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Vazba eIF3 v komplexu s eIF5 a eIF1 na ribosomální podjednotku 40S je doprovázena dramatickými strukturními změnami

Binding of eIF3 in complex with eIF5 and eIF1 to the 40S ribosomal subunit is accompanied by dramatic structural changes

Disertační práce

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Doctoral thesis

Supervisor: Mgr. Leoš Valášek, Ph.D.

Prague, 2019

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 11. 7. 2019

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Mgr. Jakub Zeman

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List of abbreviations

cryo-EM	cryogenic electron microscopy
CTD	C-terminal domain
eIF	eukaryotic initiation factor
EM	electron microscopy
FL	full-length
HLD	Hcr1-like domain
IRES	internal ribosomal entry site
Met-tRNA$_i$	initiator methionyl-tRNA
MFC	multifactor complex
MPN	Mpr1, Pad1 N-terminal domain
MS	mass spectrometry
NMD	nonsense-mediated decay
NMR	nuclear magnetic resonance
NTD	N-terminal domain
PCI	domain present in 26S proteasome, COP9, and initiation factor 3
PIC	preinitiation complex
REI	reinitiation
RPEs	reinitiation-promoting elements
RRM	RNA recognition motif
RTIDs	readthrough-inducing drugs
rti-tRNAs	readthrough-inducing tRNAs
SEC	size-exclusion chromatography
smFRET	single-molecule fluorescence resonance energy transfer
TC	ternary complex
uORF	upstream open reading frame
UTR	untranslated region
XL-MS	chemical cross-linking coupled to mass spectrometry
YLC	yeast-like core

Abstract

In eukaryotic translation, eukaryotic initiation factors (eIFs) are at least as important as the ribosome itself. Some of these factors play different roles throughout the entire process to ensure proper assembly of the preinitiation complex on mRNA, accurate selection of the initiation codon, errorless production of the encoded polypeptide and its proper termination. Perhaps, the most important factor integrating signals from others and coordinating their functions on the ribosome is eIF3. In *Saccharomyces cerevisiae*, eIF3 is formed by five subunits. All these subunits contain structural motifs responsible for contact with ribosomal proteins and RNAs. In addition to these highly structured parts, the rest of eIF3 is unstructured and very flexible. Therefore, despite the recent progress thanks to the use of a cryo-electron microscopy, a precise structure and position of eIF3 on the 40S ribosomal subunit are still not known. Also, the presence of eIF3 on 80S during early elongation and its role in reinitiation and readthrough are not fully understood.

In order to crack mysteries of yeast eIF3, we used x-ray crystallography, chemical cross-linking coupled to mass spectrometry, and various biochemical and genetic assays.

We demonstrated that eIF3 is very compactly packed when free in solution. This finding is in sharp contrast with the situation when eIF3 interacts with the 40S and embrace it almost completely from both the mRNA entry and exit channels. Considering that eIF3 association with its major interacting partners, namely eIF1 and eIF5, do not seem to dramatically change the globular shape of ribosome-free eIF3, we conclude that it is most probably the initial contact of eIF3 with the 40S that triggers its dramatic structural rearrangement. Importantly, using the same approach we determined the so far unknown binding site of eIF5 on 40S.

With the help of the newly developed pull-down assay, we also demonstrated that eIF3 stays bound on ribosomes elongating and terminating on short upstream open reading frames and promotes reinitiation in both yeast and mammals. On top of that, we designed and verified an *in vivo* assay for the comprehensive study of translational readthrough.

This thesis thus markedly extends the knowledge of yeast eIF3, its geometry, structural rearrangements provoked by its different binding partners, and its roles in reinitiation and readthrough.

Abstrakt

Eukaryotické iniciační faktory (eIF) jsou pro průběh eukaryotické translace minimálně stejně důležité jako ribosom. Některé tyto faktory mají rozdílné úlohy napříč celou translací v zajištění korektního složení preiniciačního komplexu na mRNA, přesném výběru iniciačního kodonu, bezchybné produkci příslušného polypeptidu a jejím řádném ukončení. Patrně nejdůležitějším z těchto faktorů je eIF3, který integruje signály od ostatních faktorů a koordinuje jejich funkci na ribosomu. V případě *Saccharomyces cerevisiae* je eIF3 tvořen pěti podjednotkami. Všechny tyto podjednotky obsahují strukturní motivy zodpovědné za kontakt s ribosomálními proteiny a molekulami RNA. Kromě těchto vysoce strukturovaných částí je zbytek eIF3 nestrukturovaný a velmi flexibilní. Z toho důvodu nejsou i přes současný pokrok v kryoelektronové mikroskopii doposud známy ani přesná struktura eIF3, ani jeho přesná poloha na ribosomální podjednotce 40S. Rovněž přítomnost eIF3 na ribosomu 80S během časné elongace a role eIF3 v reiniciaci a pročítání stop kodonu nejsou zatím zcela prostudovány.

K rozluštění těchto tajemství eIF3 jsme použili rentgenovou krystalografii, chemické zesítění spojené s hmotnostní spektrometrií a rozličné biochemické a genetické metody.

Naše práce ukazuje, že je eIF3 velmi kompaktně sbalený, pokud se nachází volný v roztoku. Toto zjištění je v ostrém kontrastu se situací, kdy se eIF3 váže na 40S a téměř kompletně ji objímá od kanálu pro vstup mRNA až po kanál pro její výstup. Jelikož vazba eIF3 s jeho hlavními interakčními partnery eIF1 a eIF5 zřejmě nemá zásadní vliv na jeho globulární tvar, usuzujeme, že až kontakt s 40S spouští jeho dramatickou strukturní přeměnu. Za použití stejného přístupu jsme též blíže určili dosud neznámé vazebné místo eIF5 na 40S.

Rovněž jsme popsali, že eIF3 zůstává navázán na ribosom během elongace a terminace na krátkých otevřených čtecích rámcích a podporuje následnou reiniciaci shodně v kvasinkách i v savčích buňkách.

Tato dizertační práce tak výrazně rozšiřuje dosavadní znalost kvasinkového eIF3, jeho uspořádání, strukturních změn navozených vazbou interakčních partnerů a jeho rolí v reiniciaci a pročítání stop kodonu.

1 Introduction

Gene expression is a fundamental process during which the genetic information encoded in DNA is first transcribed into a messenger RNA and subsequently translated into a chain of amino acids, giving rise to a new protein molecule. This basic flow of genetic information was named as the central dogma of molecular biology by Francis Crick between years 1957 and 1958 and re-stated in 1970 (Crick, 1958, 1970). Since then, different ways of information transfer were discovered thanks to studying viruses and chemical modifications of nucleic acids and proteins (reviewed in Koonin, 2012).

Gene expression is regulated on multiple levels to limit energy wasting and ensure proper functioning of an organism. Since translation machinery uses existing mRNAs, regulation on translation level allows rapid changes in concentration of proteins.

Despite the accepted theory that history of life on Earth begun as RNA world, the vast majority of functions in living cells are provided by proteins. Translation of the mRNA-encoded information into a protein is a multistep process consisting of initiation, elongation, termination, and ribosomal recycling as presented in Figure 1. Most of the regulation takes place during the initiation phase making it the most critical step of the whole translation process (reviewed in Hinnebusch, 2011; Aitken and Lorsch, 2012; Valášek, 2012; Dever et al., 2016). Translation initiation in eukaryotes requires participation of numerous proteins and protein complexes called eukaryotic initiation factors (eIFs). At least a dozen of those eIFs are required to assembly elongation-competent 80S ribosomes. This is in sharp contrast to bacteria, where only three main factors are necessary.

