs1, if delivered to the ribosome in the MFC, must translocate from eIF3 to the P-site. mRNA is delivered to the 43S PIC in a complex with eIF4F (composed of eIF4A, E and G), eIF4B (and/or eIF4H in mammals) and PABP in an ATP-dependent reaction creating a "landing pad" close to the mRNA's cap structure that is bound by eIF4E (the interaction between eIF4G and PABP is shown as a dotted line for simplicity). As a result, the 48S PIC is formed and scanning for AUG commences. The actual attachment of mRNA to the ribosome is believed to be mediated via the eIF4G – eIF3 interaction in mammals (dotted line "M") that seems to be bridged via eIF5 in yeast (dotted line "Y"; this line is not shown in all cartoons for simplicity). During scanning, all secondary structures that could impede the movement of the PIC in the 5' to 3' direction are melted with help of helicase eIF4A and its co-factors eIF4B or eIF4H at the expense of ATP. Also, eIF5 stimulates GTP hydrolysis on eIF2 (GAP activity), however, the resulting Pi is not released until the AUG is located. Upon AUG recognition, eIF1 as a gatekeeper is either ejected from the ribosome or could move back to eIF3 to allow Pi release triggering reciprocal conformational switch to the closed form of the PIC that arrests scanning. eIF5B then promotes subunit joining that kicks out all interface-side-bound eIFs with the exception of eIF1A, and the solvent-side-bound eIF3 and eIF4F (interactions between eIF3 and two "solvent-side" ribosomal proteins RPS0 and RACK1/ASC1are indicated). GTP hydrolysis on eIF5B stimulated by the GTPase activating center (GAC) of the large subunit triggers coupled release of eIF5B and eIF1A rendering the resulting 80 initiation complex ready to elongate. It is believed that eIF3 and eIF4F can stay 80S-bound for at least a few elongation cycles thanks to their location on the back of the 40S subunit. eIF2•GDP is released in a binary complex with eIF5 that competes with and thus partially inhibits the action of the GEF eIF2B to exchange GDP for GTP on eIF2 (GDI activity). Upon this exchange, eIF2•GTP is ready to form a new TC that can enter the entire cycle all over again. Two "Translational control (TC) points" are indicated by yellow arrows and the mechanism of their action by yellow cross lines; the first targets the eIF4E–eIF4G interaction and the other the GTP/GDP exchange on eIF2 by phosphorylating its α subunit.

2.2. Translation initiation factor eIF3

Initiation factor eIF3 is the largest protein complex among initiation factors that plays a role in nearly every single step of translation initiation as mentioned above. Besides that, it is also involved in reinitiation (a regulatory mechanism explained in the later chapter) (Hinnebusch 2006, this PhD. thesis), in termination and post-termination ribosomal recycling (Beznosková et al., 2013; Pisarev, Hellen, & Pestova, 2007) and in the nonsense-mediated decay pathway (Isken et al., 2008).

In budding yeast, eIF3 comprises five core essential subunits (a/Tif32, b/Prt1, c/Nip1, i/Tif34 and g/Tif35) and one non-core subunit (j/Hcr1). These all have corresponding orthologs in the more complex mammalian eIF3, which contains seven additional non-conserved subunits (eIF3d, e, f, h, k, l and m) (reviewed in Valášek 2012).

Thanks to a very extensive effort of mainly Hinnebusch's and Hershey's laboratories, the composition of yeast and mammalian eIF3 subunits, their mutual interactions as well as their interactions with other initiation factors within the MFC were described in great depth. The analysis of interactions capitalized on combining the results from the

two-hybrid screens, *in vitro* binding assays and purification of MFC subcomplexes formed *in vivo* by affinity-tagged eIF3 subunits lacking various predicted binding domains. The results of these studies are summarized in Valášek et al. 2003; the details can be found in the following individual publications (Verlhac et al. 1997; Asano et al. 1998; Asano et al. 1999; Asano et al. 2000; Phan et al. 1998; Vornlocher et al. 1999; Valášek et al. 2001; Valášek et al. 2002). These studies revealed that in yeast each of the three largest subunits of eIF3 (a/Tif32, b/Prt1 and c/Nip1) has a binding site for the other two subunits, and that the extreme C-terminal domain (CTD) of Prt1 additionally interacts with eIF3 subunits i/Tif34 and g/Tif35. j/Hcr1 binds simultaneously to both the N-terminal domain (NTD) of Prt1 and the Tif32-CTD.

As for the contacts of eIF3 within the MFC, eIF1 is tethered to the MFC through interactions with the Tif32-CTD and Nip1-NTD. In addition to eIF1, the Nip1-NTD also binds to the CTD of eIF5. The β-subunit of eIF2 makes two critical contacts with eIF3, a direct interaction with the extreme CTD of Tif32 and an indirect association with the Nip1-NTD via the eIF5-CTD (Valášek et al., 2003). Our model in Fig. 2 visualizes the predicted arrangement of eIF3 subunits as well as all MFC-binding partners according to the known interactions.

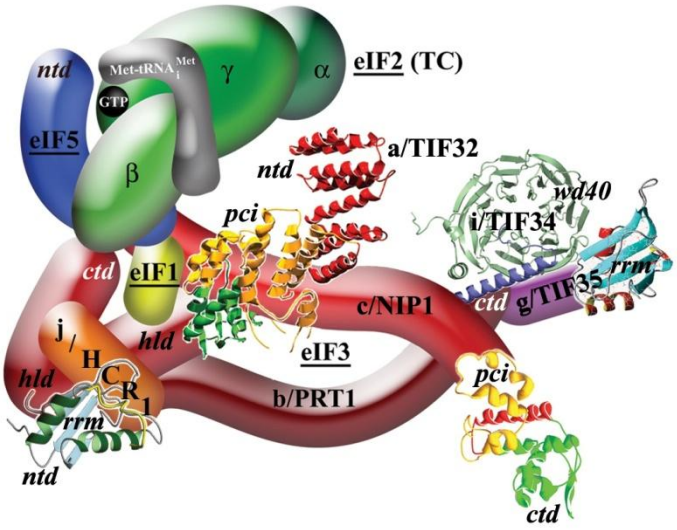
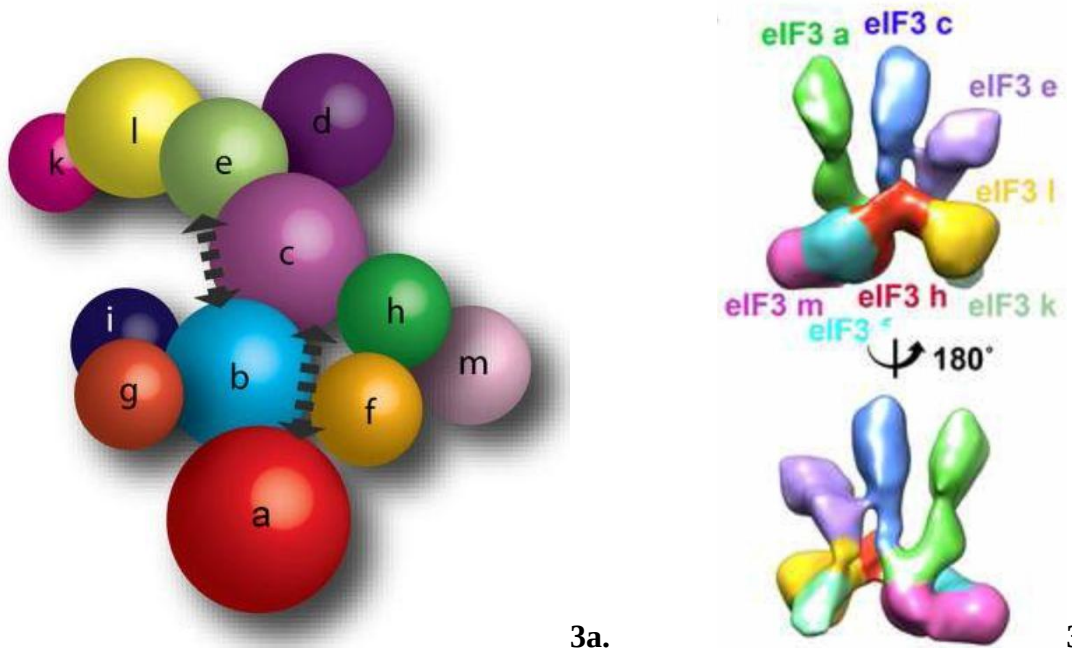