The main aim of my postgraduate study was to gain more information about the structure of the protein complex eIF3 from the budding yeast *Saccharomyces cerevisiae*. After several attempts to obtain crystals suitable for x-ray crystallography have failed, I changed my approach and employed chemical cross-linking coupled to advanced mass spectrometry (XL-MS). This method gave me an exciting, yet limited understanding about the geometry of eIF3 in its different states. Besides that, together with my colleagues I collaborated on projects dealing with roles of eIF3 in reinitiation and stop codon readthrough. I provided them with my knowledge of protein biology and structural bioinformatics. Nevertheless, this thesis is almost exclusively focused on the yeast eIF3 structure and its role in translation initiation.

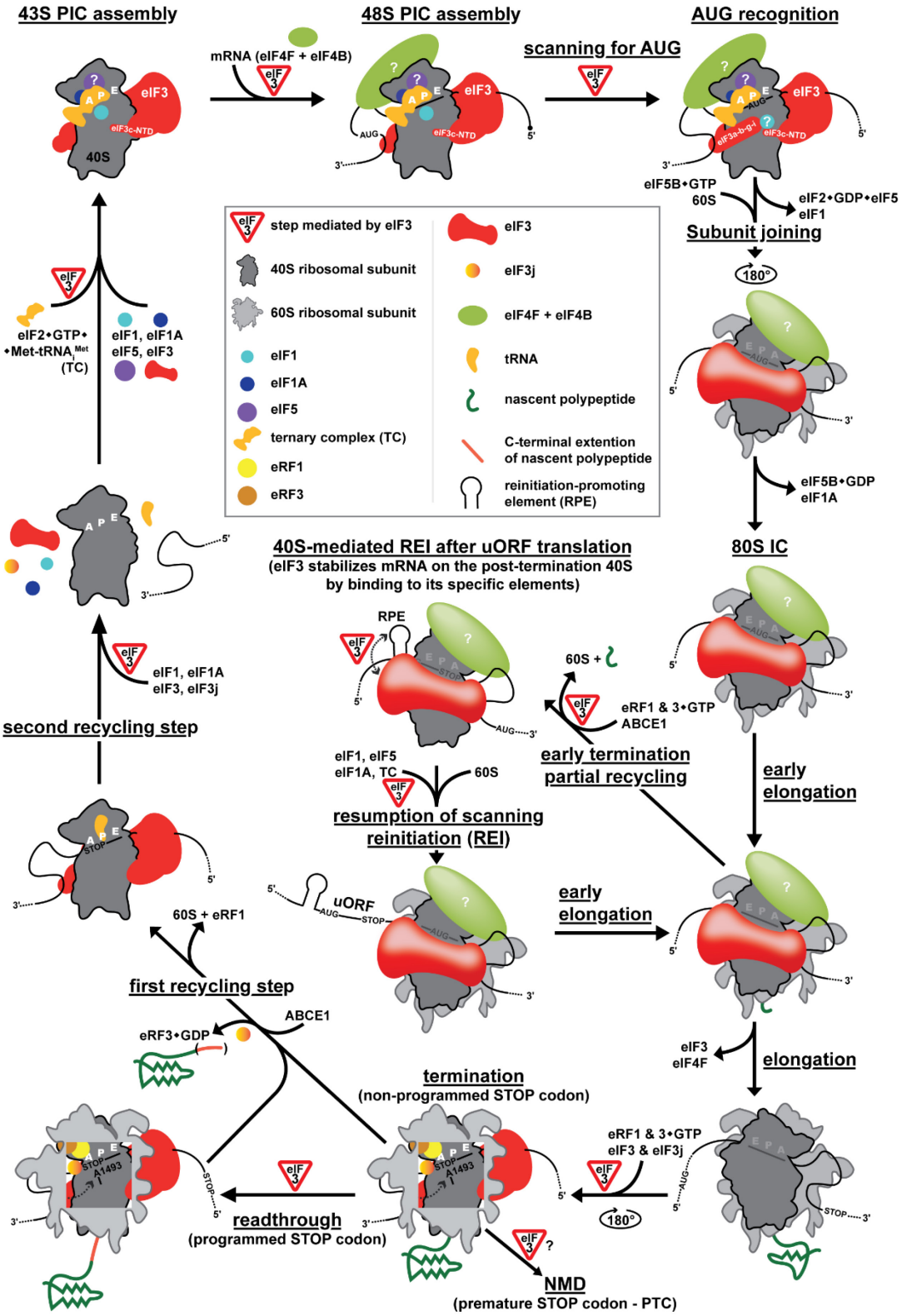

Figure 1: Schematics of the entire translation cycle with 'detours' for reinitiation, nonsense-mediated decay (NMD) pathway, and programmed stop codon readthrough. The role of eIF3 at individual steps is highlighted. For details, please see the main text.
Adapted from (Valášek et al., 2017).

1.1 Eukaryotic translation initiation

Translation cycle begins with initiation. During this step, mRNA is brought to the ribosome in a way that assures the proper identification of the start of the coding sequence (reviewed in Valášek, 2012; Hinnebusch, 2017). This is ensured by the initiator methionyl-tRNA (Met-tRNA$_i$) whose CAU anticodon is complementary to the canonical initiation AUG codon. Ternary complex (TC) consisting of Met-tRNA$_i$, eIF2, and GTP bound to the γ subunit of eIF2 (Erickson and Hannig, 1996) binds to 40S together with other eIFs such as eIF1, 1A, 3, and 5 (reviewed in Hinnebusch et al., 2007; Pestova et al., 2007). As a result of this step, the so-called 43S pre-initiation complex (PIC) is formed. Factors eIF1, 1A, 3, and 5 subsequently prepare 40S for mRNA docking by opening the mRNA binding channel. Importantly, mRNA is loaded to 40S pre-bound by eIF4F, eIF4B, and poly(A)-binding protein PABP1 (Mitchell et al., 2010) with the help of eIF3. The trimeric complex eIF4F includes the scaffold protein eIF4G, the helicase eIF4A, and eIF4E binding the 5′ 7-methylguanosine cap of mRNA. Binding of the 43S PIC to mRNA near its cap structure forms the 48S PIC. The 48S PIC subsequently starts to scan the sequence of nucleotides downstream of the cap in order to find the initiation codon in optimal context (Kozak, 1986). In order to ensure a smooth movement along the 5′ untranslated region (UTR) up to the start codon recognition, mRNA secondary structures have to be unwound. This task is performed by helicases such as eIF4A or Ded1, and in higher eukaryotes Dhx29 (Rogers et al., 1999; Iost et al., 1999; Pisareva et al., 2008). The AUG recognition causes conformational changes in the PIC. As a result, hydrolysed GTP is released from eIF2, 40S mRNA binding channel is closed, and most of the initiation factors are ejected from the 48S PIC (reviewed in Hinnebusch, 2014). GTP-bound eIF5B mediates 60S joining (Pestova et al., 2000) and subsequently dissociates together with eIF1A producing an elongation-competent 80S ribosome (Fringer et al., 2007).

1.1.1 Comparison of translation initiation between *S. cerevisiae* and higher eukaryotes

In general, translation initiation in *S. cerevisiae* is remarkably similar to translation initiation in mammals. This fact was demonstrated many times by substituting mammalian eIFs for their yeast homologues and *vice versa* (Altmann et al., 1989; Jaramillo et al., 1990; Schwelberger et al., 1993). However, there are still smaller or bigger sequential and/or structural differences among these homologues caused by additional regulatory roles of eIFs in higher eukaryotes. This phenomenon will be demonstrated by the case of eIF3 in chapter 1.2.

A stable multifactor complex (MFC) comprising eIF1, TC, eIF3, and eIF5 is formed in yeast in the absence of 40S both *in vitro* and *in vivo* stabilising the 43S PIC formation (Asano et al., 2000; Valášek et al., 2002; Singh et al., 2004). The same multifactor complexes were later described in plants and mammals as well (Dennis et al., 2009; Sokabe et al., 2012).

In mammalian *in vitro* reconstituted systems, purified 40S, eIFs 1, 1A, and 3 were sufficient to promote scanning on unstructured 5′ UTRs and locate the initiation codon without any requirement for ATP. In contrast, the presence of even weak secondary structures in 5′ UTRs required RNA helicases (eIF4F) and ATP (Pestova and Kolupaeva, 2002). In yeast, also eIF5 is necessary for this minimal PIC translation initiation, at least *in vivo* (Yamamoto et al., 2005; Cuchalová et al., 2010).