Figure 2. A 3D model of eIF3 and its associated eIFs in the MFC (Valášek et al., unpublished data). ntd, N-terminal domain ; ctd, C-terminal domain; hld, HCR1-like domain; rrm, RNA recognition motif; pci, PCI domain; TC, ternary complex. The NMR structure of the interaction between the RRM of human eIF3b (green and light blue) and the N-terminal peptide of human eIF3j (yellow) (Elantak et al., 2010), the NMR structure of the C-terminal RRM of human eIF3g (red and sky-blue, PDB accession code 2CQ0) (Cuchalová et al., 2010), the X-ray structure of the yeast i/TIF34 – b/PRT1-CTD complex (Herrmannová et al., 2011), the 3D homology model of the c/NIP1-CTD (Kouba et al., 2012) and the crystal structure of the a/TIF32-NTD (residues 276-494) extended by a homology-based prediction of residues 1-275 (S. Khoshnevis unpublished data) were used to replace the original schematic representations of the corresponding molecules.

Analysis of the composition and spatial arrangement of mammalian eIF3 has been a subject of thorough investigation by several labs for nearly two decades now, yet its

progress is far from complete. Perhaps due to larger complexity of this factor in higher eukaryotes, different scientific methods yielded different views of organization of all 13 subunits within eIF3. *In vitro* reconstitution of human eIF3 from baculovirus expression system proposed that the functional core (3a, 3b and 3c) contains also three non-conserved subunits 3e, 3f and 3h instead of 3i and 3g in yeast (Masutani et al., 2007). Other study, using solution disruption assays and tandem mass spectroscopy, suggested existence of three stable eIF3 modules, one of which containing 3a, 3b, 3i and 3g closely resembled the core of yeast eIF3 (Zhou, Sandercock et al., 2008) (Fig. 3a). And a recent comprehensive cryo-EM study revealed an octameric organization of eight core subunits (a, c, e, f, h, k, l and m) containing the PCI and MPN domains, with which the remaining 5 subunits (b, g, i, d and j) interact. The whole structure of eIF3 thus bears a close resemblance to that of the proteasome lid and COP9 signalosome (Querol-Audi et al. 2013) (Fig. 3b). Still, much more work is needed to reveal the real structure of mammalian eIF3 with true contacts between individual subunits.



3a. **3b.**

Figure 3. Models of mammalian eIF3. **3a.** A model of the human eIF3 (Zhou, Sandercock et al., 2008). The 13-subunit complex can be maintained intact in the gas phase, enabling to establish its stoichiometry and its overall subunit architecture via tandem mass spectrometry and solution disruption experiments. Dissociation takes place as a function of ionic strength to form three stable modules eIF3(c:d:e:l:k), eIF3(f:h:m), and eIF3(a:b:i:g). The remaining subunit eIF3j, a labile subunit, attaches to the complex via eIF3b (not shown in the model). These modules are linked by interactions between subunits eIF3b:c and eIF3c:h. Arrows denote additional interactions not readily represented in this model. The interaction map was confirmed with the homologous yeast eIF3 complex that contains the five core subunits found in the human eIF3 and the data were supplemented with results from immunoprecipitation. **3b.** A model of human eIF3 core (Querol-Audi et al., 2013). The core comprises 8 subunits (a, c, e, f, h, k, l and m) with PCI or MPN domains and remaining 5 subunits (b, g, i, d and j) interact with it (not shown in the model). It was previously reported a negative stain 3D reconstruction of the reconstituted eIF3 octamer core (Sun et al., 2011). In order to improve the reliability and resolution of eIF3 PCI/MPN octamer reconstructions, the cryo-EM data of frozen hydrated samples were collected. Using 200,000 particle images, the cryo-EM structure of the PCI/MPN 8mer with a resolution of ~15 Å was obtained. As with the prior negative stain EM reconstruction, the eIF3 PCI/MPN octamer bears also a striking resemblance to natively purified intact eIF3.

In the following two chapters I will focus on detailed description of only two subunits of yeast eIF3, as they are relevant for this thesis.

2.2.1. The a/Tif32 subunit of eIF3

The a/Tif32 subunit of eIF3 is the largest protein of eIF3 in all species with 964 amino acid residues and 110 kDa in yeast. Systematic effort was devoted to mapping the binding domains with its three interaction partners within eIF3. The binding domain for Nip1 is in its N-terminal half (between residues 200-600) (Valášek et al., 2002), followed by a special region comprising residues 490-790 that displays ~25% identity with the entire sequence of Hcr1 and serves as a binding site for Prt1. Due to this high similarity, this sequence was designated Hcr1-like domain (HLD). It contains an important sequence motif conserved among Tif32 and Hcr1 homologs called the KERR motif (K^{721}-X_5-E^{727}-R^{728}-X_2-R^{731} in *S. cerevisiae* Tif32). A site-directed substitution of the entire KERR motif in Tif32 is lethal and partial mutations evoke reduced efficiency of scanning and/or AUG recognition (Chiu et al., 2010). Overexpression of Hcr1 is able to compensate for some defects produced by mutation in this motif suggesting functional interconnection and partial interchangeability between these two proteins. The HLD is not the only binding domain for Prt1 in Tif32; the extreme C-terminus of Tif32 also contributes to the overall binding affinity between these two proteins (Valášek et al., 2001). Tif32 can also directly interact with Hcr1, but the specific domains involved in their contact were not precisely determined (Chiu et al., 2010; Valášek et al., 2001).

Besides these contacts, the C-terminal half of the HLD domain (residues 642-791) was shown to contribute to eIF1 binding to eIF3 as well as to the MFC. Finally, the extreme C-terminus of Tif32 serves as a direct anchor for stable association of eIF2 with the MFC, besides its other contact with Nip1-bound eIF5. Detail analysis revealed that the Tif32-CTD interacts directly with eIF2 *in vivo* and *in vitro* via its β-subunit (Valášek et al., 2002).

In addition to that, Tif32 also directly interacts with several components of the small ribosomal subunit. First, the extreme CTD of Tif32 (residues 791-964) binds specifically to helices 16-18 of domain I of 18S rRNA (Valášek et al., 2003). Second, the same region also interacts with ribosomal proteins Rps2 and Rps3, both of which are situated close to the mRNA entry channel on the solvent side of ribosome (Chiu et al., 2010). Occurrence of these interactions strongly indicates that the Tif32-CTD can modulate the mRNA entry channel latch, formed by helices 18 and 34 of 18S rRNA (Passmore et al., 2007), as a way of influencing the transition from open to closed PIC conformations and thus promote mRNA recruitment, scanning and AUG recognition.

The last segment of Tif32 left to be described is actually the most relevant for the scope of my thesis – it is its extreme N-terminus. Deletion of the extreme N-terminus of Tif32 (in *tif32-Δ8*) had a minimal impact on the MFC composition, strongly indicating that it is not involved in any protein-protein interactions within this complex, but severely reduced eIF3 binding to the 40S ribosome (Valášek et al., 2003). In addition, it was demonstrated that the N-terminal half of Tif32 together with Nip1 and eIF5 constitutes

the so called minimal 40S-binding unit (MBU) of eIF3; i.e. the minimal part of the MFC that is still able to bind to the small ribosomal subunit. This strongly suggested that the NTD of Tif32 directly interacts with the 40S subunit and that this interaction is crucial for the MFC loading to the ribosome. Consistent with this idea, specific interactions between the Tif32-NTD and small ribosomal proteins Rps0A and Rps10A were identified (Valášek et al., 2003).

2.2.2. The c/Nip1 subunit of eIF3

c/Nip1 represents the second biggest subunit of eIF3. One of its main tasks is to interconnect several initiation factors in the MFC and thus promote their association with the 40S ribosome, because it contains binding sites not only for two other eIF3 subunits, Tif32 and Prt1, but also for eIF1 and eIF5. The latter two factors were implicated in selecting AUG as the start codon (Huang et al., 1997) and this role plus their closely related functions in scanning may all be coordinated by their association with Nip1 (see also bellow) (Asano et al., 2000; Asano et al., 1999; Karásková et al., 2012; Phan et al., 1998; Valášek et al., 2004).

In particular, it is the extreme N-terminus of Nip1 that connects eIF3 with eIF1 and eIF5. Deletion of its first 156 residues was shown to completely eliminate binding of eIF1 and eIF5 with the rest of eIF3 *in vivo*, and since eIF5 represents one of the bridges between eIF2 and eIF3, binding of eIF2 was also eliminated (Valášek et al., 2002). The same study revealed that deletion of immediately following residues 157-371 in Nip1 abolished binding of Tif32 and another internal deletion of residues 371-570 greatly diminished association of Nip1 with Prt1, and consequently also with Tif34 and Tif35, which both exclusively interacts with the extreme CTD of Prt1. Thus it seems that Nip1 serves as some sort of a scaffold protein for eIF3 and MFC assembly with at least four different binding sites following each other (Valášek et al., 2002).

In support of this idea, even the remaining C-terminal one third of Nip1 was shown to be engaged in important interactions. Notably, Nip1 residues 651 through 783 fold into the canonical PCI domain that interacts with RNA including 18S rRNA and thus it was proposed to promote eIF3 association with 40S ribosomes (Kouba et al., 2012). This discovery was rather surprising for us because PCI domains typically mediate protein-protein and not protein-RNA interactions to build large multiprotein assemblies like the 26S proteasome lid, COP9 signalosome and eIF3 (Pick, Hofmann, & Glickman, 2009). Besides Nip1, yeast eIF3 contains only one additional PCI-containing subunit in Tif32, whereas mammalian eIF3 is composed of 6 PCI subunits (a, c, e, k, l, m).

The extreme CTD of Nip1 then interacts with the small ribosomal protein Asc1/Rps33 (its mammalian ortholog is called RACK1) (Kouba et al., 2012). Asc1 and RACK1 proteins are both members of the WD40 (Trp-Asp) repeat scaffold protein family that adopts a typical seven-bladed β-propeller structure. Asc1/RACK1 is located on the head of the 40S ribosomal subunit near the mRNA exit channel and makes extensive contacts with helices h39 and h40 of 18S rRNA and ribosomal proteins Rps16, 17, and 3 (Rabl et al., 2011; Sengupta et al., 2004). The interaction between Nip1 and Asc1 was

localized to the last 60 amino acid residues of Nip1 and to the blades 1-3 in β-propeller of Asc1 and implicated in stabilizing eIF3 in the PICs (Kouba et al., 2012).
The Nip1 role in AUG recognition was studied by introducing a series of systematic mutations into its NTD followed by examination of their effects on the efficiency and fidelity of translation initiation. In particular, the first 160 residues were divided into 16 10-residues segments (boxes) and each of them was replaced by 10 alanines or 10 amino acid residues of opposite charge (for example box6 contains Glu-rich stretch, therefore it was substituted with a stretch of nine arginine residues to convert it from a highly acidic to a highly basic segment → Box6R). Six box mutations were found to impair the Nip1-NTD interaction with eIF5 and/or eIF1 to varying degree and, consequently, also their affinity for the 40S ribosome.
As a result, three Nip1-NTD boxes, namely Box2, Box6R and Box12 were found to severely impair functions of eIF1 and eIF5 in regulating the AUG selection and, in addition, Box6R and Box15 decreased the rate of TC binding to 40S subunits and thus disrupted translational control of *GCN4* expression (see below) (Valášek et al., 2004).