1.2 The eIF3 complex

Translation initiation factor 3 is the largest and most complex of all eIFs. In years after its discovery in the 1970s, it was shown that eIF3 is a multisubunit protein complex (Schreier and Staehelin, 1973; Freienstein and Blobel, 1975; Benne and Hershey, 1976). In *S. cerevisiae*, eIF3 is composed of five subunits: a/Tif32, b/Prt1, c/Nip1, i/Tif34, and g/Tif35 (Figure 2A) (Asano et al., 1998). All these subunits have orthologues in the more complex mammalian eIF3, which contains 12 subunits (eIF3a-m; Figure 2B) (reviewed in Hinnebusch, 2006; Valášek, 2012). For many years, the yeast j/Hcr1 (Valášek et al., 1999) and its mammalian orthologue eIF3j (Block et al., 1998) were considered as the sixth and thirteenth subunit of eIF3, respectively. Nonetheless, recent evidence strongly indicates that they rather represent eIF3-associated factors having mostly eIF3-independent roles. Hcr1 was proposed to have a more important role in termination than in initiation (Beznosková et al., 2013), and eIF3j was found to block mRNA binding to 40S by interacting with ribosomal decoding centre and eIF1A (Fraser et al., 2007), whereas eIF3 is one of key factors promoting mRNA recruitment to the 43S PIC.

Thanks to the intensive research done mainly in budding yeast, eIF3 has been demonstrated to participate in nearly every step of translation initiation. Some domains of eIF3 stimulate the TC and mRNA recruitment to the PIC and control the processivity of scanning and the fidelity of the start codon selection (Valášek et al., 2002, 2004; Nielsen et al., 2004; Jivotovskaya et al., 2006; ElAntak et al., 2010; Chiu et al., 2010; Cuchalová et al., 2010; Herrmannová et al., 2012; Karásková et al., 2012; Khoshnevis et al., 2014; Aitken et al., 2016; Obayashi et al., 2017); eIF3 also keeps both ribosomal subunits apart (Kolupaeva et al., 2005), but, as we also recently demonstrated, stays bound to the 80S even during early elongation stage

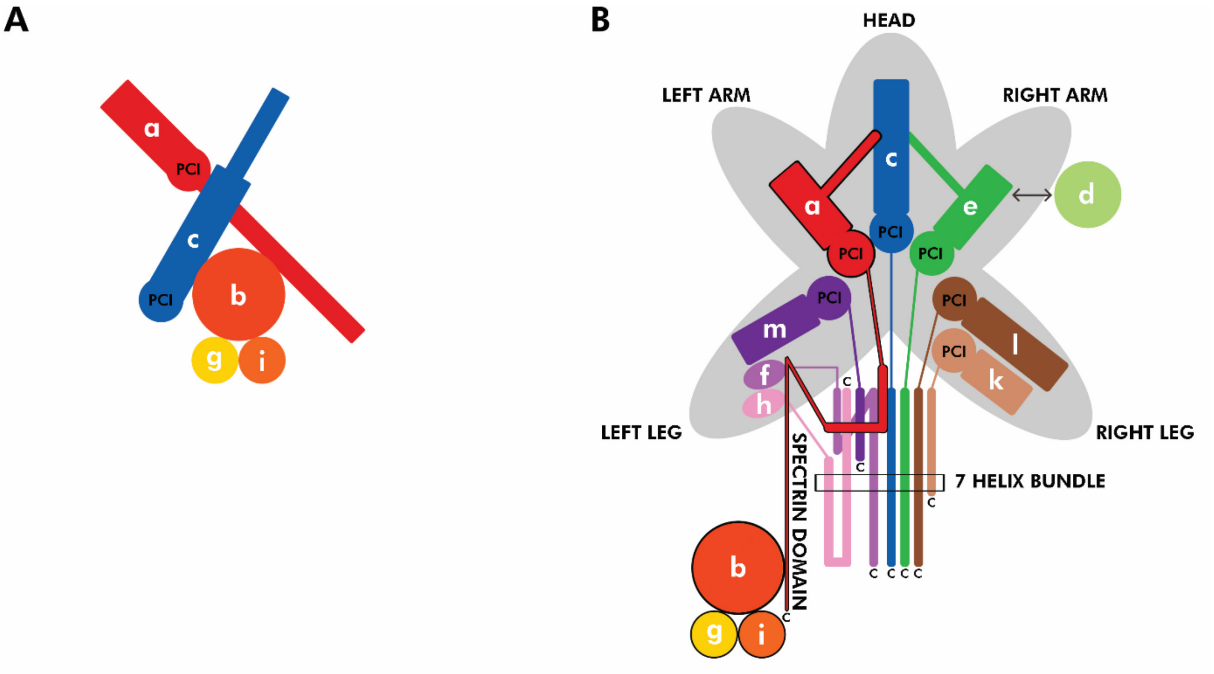

Figure 2: Schematic models depicting structural organization of the yeast and mammalian eIF3 complexes. **(A, B)** In *S. cerevisiae*, the eIF3 complex is formed by five subunits (A), whereas in mammals it consists of 12 subunits (B). These schematics illustrate both similarities as well as differences between budding yeast and mammalian eIF3. One of the main structural domains shared by several eIF3 subunits – the PCI (for Proteasome, COP9, Initiation factor 3) domain – is shown in bold in both panels. (B) The so-called octamer with its anthropomorphic shape is highlighted in grey.
Adopted from (Zeman et al., 2019)

(Szamecz et al., 2008; Munzarová et al., 2011; Mohammad et al., 2017). In addition to these roles in the general translation initiation mechanism, eIF3 has roles in selective mRNA translation initiation as well. Distinct groups of human mRNAs were found to bind eIF3 with their 5' UTR specific structural elements. These mRNAs encoding proteins involved in the cell cycle, differentiation or apoptosis are thus involved in eIF3-specific, cap-dependent activation or inhibition of translation initiation (Lee et al., 2015). In two later studies, eIF3d itself and eIF3l as part of the entire eIF3 complex were shown to be able to bind directly to 5' cap (Kumar et al., 2016; Lee et al., 2016). These results suggest that eIF3 could interact with numerous mRNAs coding for regulatory factors in an unusual way involving 5' cap and/or 5' UTR secondary structures and mediates their loading to the 43S PIC. In fact, such a case has been reported recently and authors estimate that this alternate form of cap-dependent translation initiation mediated by eIF3d is responsible for translation of up to 20% of cellular mRNAs (de la Parra et al., 2018). Another special type of translation initiation is an internal ribosomal entry site (IRES)-mediated initiation. In case of the cellular mRNA encoding the X-linked inhibitor of apoptosis (XIAP), binding of eIF3 together with PABP was shown to help the 40S

recruitment to XIAP IRES and promote IRES-mediated translation (Thakor et al., 2017). In the case of many viral IRESs, it was believed for a long time that eIF3 also promotes IRES-mediated translation (Otto and Puglisi, 2004). However, using cryo-EM, it was shown that Hepatitis C virus (HCV)-like IRESs actually displace eIF3 from its regular binding site on 40S and that eIF3 may in fact serve as an IRES-inhibitor (Hashem et al., 2013b), at least in this case. Due to the great diversity of so far identified IRESs, it is therefore conceivable that eIF3 have stimulatory effect on some classes but inhibitory on others.

In contrast with its given name, eIF3 was shown to participate not only in initiation but in virtually every step of translation (Figure 1) including termination and ribosomal recycling (Pisarev et al., 2007, 2010), stop codon readthrough (Beznosková et al., 2013), reinitiation (Park et al., 2001; Szamecz et al., 2008; Roy et al., 2010; Cuchalová et al., 2010; Munzarová et al., 2011; Mohammad et al., 2017), or nonsense-mediated decay (NMD) pathway (Morris et al., 2007; Isken et al., 2008; Choe et al., 2012; Flury et al., 2014). Three studies shedding light on roles of eIF3 in reinitiation and stop codon readthrough are part of this thesis.