2.3. Regulation of translation

Translation is a robust process that takes care of timely and spatially organized synthesis of all proteins that the cell requires at the given time and at the given amount. That is why it has to be tightly regulated to avoid wasting material and energy. In eukaryotes, we can distinguish two main categories of mechanisms that regulate translation initiation: (i) processes that impact eIFs (or ribosomes) and therefore affect all scanning-dependent initiation events, and (ii) processes that have an impact on the mRNA itself. The first category involves the control of availability of active eIF2 or eIF4F factors by reversible protein phosphorylation or specific irreversible proteolysis of eIF4G. The second category includes sequence-specific RNA-binding proteins or microRNAs (miRNAs) that cause silencing or degradation of certain mRNAs. Beyond these categories, translation can also be influenced by the structure and/or "composition" of the mRNA itself, for example, if it contains short uORFs (upstream ORFs) that usually prevent the expression of the main gene (reviewed in Sonenberg and Hinnebusch 2009; Jackson, Hellen, and Pestova 2010).
The aforementioned types of regulation were mostly described only in higher eukaryotes, preferentially in mammals. Perhaps the best-characterized mechanism of translational control during stress is the phosphorylation of eIF2 on Ser51 of its α-subunit. Four eIF2α kinases have been characterized: HRI (haem-regulated kinase occurring probably only in erythroid cells), PKR (activated by double-stranded RNAs typically during the antiviral response), PERK (responding the endoplasmic reticulum stress) and GCN2 (activated by amino acid starvation). In yeast, the only eIF2 kinase is Gcn2 (Dever, Dar, & Sicheri, 2007).
Initiation factor eIF2 contains three subunits (α, β, γ). While the γ-subunit serves as a binding site for GTP and Met-tRNA$_i^{Met}$ and the β-subunit probably contributes to interaction with RNA and eIF5 and eIF2B, the α-subunit is critical for regulation of

eIF2 activity. As mentioned above, the α-subunit contains phosphorylation site in Ser51 that is under control of Gcn2 and other kinases.
Phosphorylation of the α-subunit can occur in both GTP and GDP-bound forms of eIF2. Phosphorylated eIF2-GTP is still fully capable of forming an initiation-competent ternary complex, but following its release from ribosome, phosphorylated eIF2-GDP tightly binds to and thus sequesters the guanine-nucleotide exchange factor eIF2B, abrogating its activity. Thus, phosphorylation of the α-subunit converts eIF2 from a substrate to a competitive inhibitor of eIF2B. It was shown that eIF2 is in excess over eIF2B in all examined organisms. As a consequence, phosphorylation of even a small percentage of eIF2α results in a complete sequestration of eIF2B, which leads to a rapid decrease of active TC and inhibition of protein synthesis in general.

2.3.1. Upstream ORFs

Eukaryotic mRNAs are usually considered to be monocistronic; i.e. containing one major open reading frame (ORF) flanked by sequences commonly known as 5' and 3' untraslated regions (5' and 3' UTR). These UTRs often significantly contribute to regulation of translation efficiency, mRNA stability and subcellular distribution of a given mRNA. 5' UTRs for example very often contain short upstream ORFs that quite frequently serve as an alternative translation initiation sites and thus influence the robustness of expression of the main gene. In particular, about 45-50% of mammalian genes and about 13% of yeast genes were predicted to carry at least one functional uORF upstream of the main protein coding ORF (Calvo, Pagliarini, & Mootha, 2009; Jackson et al., 2010). The real number of such uORF-containing transcripts, however, remains to be determined experimentally because despite of accumulation of various nucleotide sequence databases, a precise determination of the 5' UTR sequences in existing transcripts is problematic.
Several aspects determine whether or not the presence of uORF influences the expression of the main gene. For example, the sequence context of its uORF AUG (uAUG), its length, its distance from the 5' cap and to the following ORF or uORF, nucleotide composition and secondary structures of the sequences immediately preceding and following uORF and sometimes also a nature of a peptide that they encode (reviewed in Hood et al., 2009; Vilela & McCarthy, 2003). We can distinguish at least four different ways of how the uORF translation can affect translation from a downstream start codon.
The first and most common is a prevention of a downstream re-initiation event. Ribosomes initiate at uAUG of uORF that, however, does not allow resumption of scanning after its own translation because both ribosomal subunits get recycled. The probability of re-initiating at a downstream start codon is then correspondingly reduced. The main factor that determines the recognition of the upstream start codon is its immediate sequence context (Kozak sequence) (Kozak, 1978). When uAUG is in a relatively poor initiation context, there is an increased chance that ribosomes will reach downstream start codon by leaky scanning. Recognition of such uORF can be also

dependent on actual cell conditions, which may alter the degree of programmed leaky scanning (Palam, Baird, & Wek, 2011).

The second, relatively very rare mechanism is exerted by uORFs that allow resumption of scanning after their own translation because only the large ribosomal subunits get recycled, whereas the small ribosomal subunit is stabilized on the mRNA post-terminally. Re-scanning 40S subsequently reacquires a new ternary complex to be able to reinitiate on the next AUG start codon. There are several factors that determine the degree of permissiveness of given uORF for efficient reinitiation. One of them is its length that must be kept short. It is, however, not the length *per see* that is critical but the actual elongation rate. If it is slow, uORF must be very short because elongating 80S carries several transiently associated eIFs that are absolutely essential for efficient reinitiation, and the longer the uORF is or the slower the elongation rate is, the bigger is the probability that these factors will drop off (Kozak, 2001). In yeast, uORFs longer than 35 codons no longer promote efficient reinitiation (Rajkowitsch et al., 2004).

Besides the length of the coding sequence, the nature of flanking sequences is also very important. Earlier work on yeast *GCN4* mRNA suggested that AU-content within the 10-nucletide stretch immediately following the stop codon might correlate with the efficiency of reinitiation (Hinnebusch 1997). Later, sequences upstream of the start codon were also found to be critically required for efficient reinitiation (Grant, Miller, and Hinnebusch 1995). Whereas the role of the downstream sequences is totally unknown, the upstream sequences ensure stabilization of the post-termination 40S subunit on the mRNA, as we discovered in this work (see below).

The last but not least factor influencing reinitiation efficiency is the intercistronic distance between uORF and the main ORF (or next uORF). Sufficient intercistronic distance is necessary for recruitment of a new ternary complex that enables the scanning 40S ribosome to recognize and reinitiate on the next AUG. Indeed, presence of stable secondary structures within the intercistronic distance also has its impact on reinitiation efficiency (Dever, 2002). The best-studied example of the reinitiation mechanism is the one occurring on the *GCN4* mRNA in *S. cerevisiae* (Hinnebusch, 2005) (see below). Besides this, there are only a handful of examples where uORFs display an experimentally proven REI-permissive character, such as the yeast transcription factor *YAP1* (Vilela et al., 1998), mammalian ortholog of *GCN4* ATF4 (Vattem & Wek, 2004) and the bZIP transcriptional regulator ATF5 (Zhou, Palam et al., 2008).

The third mechanism of translational control exerted by uORFs involves translational arrest of 80S ribosomes either during their elongation or termination; it is so called translational stalling. Stalling is usually caused by the action of a nascent peptide encoded directly by a given uORF. Such a “stall” then acts as a barrier to the ribosomes scanning from the 5’ cap so that they cannot reach a start codon which is downstream of that uORF.

The last type of the uORF-mediate translational control affects mRNA stability via the Nonsense Mediated Decay (NMD) pathway. NMD is triggered by the ribosome occupancy of a premature translation termination codon within the coding sequence of the main gene, which can be “mistaken” for the stop codon of an upstream uORF.

Large-scale analyses of *S. cerevisiae* transcriptome indicated that mRNAs affected by NMD are very often uORF-containing transcripts (Guan et al., 2006; He et al., 2003).

2.3.2. *GCN4* as a model of translational control via REI

Yeast *GCN4* gene encodes a transcriptional activator of various biosynthetic genes. Gcn4p stimulates transcription of more than 30 amino acid biosynthetic genes, representing 12 different pathways, and also genes encoding various aminoacyl-tRNA synthetases and pathway-specific activators. It can also induce expression of purine biosynthetic enzymes, genes involved in biosynthesis of amino acid precursors or vitamins, and genes encoding peroxisomal components, mitochondrial carrier proteins, amino acid transporters or autophagy proteins. The elevated levels of biosynthetic enzymes allow for increased amino acid production, high-level tRNA charging, and a restoration of optimal translation rates in the cell. All these actions belong to a regulatory response known as General amino acid control (GAAC) in yeast, which is triggered by starvation or stress conditions. It is most often induced by amino acid deprivation caused by inhibition of some biosynthetic enzymes. The key trans-acting factor of the GAAC response is the protein kinase Gcn2. For review, see (Hinnebusch, 2005).

Expression of *GCN4* paradoxically increases when general translation is reduced. The mechanism of *GCN4* induction is brilliant because it combines two regulatory events that usually serve to down-regulate translation. These events, in case of *GCN4*, conversely enable very efficient expression of the Gcn4 protein that in turn helps to overcome the stress situation in the cell.

The *GCN4* transcript contains four upstream ORFs (uORF1-4) that are two or three codons long and all very efficiently translated. The first two of them (uORF1 and uORF2 occurring very close to each other) support very efficient reinitiation whereas the remaining two do not (for details see below) (Gunišová & Valášek, 2014). Ribosomes translate uORF1, resume scanning downstream and as soon as they reacquire new ternary complex, they restore their competence to initiate on the next AUG.

In non-starvation conditions, nutrients are not limited and ternary complexes are produced in high amount. 40S ribosomes scanning downstream from uORF1 thus reacquire the TC relatively rapidly and preferentially reinitiate at one of the last two REI-non-permissive uORFs, none of which supports efficient REI. The main *GCN4* ORF is thus never reached by the scanning 40S ribosomes and remains unexpressed (repressed).

In starvation conditions, nutrients are limited and deacylated tRNAs accumulate. This situation is sensed by the protein kinase Gcn2 that phosphorylates Ser51 of the α-subunit of eIF2. Phosphorylation inhibits the function of guanine-nucleotide exchange factor eIF2B, as outlined above, which diminishes production of ternary complexes. As a result, 40S ribosomes scanning downstream from uORF1 must travel for a longer period of time till they reacquire the new TC. This significantly increases the chance that the last two REI-non-permissive uORFs are bypassed and ribosomes reach the

GCN4 start codon. Thus, whereas the global protein synthesis is significantly down-regulated, *GCN4* expression is concurrently induced (derepressed).

Regulation of translation of the *GCN4* mRNA can be simply and elegantly studied by fusing the main *GCN4* ORF in frame with the *lacZ* gene followed by site-directed mutagenesis of the desired 5' UTR sequences. The level of expression is then measured and calculated from β-galactosidase activities of individual constructs. Starvation conditions are mimicked by the 3-aminotriazole (3-AT) treatment that inhibits histidine biosynthetic pathway and evokes amino acids starvation (Mueller & Hinnebusch, 1986). A similar effect can be achieved with sulfometuron methyl, which inhibits Ile, Val, and Leu biosynthesis (Hinnebusch, 2005). Mutations that fail to derepress *GCN4* and lead to its constitutive repression even under starvation conditions cause the Gcn^- phenotype (general control non-derepressible). On the contrary, mutations that constitutively express *GCN4* gene cause the Gcd^- phenotype (general control derepressed).

In general, each upstream AUG codon in the *GCN4* leader can repress translation; however, the two 3' proximal AUG codons are much more inhibitory than the two 5' proximal AUG codons (Mueller & Hinnebusch, 1986). The presence of uORF3 or uORF4 alone causes strong and constitutive repression of *GCN4*. The solitary uORF4 reduces *GCN4* translation to only 1% of the level seen in the absence of all four uORFs, under both starvation and non-starvation conditions. In contrast, uORF1 alone reduces *GCN4* translation by only 50% (Mueller et al., 1988), presumably because half of the ribosomes that translate uORF1 are able to resume scanning and reinitiate at *GCN4*. Surprisingly, the effect of uORF2 was not studied until very recently, perhaps because it was shown that the minimalistic set up of uORF1 and uORF4 was sufficient for nearly wild-type regulation of *GCN4* expression (Mueller & Hinnebusch, 1986). Nevertheless, detailed analysis of uORF2 revealed that it is not only nearly as efficient for REI as uORF1, but also functions in an analogous *modus operandi* to uORF1 and thus backs up its regulatory role in the so called fail-safe mechanism of the *GCN4* translational control (Gunišová & Valášek, 2014).

Derepression of *GCN4* fully depends on uORF1 and its ability to retain the small ribosomal subunit during termination on its stop codon to resume scanning for REI downstream. Exclusion of uORF1 from the *GCN4* mRNA leader or extension of its coding sequence into the beginning of the *GCN4* coding region so that both ORFs overlap leads to non-inducible phenotype (Grant, Miller, & Hinnebusch, 1994; Mueller & Hinnebusch, 1986).

A crucial feature determining the REI-permissive character of uORF1 is its sequence context. Reinitiation permissiveness of uORF1 is lost when its third (and last) coding triplet and/or 10 nucleotides immediately following its stop codon were replaced with the corresponding nucleotides from inhibitory uORF4. In fact, these mutations converted uORF1 into a strong translational barrier similar to uORF4 (Miller & Hinnebusch, 1989). It was also shown that efficient reinitiation is not associated with a particular amino acid or isoacceptor tRNA in the position of the last coding codon (Grant & Hinnebusch, 1994).

Equally important is the 5' sequence context of uORF1. Site-directed substitution of or progressive deletions in the 5' UTR of uORF1 dramatically impaired efficiency of REI.

This suggested that the nearly 230 nt long leader of uORF1 contains at least two regions that critically contribute to efficiency of reinitiation by an unknown mechanism back then. The first region was predicted to occur ~60 nt upstream of uORF1, the second region was thought to lie between nt -181 to -141 relative to AUG of uORF1 (Grant et al., 1995).

Finally, it should also be mentioned that besides four uORFs, the *GCN4* leader also contains two non-AUG uORFs mapping in front of uORF1 (Ingolia et al., 2009). Both seem to be translated but their biological function is unknown, as they were not implicated in regulation of *GCN4* expression *in vivo* (Zhang & Hinnebusch, 2011).