This thesis is mainly focused on yeast eIF3, therefore only subunits of yeast eIF3 are described in details. Nevertheless, basic structural information about mammalian eIF3 is provided as well.

1.2.1 The a/Tif32 subunit

The largest subunit of yeast eIF3 is a/Tif32 with molecular weight of 110.3 kDa. Under the name Rpg1, it was originally characterized as a factor required for passage through the G1 phase of the cell cycle (Kovarik et al., 1998).

The N-terminal domain (NTD; amino acids 1-321) is formed by several alpha helices and is followed by the so-called PCI domain (amino acids 321-496) (Khoshnevis et al., 2014). The PCI domain is a structural motif shared by proteasome, COP9 signalosome, and eIF3. In all these protein complexes, PCI domains serve as a scaffold enablingbinding to each other and to other proteins (Aravind and Ponting, 1998; Hofmann and Bucher, 1998; Scheel and Hofmann, 2005). The N-terminal part of a/Tif32 binds the C-terminal domain (CTD) of c/Nip1 and ribosomal protein uS2 (Valášek et al., 2002; Kouba et al., 2012a; Khoshnevis et al., 2012). Truncation of the first 200 amino acid residues of a/Tif32 resulted in a reduction of MFC binding to 40S *in vivo* suggesting a very important role of a/Tif32-NTD in stabilizing the eIF3-40S complex (Valášek et al., 2003). Later, it was proposed that the N-terminal part of a/Tif32 bound to uS2 forms an extension of the mRNA exit channel since uS2 is located near this

channel (Munzarová et al., 2011). This is in agreement with the fact that both yeast a/Tif32 and mammalian eIF3a interact with mRNAs emerging from the mRNA exit channel (Pisarev et al., 2008; Aitken et al., 2016), which is in specific cases highly important for a gene-specific regulatory mechanism called reinitiation (REI) (Szamecz et al., 2008; Munzarová et al., 2011).

The C-terminal part of a/Tif32 contains the Hcr1-like domain (HLD, amino acids 625-869) that shares 25 % sequence identity with Hcr1. HLD interacts with Hcr1, b/Prt1, and eIF1 (Valášek et al., 2001, 2002). The very C-terminal end of a/Tif32 interacts with eIF2 (Valášek et al., 2002), ribosomal proteins uS3 and uS5 (Chiu et al., 2010), and helices 16-18 of the 18S rRNA (Valášek et al., 2003). These ribosomal proteins and helices form the mRNA entry channel, so it is not surprising that the a/Tif32-CTD was shown to promote mRNA recruitment, scanning, and influence the start codon selection (Chiu et al., 2010).

1.2.2 The b/Prt1 subunit

The third largest subunit of yeast eIF3 is b/Prt1, an 88.1 kDa protein. The DNA fragment containing *PRT1* was isolated and cloned as a first gene required for the initiation of protein biosynthesis in *Saccharomyces cerevisiae* by complementation of the temperature-sensitive *prtl-1* mutation (Keierleber et al., 1986).

The N-terminal part of b/Prt1 contains an RNA recognition motif (RRM, amino acids 71-166) that mediates interactions with a/Tif32-HLD, Hcr1-NTD, and 40S. The precise binding site on 40S is not known yet (Valášek et al., 2001; ElAntak et al., 2007, 2010; Chiu et al., 2010).

The central part of b/Prt1 was originally believed to be composed of fourteen WD40 repeats folded into two seven-bladed β-propellers (Marintchev and Wagner, 2004) until a crystal structure from *Chaetomium thermophilum* revealed that this propeller is unusually formed by nine WD40 repeats and this unique architecture is common to all eIF3b orthologues (Liu et al., 2014). Via its β-propeller domain, p/Prt1 binds to c/Nip1, ribosomal protein uS4, and the 18S rRNA helix h16 (Valášek et al., 2002; Liu et al., 2014).

The C-terminal part folds into a long α-helix (amino acids 691-729) with unstructured C-terminal tail and is required for binding of i/Tif34 and g/Tif35 (Asano et al., 1998; Herrmannová et al., 2012). Mutation of residues mediating the interaction with i/Tif34 eliminates also g/Tif35 from MFC *in vivo* and leads to severe leaky scanning phenotype caused by the presence of aberrant PICs (Herrmannová et al., 2012).

1.2.3 The c/Nip1 subunit

The second largest subunit of yeast eIF3 is c/Nip1 with molecular weight of 93.2 kDa. Since it was discovered during a study screening for temperature-sensitive mutants manifesting defects in nuclear import, it was named Nip1 which stands for Nuclear import 1 (Gu et al., 1992).

The N-terminal part of c/Nip1 contains binding sites for eIF5 and eIF1 and thus regulates AUG recognition and serves as a nucleation centre for MFC assembly and 43S PIC formation (Greenberg et al., 1998; Asano et al., 2000; Valášek et al., 2002, 2003; Kouba et al., 2012b; Karásková et al., 2012; Obayashi et al., 2017). Mutations in the c/Nip1-NTD impairing its contact with eIF1 are causing the so-called Sui$^-$ phenotype (Sui, suppressor of initiation codon mutation) which allows selection of near-cognate codons instead of AUG as a start codon (Valášek et al., 2004), whereas mutations impairing binding of eIF5 lead to defects in TC recruitment (Karásková et al., 2012). The following part of c/Nip1 contains a binding site for the PCI domain of a/Tif32 (Valášek et al., 2002, 2003).

The central part of c/Nip1 folds into several α-helices (amino acids 251-608) and contains a binding site for b/Prt1 (Valášek et al., 2002; Khoshnevis et al., 2012).

Immediately after this helical region is located the C-terminal PCI domain (amino acids 608-783). However, deletion of this PCI domain does not influence the integrity of eIF3 (Khoshnevis et al., 2012). The c/Nip1-PCI binds non-specifically to RNA and blades 1-3 of ribosomal protein Rack1 (Kouba et al., 2012b).

1.2.4 The i/Tif34 subunit

With the molecular weight of 38.7 kDa, i/Tif34 is the second smallest subunit of yeast eIF3. It was identified as an important factor in translation initiation, cell cycle progression, and yeast mating (Naranda et al., 1997; Verlhac et al., 1997). It adopts a canonical seven-bladed β-propeller structure with a short C-terminal α-helix. Binding partners of i/Tif34 are b/Prt1 via blades 5 and 6 (Herrmannová et al., 2012) and g/Tif35-NTD via blades 1, 6, and 7 (Erzberger et al., 2014). Mutation in blade 6 causes a slow scanning phenotype but has no effect on eIF3 integrity (Cuchalová et al., 2010).

1.2.5 The g/Tif35 subunit

The smallest subunit of yeast eIF3 is the 30.5 kDa protein g/Tif35.

The N-terminal third of g/Tif35 contains a zinc finger domain and is responsible for interaction with i/Tif34 and b/Prt1 (Asano et al., 1998). Otherwise weak binding of b/Prt1 to

g/Tif35 is stabilised by the presence of i/Tif34 (Herrmannová et al., 2012; Khoshnevis et al., 2012).

The C-terminal third folds into the RRM (amino acids 183-273) which non-specifically binds both rRNA and mRNA, but was shown to be not essential (Hanachi et al., 1999). The structure of human eIF3g-RRM was originally solved by K. Tsuda et al. (unpublished data, PDB accession code 2CQ0), and because there is a high sequence homology with yeast g/Tif35-RRM, it can be assumed that they share a common structure (Cuchalová et al., 2010).

The interactions of g/Tif35 with ribosomal proteins uS3 and uS10 were described and since are these proteins located near the ribosomal mRNA entry channel, this interactions are associated with roles of g/Tif35 in promoting reinitiation and scanning through stable secondary structures on mRNAs (Cuchalová et al., 2010; Aitken et al., 2016).

1.2.6 The loosely associated factor Hcr1

Hcr1 is a 29.5 kDa protein originally isolated as a high copy suppressor of the temperature-sensitive phenotype of the *rpg1-1* allele of *TIF32* (Valášek et al., 1999).

The N-terminal part of Hcr1 interacts with the RRM domain of b/Prt1 and its deletion causes a leaky scanning phenotype which can be suppressed by eIF1A overexpression (ElAntak et al., 2010). The C-terminal part of Hcr1 interacts with ribosomal proteins uS5 and uS12 located in the vicinity of the mRNA entry channel and with a/Tif32. Both parts of Hcr1 are required for binding to the a/Tif32-HLD (Chiu et al., 2010).

As already mentioned, Hcr1 is not considered as a subunit of eIF3 anymore because of its different roles as a separate factor, especially in translation termination. It binds to Rli1, a protein critically promoting translation termination and ribosomal recycling (Khoshnevis et al., 2010; Barthelme et al., 2011; Shoemaker and Green, 2011). In our laboratory, it was shown that deletion of *HCR1* increased termination codon read-through which was suppressible by overexpression of *RLI1*. Also, *RLI1* fully suppressed the slow growth phenotype of the hcr1Δ strains. Therefore, it was proposed that a defect in translation termination, and not initiation, is the major contributor to the slow growth phenotype of *hcr1* deletion strains (Beznosková et al., 2013).

1.3 Structure of free eIF3

Despite the fact that eIF3 was discovered many years ago, its structure was shrouded in mystery for a long time and not even today is fully known. Originally, contacts among individual subunits of yeast eIF3 were very thoroughly studied using pull-down assays, analytical size-exclusion chromatography (SEC), and *in vivo* mutations, while methods like nuclear magnetic resonance (NMR) spectroscopy and x-ray crystallography were applied later (Asano et al., 1998, 2000; Phan et al., 2001; Valášek et al., 2001, 2002, 2003; ElAntak et al., 2010; Herrmannová et al., 2012; Kouba et al., 2012b; Khoshnevis et al., 2014). The overall geometry of yeast eIF3 was described very well, but structures of only individual domains were solved (Figures 3 and 4C), in many cases just thanks to their homology with already resolved mammalian counterparts. Otherwise, there were no successful attempts to crystalize neither yeast nor mammalian eIF3 and only low-resolution single particle electron microscopy (EM) analyses of negatively stained samples were available (Figure 4A, B) (Behlke et al., 1986; Khoshnevis et al., 2012). The causes of this failures were ascribed to the great flexibility of eIF3 which is needed for its rearrangement in different roles and situations throughout the translation cycle. This flexibility is mostly provided by unstructured parts of individual subunits and rearrangement of the whole complex. Hence, the original purpose of my project was to overcome these issues using different methods and modified complexes in order to shed more light on the structure of free yeast eIF3.

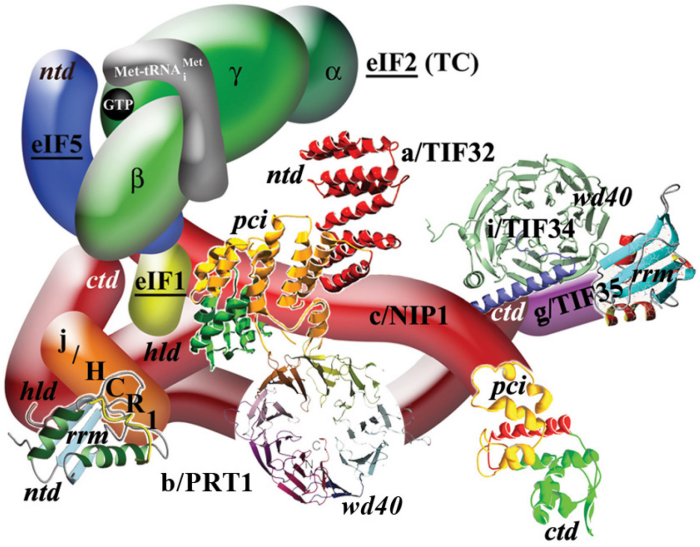

Figure 3: A 3D model of eIF3 and its associated eIFs in the multifactor complex (MFC)
A schematic model of MFC originally published by Valášek *et al.* (2002) supplemented with real protein structures of individual domains.
ntd, N-terminal domain; ctd, C-terminal domain; hld, HCR1-like domain; rrm, RNA recognition motif; pci, PCI domain; TC, ternary complex; wd40, β-propeller domain.

The assembly of yeast eIF3 as well as more complex eIF3 of mammals and *Neurospora crassa* most likely starts with the creation of the eIF3 nucleation core formed by eIF3a and

eIF3b subunits. They interact with each other via the N-terminal RRM of eIF3b and C-terminal spectrin domain (HLD in yeast) of eIF3a (Valášek et al., 2001, 2002; Khoshnevis et al., 2012; Dong et al., 2013). The very C-terminal part of eIF3b then binds eIF3g and eIF3i. In yeast, their mutual interactions fortify the so-called eIF3b-g-i module (Asano et al., 1998). In the case of mammalian eIF3, the spectrin domain of eIF3a plays a similar role in stabilizing the eIF3b-i interaction (Dong et al., 2013). In yeast, N-terminal PCI domain of a/Tif32 and C-terminal domain of b/Prt1 bind c/Nip1 to complete the whole protein complex assembly (Figure 2A) (Phan et al., 2001; Valášek et al., 2002; Khoshnevis et al., 2014). In mammals and other higher eukaryotes, six subunits containing the PCI domain (a, c, e, k, l, m) and two subunits containing the MPN (Mpr1-Pad1 N-terminal) domain (f, h) bind together in a horseshoe shape to form the so-called octamer (Figure 2B). *In vivo*, the formation of the eIF3a-b nucleation core is probably a prerequisite for the human octamer assembly (Wagner et al., 2016). Based on the research of our group, it seems that the formation of the yeast-like core (YLC) consisting of eIF3a-b-g-i subcomplex precedes the nucleation of the octamer as YLC was also shown to exist free in the cytoplasm of human cells (Wagner et al., 2014, 2016).