3. Aims of the study

Specifically, I aimed to:

- further characterize the role of the N-terminal domain of Tif32 (eIF3a in yeast) in stimulating association of eIF3 and other MFC components with the 40S ribosome;
- understand the role of the Tif32-NTD in post-termination retention of 40S ribosomes sitting at the uORF1 stop codon on the *GCN4* transcript;
- identify specific *in cis* elements within the 5' sequences of uORF1 as well as particular residues in the Tif32-NTD that are responsible for high efficiency of reinitiation (REI) past *GCN4* uORF1;
- characterize the interaction between the Tif32-NTD and a small ribosomal protein Rps0A in great detail by analyzing mainly the Rps0A contribution to the stable 40S-binding of eIF3;
- further characterize the function of the N-terminal domain of Nip1 (eIF3c in yeast) in translation initiation by pinpointing its critical residues that either promote the assembly of pre-initiation complexes (PICs) or ensure stringent selection of the AUG start codon.

4. Methods

4.1. List of methods

Yeast and bacteria cells cultivation

Nucleic acids procedures

Protein procedures, Western blot analysis

Genetic modification of yeast cells

Polysome profile analysis

Sucrose gradient separation

β-galactosidase assay

Ni^{2+} chelation chromatography, GST-pull down assay

RNA structure probing

4.2. Principle of methods

All the yeast cultures (*Saccharomyces cerevisiae*) were cultivated in liquid or on solid media using commonly: rich medium YPD (yeast extract, pepton, dextrose) or minimal medium SD (synthetic defined).
Bacterial cultures (*Escherichia coli*) used for propagation of plasmids and DNA cloning were cultivated in liquid lysogeny broth (LB) medium or on corresponding solid medium.
The DNA was manipulated by the isolation of DNA, restriction of DNA by restriction endonucleases, ligation, polymerase chain reaction – PCR, electrophoretic separation of DNA in order to prepare a desired DNA molecule suitable for cloning. The RNA was isolated from the cells for Northern blot analysis.
The protein manipulations included protein isolation, electrophoretic separation of proteins and Western blot analysis.
Yeast strains were prepared by transformation of plasmid molecule(s), plasmid shuffling in selective media and by integration of deletion or fusion DNA cassettes into yeast genomes. Plasmids were multiplied in bacterial cells.
Polysome profile analysis allowed monitoring of translation and especially its initiation phase. Cells were grown into exponential phase and the translating ribosomes were locked on mRNA by the cycloheximide treatment. Thus pretreated cells were lysed and the whole-cell extracts (WCEs) were resolved by the velocity sedimentation through 5–45% sucrose gradients. The resulting gradients were collected and scanned at 254 nm to visualize the ribosomal species. A typical polysome profile showed peaks corresponding to free 40S and 60S ribosomal species and mRNAs containing different

numbers of 80S elongating ribosomes appearing as monosomes, disomes, etc. (Valášek et al., 2007).

Sucrose gradient separation provided the best available approximation of the native composition of 43S/48S pre-initiation complexes *in vivo*. Cells were grown into exponential phase and cross-linked with formaldehyde prior to harvesting to stabilise the native pre-initiation complexes. WCEs were prepared and separated on a 7.5–30% sucrose gradient by high velocity centrifugation. Fractions were collected and subjected to Western blot analysis where the composition of the complexes were examined by antibodies (Valášek et al., 2007).

β-galactosidase assay was used for determination of expression level of desired ORF that was fused with *lacZ*. Cells were cultivated to appropriate OD and WCEs were prepared. The β-galactosidase activities were measured in the WCEs and expressed in units of nmol of o-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg of protein (Grant & Hinnebusch, 1994; Mueller & Hinnebusch, 1986).

Ni^{2+} chelation chromatography revealed *in vivo* interaction of the His_8-tagged protein. WCEs were prepared from the selected strains and incubated with Ni^{2+}Sepharose that specifically bound the His_8-tagged protein. The bound proteins were eluted and subjected to Western blot analysis with the antibodies of expected binding partners (Nielsen & Valášek, 2007).

GST-pull down assay was able to show interactions between GST-fussed protein and *in vitro* synthesized ^{35}S-labeled polypeptides. Proteins fused to GST were expressed in *E. coli* and immobilized on Glutathion-Sepharose beads. This was incubated with ^{35}S-labeled polypeptides synthesized *in vitro*. The beads were washed properly and bound proteins were separated by SDS-PAGE. The gel was first stained against proteins and the autoradiography followed to visualise the labelled polypeptide (Valášek et al., 2001).

RNA structure probing allowed determining of the secondary structure of RNA molecule. The RNA was radiolabeled at 5' end and subjected to limited digestion with RNase T1 (cleaves after single-stranded G residues) or RNase V1 (cleaves within double-stranded RNA). The digested products were then separated on the polyacrylamide sequencing gel and detected by the autoradiography (Munzarová et al. 2011 in this PhD. thesis).

5. Presented publications

Individual publications are enclosed at the end of this thesis.

Publication I

The eIF3a Cooperates with Sequences 5' of uORF1 to Promote Resumption of Scanning by Post-Termination Ribosomes for Reinitiation on GCN4 mRNA
Béla Szamecz, Edit Rutkai, Lucie Cuchalová, **Vanda Munzarová**, Anna Herrmannová, Klaus H. Nielsen, Laxminarayana Burela, Alan G. Hinnebusch, Leoš Valášek
Genes Dev. 2008 Sep 1; 22(17):2414-25.
PMID: 18765792

IF_{2008} 13,623

IF_{2013} 12,639

Contribution of the author: 15%; I was involved in cloning and also contributed to the design of several experiments that I also performed.

Publication II

Translation Reinitiation Relies on the Interaction between eIF3a/TIF32 and Progressively Folded cis-Acting mRNA Elements Preceding Short uORFs
Vanda Munzarová, Josef Pánek, Stanislava Gunišová, István Dányi, Béla Szamecz, Leoš Shivaya Valášek
PLoS Genet. 2011 Jul; 7(7):e1002137
PMID: 21750682

IF_{2011} 9,532

IF_{2013} 8,167

Contribution of the author: 80%; I conceived and designed the experiments, performed majority of them, analyzed the data and also contributed to writing the publication.

Publication III

Small Ribosomal Protein RPS0 Stimulates Translation Initiation by Mediating 40S-Binding of eIF3 via Its Direct Contact with the eIF3a/TIF32 Subunit
Tomaš Kouba, István Dányi, Stanislava Gunišová, **Vanda Munzarová**, Vladislava Vlčková, Lucie Cuchalová, Andreas Neueder, Philipp Milkereit, Leoš Shivaya Valášek
PLoS One. 2012; 7(7):e40464
PMID: 22792338

IF_{2012} 3,730

IF_{2013} 3,534

Contribution of the author: 20%; I contributed to the design of several experiments that I also performed.