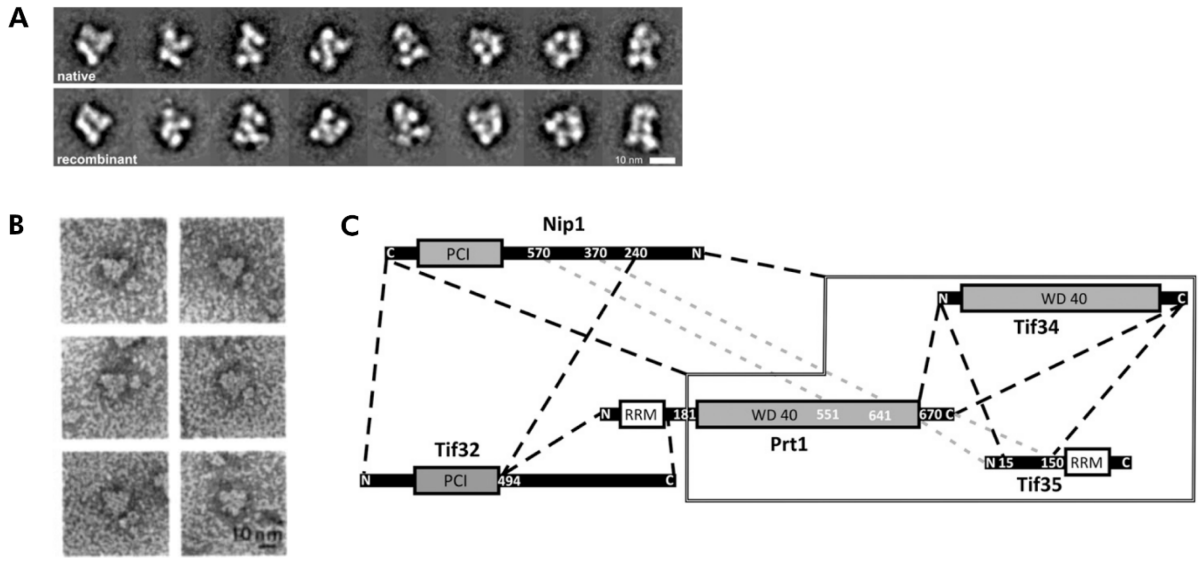

Figure 4: Structural information about free eIF3 complexes
(A) Single particle EM analysis of negatively stained native (upper row) and *in vitro* reconstituted (lower row) eIF3 complexes.
(B) Electron micrographs of negatively stained glutaraldehyde-fixed eIF3 from rat liver.
(C) Yeast eIF3 interaction map. Subcomplexes that are stable according to the SEC analysis are depicted by dashed lines. The interaction of c/Nip1 with b/Prt1-i/Tif34-g/Tif35 subcomplex is represented by a solid line around this subcomplex. Binding of $Nip1^{370-570}$ to $Prt1^{CTD}$ was observed by Valášek *et al.* (2002), while the binding of $Tif35^{CTD}$ to $Prt1^{CTD}$ was concluded from indirect observations. Therefore, hese two interactions are represented by the grey dashed lines.
Adapted from (Behlke et al., 1986 (B); Khoshnevis et al., 2012 (A, C))

1.4 Structure of eIF3 bound on 40S

Shortly after its discovery, mammalian eIF3 was shown to bind to the platform of 40S (Figure 5) (Emanuilov et al., 1978). The first 3D reconstruction at the resolution of 48 Å confirmed that eIF3 binds to the solvent-exposed side of the 40S platform, but also showed that it reaches the 40S intersubunit side as well (Srivastava et al., 1992). It took more than 20 years to obtain structures of mammalian 43S PIC in a resolution high enough to predict the position of the YLC subunits (Hashem et al., 2013a) and to even assign densities to the individual subunits (Des Georges et al., 2015). Thanks to these and other structural and biochemical studies, nowadays it is already well established that mammalian eIF3 body binds to the 40S solvent-exposed side and projects some of its domains onto the ribosomal intersubunit side. Subunits eIF3a and eIF3c contact ribosomal proteins eS1 and eS26, and uS15 and eS27, respectively, occurring near the mRNA exit channel. Near the mRNA entry channel, electron densities corresponding to the eIF3b-i module were observed in contact with uS4 (Hashem et al., 2013a; Erzberger et al., 2014; Des Georges et al., 2015). In comparison with the octamer anchoring eIF3 on 40S, the eIF3b-g-i module is rather mobile thanks to the C-terminal part of eIF3a which serves as a mechanical arm.

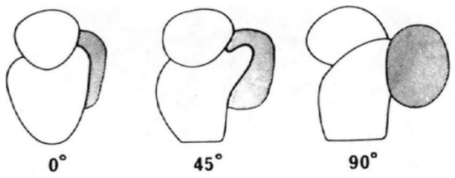

Figure 5: First model of the mammalian eIF3-40S complex
Schematic representation of the eIF3-40S complex isolated from rabbit reticulocytes based on electron microscopy images of negatively stained samples.
Adapted from (Emanuilov et al., 1978)

The integrative modelling of the yeast 40S-eIF1-eIF3 complex stabilised by cross-linking and the cryo-EM structure of also cross-linked yeast 40S-eIF1-eIF1A-eIF3-eIF3j complex showed that yeast eIF3 is embracing 40S in the same manner as the mammalian eIF3 does (Figure 6A-C) (Erzberger et al., 2014; Aylett et al., 2015). These results were confirmed by the structure of the uncross-linked partial yeast 48S PIC (Figure 6D) (Llácer et al., 2015). The eIF3a-eIF3c PCI heterodimer sits near the mRNA exit channel having the anchoring function. This structural information is in agreement with the already above mentioned biochemical experiments in yeast showing that eIF3a-NTD interacts with the ribosomal protein uS2 which is part of the mRNA exit channel (Valášek et al., 2003; Kouba et al., 2012a). The NTD of eIF3a also contacts specific mRNA elements that promote translation reinitiation and are located near the mRNA exit channel (Szamecz et al., 2008; Munzarová et al., 2011; Mohammad et al., 2017).

The eIF3b-i subcomplex represented by the RRM and both β-propellers was shown to be attached to the eIF3a-CTD with the eIF3g-NTD being sandwiched between the eIF3i β-propeller and the 40S body (Erzberger et al., 2014; Aylett et al., 2015). This position is in accord with a previous study identifying contacts between yeast g/Tif35 and uS3 and uS10 near or at the mRNA entry channel (Cuchalová et al., 2010). Identically to the mammalian eIF3 complex, this position of the eIF3b-g-i module is allowed by the flexible eIF3a-CTD. Contacts between the eIF3a-CTD and uS5, uS3, and helices 16-18 of 18S rRNA were mapped before already (Valášek et al., 2003; Chiu et al., 2010). It was also demonstrated that in this conformation, the eIF3b-g-i module extends the mRNA entry channel, interacts with incoming mRNA, and module the rate and processivity of scanning (Nielsen et al., 2004; Nielsen and Valášek, 2006; ElAntak et al., 2010; Chiu et al., 2010; Cuchalová et al., 2010). The important role of eIF3 extending both mRNA entry and exit channels was shown in a biophysical study. Alterations to the a/Tif32-CTD and the eIF3b-g-i module significantly slowed mRNA recruitment, and mutations in the eIF3b-g-i module destabilised binding of TC to the PIC. Alterations to the a/Tif32-NTD destabilised mRNA interactions with the PIC at the exit channel (Aitken et al., 2016).

The eIF3c-NTD was shown to mediate interactions with eIF1 and eIF5 in yeast (Phan et al., 1998; Asano et al., 2000; Valášek et al., 2004; Karásková et al., 2012) and proposed to coordinate AUG recognition via the contact with these eIFs (Valášek et al., 2003). Interactions between the eIF3c-NTD and eIF1, eIF2, and eIF5 within the PCI complex were proposed to enable rapid scanning arrest at the AUG codon by clearing eIF1 from the ribosomal P-site (Valášek et al., 2004; Karásková et al., 2012; Obayashi et al., 2017). This hypothesis seemed possible since eIF3 brings eIF1 to the PIC in form of MFC (Asano et al., 2000), but could only be true with the eIF3c-NTD stretching around the platform of 40S all the way to the 40S P-site. This mechanism was confirmed just recently by a cryo-EM study of yeast partial 43S and 48S PICs (Figure 7) (Llácer et al., 2018a, 2018b). During the formation of 43S PIC, TC binds in a metastable conformation (P_{OUT}) to the 40S. The head of 40S moves upwards upon mRNA recruitment and expands the mRNA entry channel. During this phase, the eIF3b-g-i module bound to the eIF3a-CTD relocates from its original position near the mRNA entry channel on the solvent side to the subunit interface, connecting eIF2γ and eIF1. Together with eIF2β contacting eIF1 and Met-tRNA$_i$, this arrangement probably stabilises the open (scanning-competent) conformation of the 48S PIC. After start codon recognition, the 40S head moves downward to secure mRNA in position, anticodon stem-loop of tRNA buries deeper in the P-site (P_{IN} state) and the contact between eIF2β, eIF1, and Met-tRNA$_i$ is lost. The N-terminal tail

(NTT) of eIF1A stabilises the AUG:anticodon duplex, and the D1 domain of eIF2α interacts with mRNA in the E-site. In addition, Met-tRNA$_i$ push eIF1 aside from its original binding site. Finally, the base pairing of the start codon with Met-tRNA$_i$ tilts Met-tRNA$_i$ towards the 40S body and weakens eIF1 binding to 40S causing eIF1 dissociation. Consequently, the eIF3b-g-i module was shown to relocate back to the solvent side of 40S and the eIF5-NTD binds at the side originally occupied by eIF1. Loop-1 and loop-2 residues of the eIF5-NTD then interact with the AUG:anticodon duplex and stabilise the conformation of Met-tRNA$_i$.

Moreover, mammalian eIF3 was proposed to interact also with eIF1A, eIF4B, and eIF4F via two binding sites on eIF4G (Méthot et al., 1996; Querol-Audi et al., 2013; Villa et al., 2013). This clearly shows how important eIF3 is during translation initiation and that its flexibility is essential to fulfil all these tasks.

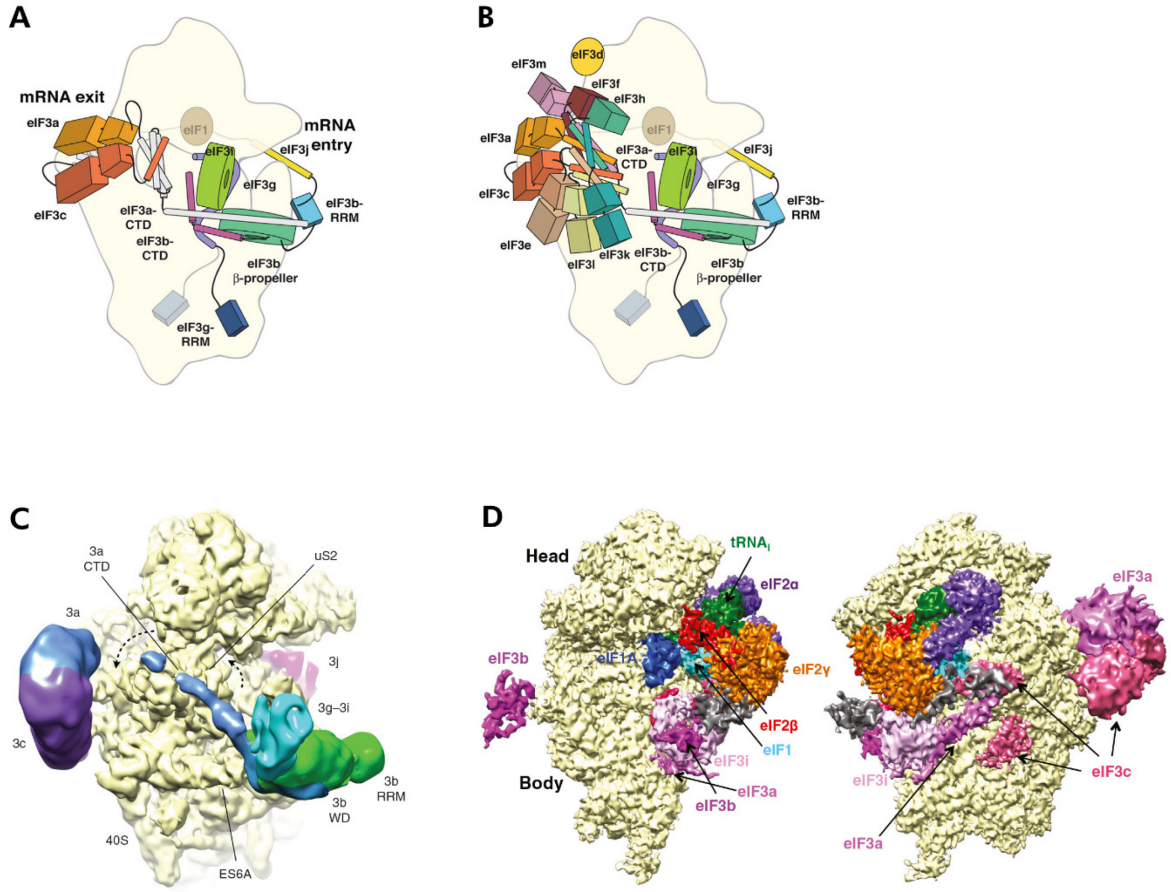

Figure 6: Models and cryo-EM structures of eIF3 bound on 40S

(A, B) Models of 40S-eIF1-eIF3 complexes in yeast (A) and mammals (B) based on cross-links and known structures.

(C) Structure of the budding yeast 40S-eIF1-eIF1A-eIF3-eIF3j complex viewed from the solvent side.

(D) The partial yeast 48S PIC in a closed state viewed from the beak side (left) and the intersubunit side (right), respectively.

Adapted from (Erzberger et al., 2014 (A,B); Aylett et al., 2015 (C); Llácer et al., 2015 (D))

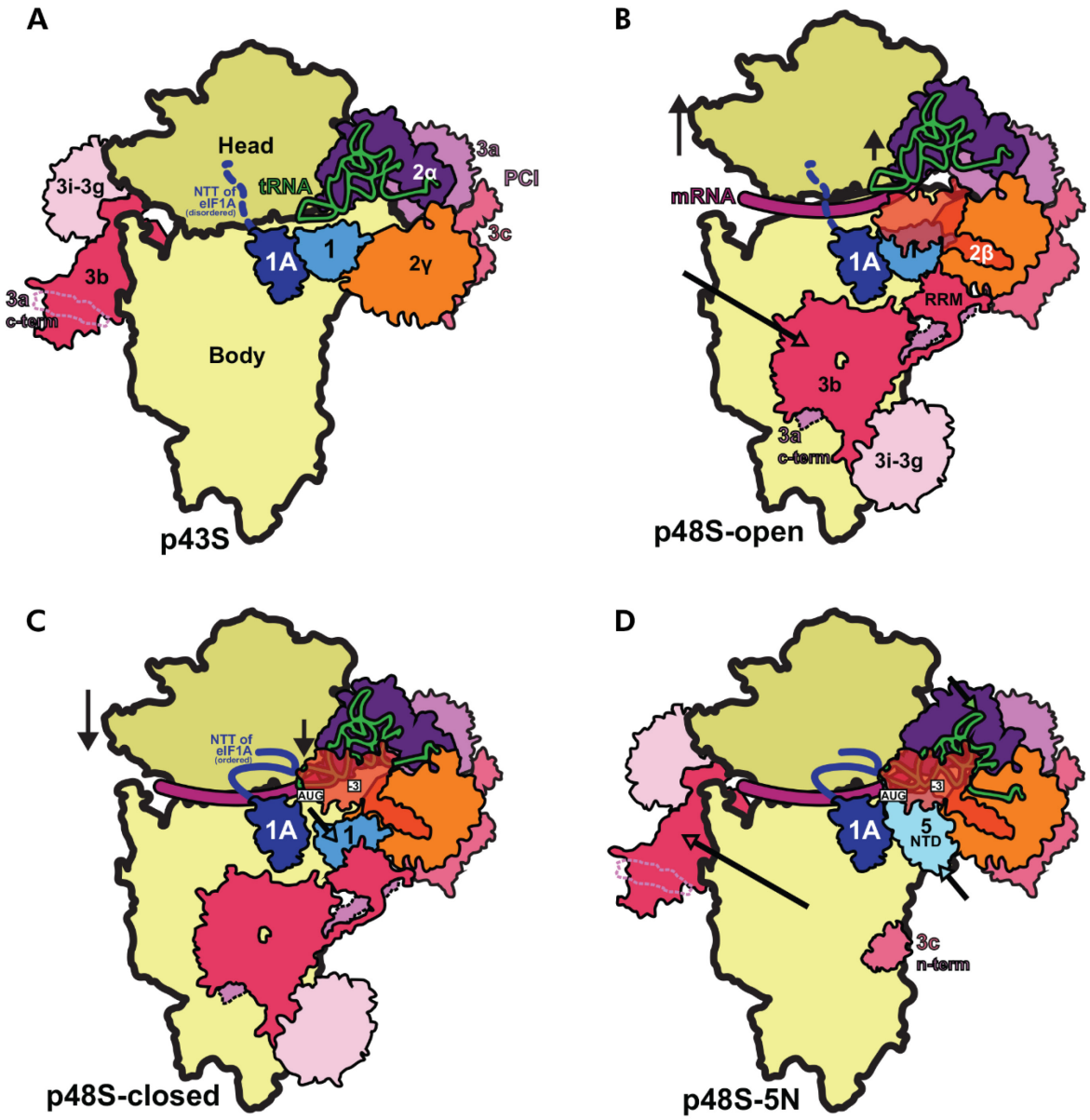

Figure 7: Schematic model of major conformational changes during initiation

(A) Formation of the 43S PIC (40S, eIF1, eIF1A, TC, and eIF3).