Publication IV

Functional Characterization of the Role of the N-terminal Domain of the c/Nip1 Subunit of Eukaryotic Initiation Factor 3 (eIF3) in AUG Recognition

Martina Karásková, Stanislava Gunišová, Anna Herrmannová, Susan Wagner, **Vanda Munzarová**, Leoš Shivaya Valášek

J Biol Chem. 2012 Aug 17; 287(34):28420-34

PMID: 22718758

IF_{2012} 4,651

IF_{2013} 4,600

Contribution of the author: 10%; I performed several experiments.

6. Summaries of my publications

Publication I follows and extends previous experimental effort of the Leoš Valášek's lab to elucidate the role of the a/Tif32 subunit of eIF3 in translation initiation in budding yeast *Saccharomyces cerevisiae*. For this purpose, it takes advantage of the viable *tif32-Δ8* mutation (N-terminal deletion of the first 200 amino acid residues) that was previously shown not to interfere with the integrity of the MFC (Valášek et al., 2002) but to completely eliminate binding of mutant eIF3 and its associated eIFs in the MFC to the 40S subunits *in vivo* when competing with the wild-type MFC (Valášek et al., 2003).

This publication confirmed the 40S binding defect of this slow growing mutant even when expressed as the only source of the Tif32 protein known to interact with small ribosomal protein Rps0A. In addition to that, *tif32-Δ8* also imparted a strong Gcn$^-$ phenotype indicative of a defect in regulation of *GCN4* expression. Detailed analysis revealed that this mutant prevented the 40S subunits to remain attached to the *GCN4* mRNA upon termination at uORF1 to resume scanning downstream. Hence, we established a novel class of Gcn$^-$ mutants that affects the ability of uORF1 to promote efficient reinitiation.

In a series of experiments we demonstrated that the NTD of Tif32 contacts specific sequences 5' of uORF1 and that this functional interaction ensures high level of REI past uORF1. Experiments with length extension of uORF1 then implicated eIF3 in being the critical factor transiently associated with elongating ribosome that is, upon termination, responsible for stabilization of post-termination 40S ribosomes on the *GCN4* mRNA. Deletion of the Tif32-NTD (in *tif32-Δ8*) or mutations in the Tif32-binding site upstream of uORF1 abolished this stabilization effect and consequently diminished resumption of scanning for REI downstream.

Publication II is closely connected with the previous one as it greatly deepens our understanding of molecular details of the contact between the Tif32-NTD and the 5' sequences of uORF1 in the *GCN4* mRNA that is instrumental in allowing high level of reinitiation after uORF1 and other uORFs similar in their nature to uORF1.

In particular, we identified four reinitiation promoting elements (RPEs) in the 5' UTR of uORF1, two of which (RPE I and IV) act in synergy in the Tif32-NTD-dependent manner. Secondary structure of the sequences upstream of uORF1 was determined computationally as well as experimentally. We revealed that this region is highly unstructured with the exception of a 9-nt long stem loop and a double-circle hairpin creating RPEs II and IV, respectively. Moreover, we were able to identify a similar double-circle hairpin in yet another REI-permissive uORF of *YAP1* that displayed the same Tif32-NTD-dependent behavior as RPE IV. Based on these findings we concluded that the molecular bases of the REI mechanism are at least in yeast most probably conserved.

Finally, we also identified two distal regions in the extreme NTD of Tif32 that are fully responsible for its REI-promoting activity in co-operation with the 5' sequences of uORF1.

Publication III explores molecular details of the contact between the Tif32-NTD and the C-terminal tail of the small ribosomal protein Rps0A that was described previously (Valášek et al., 2003). This interaction creates an important molecular bridge between eIF3 and the 40S ribosome and we investigated physiological consequences of disrupting this contact by mutating the Rps0A part of the interaction. We determined that the binding site of Rps0A in the Tif32-NTD resides somewhere between residues 200 to 400. The Tif32 binding site in Rps0A was then mapped to the last 42 amino acid residues of its acidic C-terminal tail.

Depletion of Rps0A by the degron cassette decreased binding of the MFC components to the 40S ribosome and as a consequence diminished initiation rates as could be expected given its bridging role mentioned above. Consistently, a similar defect was observed also with the *rps0a* mutant strain lacking the C-terminal tail. Together, we provided an important *in vivo* evidence for the role of Rps0A and its CTT in anchoring eIF3 to the small ribosomal subunit.

The last publication (publication IV) explores the role of c/Nip1, another subunit of eIF3, in the AUG start codon recognition by identifying critical Nip1 residues involved in this process with help of semi-random mutagenesis. Subsequent analysis of the selected mutants distinguished the residues engaged only in AUG selection from those promoting the assembly of the 43S PICs. We also specified locations of the Nip1-NTD binding sites for eIF5 (in between the residues 1-45) and eIF1 (in between the residues 60-137) and further characterized their contributions to both, assembly of PICs as well as AUG recognition.

7. Discussion

eIF3 is the most complex initiation factor among all and as such it is involved in many steps of translation. Over the last decade or two we and others have characterized its contribution to translation initiation (reviewed in Valášek, 2012), reinitiation (Gunišová & Valášek, 2014; Munzarová et al., 2011; Szamecz et al., 2008), and just recently also to termination and ribosomal recycling (Beznosková et al., 2013).

The major part of my work was focused on the a-subunit of eIF3, in yeast known as Tif32, but I also participated in studies of my colleagues exploring other eIF3 subunits such as for example Nip1. Tif32 is the largest subunit of yeast eIF3 and ensures a lot of its functions. Our work revealed that its N-terminal part is involved in (i) anchoring of eIF3 to the 40S subunit thanks to its interaction with Rps0A and (ii) reinitiation process such as that governing expression of the *GCN4* transcriptional activator.

Add ii) Our finding that the Tif32-NTD promotes efficient reinitiation after translation of *GCN4* uORF1 in cooperation with its 5' sequences was the first ever identification of a *trans*-acting factor participating in this important process. We detected a genetic interaction between the *tif32-Δ8* mutation removing the N-terminal 200 amino acids residues and mutations in the 5' sequences of uORF1, wherein the deleterious effect of *tif32-Δ8* on REI was blunted or even eliminated by mutations upstream of uORF1 and *vice versa*. This genetic epistasis clearly indicated that the NTD of Tif32 and uORF1 5' sequences functionally interact to stabilize the post-termination 40S subunit on *GCN4* mRNA and we further proposed, but have not experimentally proved yet, that they are in direct physical contact. It seems reasonable to suppose that their contact is further stabilized by simultaneous binding of eIF3 to the back side of the 40S subunit where additional interactions between the *GCN4* transcript and the ribosome can occur. A potential role in this stabilization process could be also played by the g/Tif35 subunit of eIF3, the specific mutations of which displayed a similar phenotype to *tif32-Δ8* to certain extent (Cuchalová et al., 2010). It must be also noted, however, that the proposed interaction should not be too strong to impede the rapid resumption of scanning by the post-termination 40S-eIF3 complex. One of the main goals of our laboratory is to provide *in vivo* evidence for a direct contact between the NTD of Tif32 and uORF1 5' sequences.

One of the biggest achievements of my thesis was the identification of four specific elements within the 5' UTR of uORF1, designated REI-promoting elements (RPEs), as well as two stretches of 10 amino acids residues within the Tif32-NTD that are directly responsible for mediating the genetic contact between the NTD of Tif32 and uORF1 5' sequences. Two of these four RPEs, RPE II and RPE IV, were shown to adopt a very specific 2D fold both computationally as well as experimentally that seems to be critical for their function. In addition, I also discovered a structure similar to that seen for RPEIV of *GCN4* uORF1 in the mRNA leader of another yeast transcriptional activator *YAP1*. Strikingly, this structure was also shown to work in the Tif32-NTD dependent manner to ensure efficient reinitiation after uORF preceding the main *YAP1*. Based on these findings we concluded that this type of very specific interaction is a paradigm for

a regulation of REI in general, at least in yeast. Obviously, we are now keen to expand our knowledge to higher eukaryotes and investigate whether or not a similar eIF3-mRNA contact ensures REI permissiveness also there. The best model to study this phenomenon is the functional homologue of yeast *GCN4*, mammalian ATF4.

Add i) Besides its function in reinitiation, the Tif32-NTD also plays an important role in anchoring eIF3 to the 40S subunits thanks to its interaction with Rps0A. The fact that the NTD of Tif32 is critically required for bridging the eIF3 contact with 40S was revealed while analyzing the *tif32-Δ8* mutation, which removes the first 200 residues of the Tif32 protein (Valášek et al., 2003). Hence, we anticipated that the Rps0A binding site falls into these first 200 amino acids residues that are lacking in *tif32-Δ8*. Surprisingly, we found out that the direct binding site for Rps0A lies not in the first 200 but in the next 200 residues; i.e. somewhere between the residues 201 through 400. This finding could be explained by proposing that the entire NTD of Tif32 adopts a specific fold where the Rps0A-binding site between residues 201-400 allows a stable interaction with Rps0A only if the first 200 amino acids residues ensure formation of this fold. If deleted in *tif32-Δ8*, the interaction gets too weak to be really established and eIF3 can no longer stably bind to the 40S ribosome. Either way, it seems evident that the main role of the first 200 amino acids lies in reinitiation.

The N-terminal half of Tif32 (residues 1-494) can be divided into the extreme N-terminus (residues 1-275) and the PCI domain (residues 276-494) (Khoshnevis et al., 2012). Only recently the crystal structure of the Tif32-PCI domain was solved and along with that the probable alpha-helical fold of the extreme N-terminus was computationally predicted (Khoshnevis et al., 2014). This work proposed that the extreme NTD contains several basic patches that could mediate Tif32 binding to RNA. Consistently, both REI-promoting segments of the Tif32-NTD were found to occur in these basic patches and an ability of this region to directly interact with RNA was experimentally demonstrated. In fact, the Tif32-PCI domain was the second PCI domain ever shown to interact not only with proteins but also with RNA, besides the Nip1-PCI domain (Kouba et al., 2012).

In addition to Tif32, I spent a part of my Ph.D. work characterizing also the Nip1 subunit of eIF3. In particular, we focused on the N-terminal domain of Nip1 that was, based on the previous study (Valášek et al., 2004), proposed to play two relatively distinct roles: one in assembly of preinitiation complexes and the other in the AUG start codon recognition. Previously identified segments that were thought to be responsible for these two roles without knowing any details were subjected to semi-random mutagenesis and in depth analysis. We were able to assign individual functions to individual residues and in one case we could even separate the effects of distinct amino acid substitutions within a short 8-residue segment, suggesting that the c/Nip1-NTD promotes both initiation reactions (PIC assembly and AUG recognition) independently of each other at least to a certain degree.

Based on our results we proposed that upon the MFC binding to the 40S ribosome, the Nip1-NTD helps to accommodate eIF1, eIF5 and eIF2 near the ribosomal A-site. Whereas the latter two factors remain bound in this area, eIF1 is subsequently transferred to its “scanning-competent” position near the P-site. This transfer could be a

part of a large conformational rearrangement of the 40S head that opens up the mRNA binding channel for mRNA recruitment. Upon AUG recognition eIF1 triggers reciprocal changes from the open to closed states, and instead of being directly ejected, it drifts back to the c/Nip1- NTD in the A-site. Thus, eIF3 could control timing and dynamics of the eIF1 shuffling and thus contribute to regulation of accurate start codon recognition.

8. Conclusions

My Ph.D. thesis contributed not only to the general knowledge of translation initiation by providing novel insights into structure and function of initiation factor eIF3 and its associated factors in the MFC, but also to our understanding of a gene-specific regulatory mechanism called reinitiation.

- We further characterized the slow growing *tif32-Δ8* mutant by showing that it strongly abolishes binding of the MFC to 40S ribosomal subunit without affecting its integrity.
- The *tif32-Δ8* cells also exhibited a severe Gcn$^-$ phenotype; the cause of which was found to be a previously not described: a defect in efficient reinitiation past uORF1 of the *GCN4* transcript.
- The N-terminal domain of Tif32 was found to functionally interact with the 5' sequences of uORF1 in order to stabilize the 40S subunit on *GCN4* mRNA after termination on uORF1 that is critical for efficient reinitiation.
- We identified four REI-promoting elements (RPEs) in the 5' sequences of uORF1, two of which operate in the Tif32-NTD-dependent manner.
- The secondary structure of the 5' sequences of uORF1 was determined and one identified structural motif was found also in the 5' UTR of other gene under reinitiation control in the Tif32-dependent manner, yeast transcriptional activator *YAP1*, suggesting evolutionary conservation of this mechanism.
- Two distal 10 amino acid residues-long regions in the Tif32-NTD were identified that are responsible for promoting efficient reinitiation in cooperation with two RPEs of uORF1.
- A binding site of Rps0A in the Tif32-NTD was mapped between Tif32 residues 200 through 400; Rps0A interacts with Tif32 mainly via its C-terminal 42 residues.
- Conditional depletion of Rps0A significantly decreased translation initiation rates due to a significant reduction of the MFC binding to the 40S subunits. A similar effect was observed also in the *rps0a* mutant strain lacking its C-terminal tail.
- The N-terminal domain of Nip1 was subjected to semirandom mutagenesis to investigate the molecular mechanism of eIF3 involvement in early steps of translation initiation. Three major classes of mutants were isolated that affect either the assembly of pre-initiation complexes (PIC), AUG recognition, or both.
- The Nip1 interaction site for eIF5 was localized to residues 1-45 and for eIF1 to residues 60-137. Impairing of these interactions affected the PIC formation and AUG start codon recognition, respectively.

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