(B) Loading of mRNA, upward movement of the 40S head (black arrows), reposition of the eIF3b-g-i module connected to the eIF3-CTD to the subunit-interface (pink arrow).

(C) Head movement after start codon recognition, displacement of eIF1 (black and blue arrows, respectively).

(D) Base pairing of Met-tRNA$_i$ with the start codon tilts Met-tRNA$_i$ toward the 40S body (green arrow), relocation of the eIF3b-g-i module back to the solvent side of 40S (pink arrow), the eIF5-NTD binds at the site originally occupied by eIF1 (light cyan arrow).

NTT – N-terminal tail. For details, please see the main text.

Adapted from (Llácer et al., 2018b)

2 Aims of the study

The ultimate goal of my study was to solve the structure of yeast eIF3 and its position on 40S. To achieve this challenging task, I deployed different methods and optimised them to provide better results. In addition, I collaborated with my colleagues in order to shed more light on different roles of eIF3 besides initiation.

In particular, I aimed to:

A. modify purification protocol of eIF3 subunits and *in vitro* assembly of yeast eIF3 to produce diffractable crystals of eIF3 alone as well as bound on 80S,
B. optimize chemical cross-linking and preparation of samples for mass spectrometry in order to obtain structural data of free eIF3,
C. describe more precisely binding sites of yeast eIF3 on 40S,
D. explore the role of eIF1 and eIF5 in the structural rearrangement of yeast eIF3 preceding its binding to 40S,
E. determine the degree of eIF3 persistence on 80S during early elongation phase, and
F. elucidate the role of eIF3 in reinitiation and the effect of eIF3 and tRNAs in stop codon readthrough.

3 Materials and methods

DNA sequences encoding yeast proteins were cloned into vectors and expressed in *Escherichia coli* strains. Studies on yeast were performed using different laboratory strains of *Saccharomyces cerevisiae*.

3.1 List of methods

Protein purification (immobilized metal affinity chromatography, affinity chromatography, size exclusion chromatography)

Yeast and bacteria cultivation, transformation

Molecular cloning and DNA modifications

Ribosome purification

Chemical cross-linking of protein complexes

Mass spectrometry data analysis

3D modelling

Polyacrylamide gel electrophoresis

Western blot and dot blot analysis

Electrophoretic mobility shift assay

Sucrose gradient and sucrose cushion sedimentation

Pull-down assay

In-vitro transcription and translation

Structural bioinformatics and sequence analysis

4 Results

List of publications in reverse-chronological order:

Jakub Zeman, Yuzuru Itoh, Zdeněk Kukačka, Michal Rosůlek, Daniel Kavan, Tomáš Kouba, Myrte Jansen, Mahabub Pasha Mohammad, Petr Novák, Leoš Shivaya Valášek
Binding of eIF3 in complex with eIF5 and eIF1 to the 40S ribosomal subunit is accompanied by dramatic structural changes
Nucleic Acids Research 2019
doi: 10.1093/nar/gkz570
Contribution of the author: 80%; I purified all molecules, prepared samples for MS analysis, analysed MS data, and created 3D models.

Petra Beznosková, Zuzana Pavlíková, Jakub Zeman, Colin Aitken, Leoš Valášek
Yeast applied readthrough inducing system (YARIS): An in vivo assay for the comprehensive study of translational readthrough
Nucleic Acids Research 2019
doi: 10.1093/nar/gkz346
Contribution of the author: 20%; I was involved in experimental design and bioinformatics analysis of ribosomal structures and tRNA sequences.

Mahabub Pasha Mohammad, Vanda Munzarová Pondělíčková, Jakub Zeman, Stanislava Gunišová, Leoš Shivaya Valášek
In vivo evidence that eIF3 stays bound to ribosomes elongating and terminating on short upstream ORFs to promote reinitiation
Nucleic Acids Research 2017; 45 (5): 2658 2674.
doi: 10.1093/nar/gkx049
Contribution of the author: 15%; I contributed with sucrose cushion centrifugation and electrophoretic mobility shift assay experiments conducted as independent support for the results obtained by the newly developed and herein published pull-down assay.

Leoš Shivaya Valášek, Jakub Zeman, Susan Wagner, Petra Beznosková, Zuzana Pavlíková, Mahabub Pasha Mohammad, Vladislava Hronová, Anna Herrmannová, Yaser Hashem, Stanislava Gunišová
Embraced by eIF3: structural and functional insights into the roles of eIF3 across the translation cycle
Nucleic Acids Research 2017; 45 (19): 10948–10968.
doi: 10.1093/nar/gkx805
Contribution of the author: 20%; I contributed to the manuscript preparation, especially to chapters about eIF3 structure and assembly.

Vladislava Hronová, Mahabub Pasha Mohammad, Susan Wagner, Josef Pánek, Stanislava Gunišová, Jakub Zeman, Kristýna Poncová & Leoš Shivaya Valášek
Does eIF3 promote reinitiation after translation of short upstream ORFs also in mammalian cells?
RNA Biology 2017, 14:12, 1660-1667.
doi: 10.1080/15476286.2017.1353863
Contribution of the author: 7.5%; I contributed to the manuscript preparation.

I hereby confirm that the author of the thesis, Jakub Zeman, has substantially contributed to the publications listed above. In the case of his first-author publication, he performed the major part of experimental work and contributed to the manuscript preparation.

_____
Mgr. Leoš Shivaya Valášek, PhD

4.1 Publication I

Jakub Zeman, Yuzuru Itoh, Zdeněk Kukačka, Michal Rosůlek, Daniel Kavan, Tomáš Kouba, Myrte Jansen, Mahabub Pasha Mohammad, Petr Novák, Leoš Shivaya Valášek

Binding of eIF3 in complex with eIF5 and eIF1 to the 40S ribosomal subunit is accompanied by dramatic structural changes

In this study, we revealed an overall geometry of free yeast eIF3 and its structural changes during binding to 40S. To overcome known problems with forming crystals, we decided to use chemical cross-linking coupled to mass spectrometry. Using different cross-linkers, we were able to obtain information about distances between different parts of the eIF3 complex. We used these restraints to model free yeast eIF3. Applying the same methodology, we described the shape of eIF3 in complex with its binding partners, eIF1 and eIF5, and 40S.

As opposed to the compactly packed eIF3 when free in solution, eIF3 bound on 40S is dramatically rearranged and embraces almost the whole 40S. These results are in agreement with recently published structures of partial 48S PIC obtained by cryo-EM. In order to determine the involvement of other eIFs in triggering structural changes of eIF3, we applied the cross-linking approach to the partial MFC containing eIF3, eIF1, and eIF5. Our data indicate that eIF3 in this partial MFC is even more compactly packed suggesting that the contact with 40S is most probably coercing eIF3 to reorganization.

We also tried to exploit our methodology for determining the binding of eIF5 on 40S since the exact position of this factor was not fully mapped yet. Our results are in agreement with predicted binding sites of both eIF5 domains, but in addition support the prevailing idea of eIF5 being very flexible and contacting different ribosomal proteins.

4.2 Publication II

Petra Beznosková, Zuzana Pavlíková, Jakub Zeman, Colin Aitken, Leoš Valášek
Yeast applied readthrough inducing system (YARIS): an *in vivo* assay for the comprehensive study of translational readthrough

Based on observations of premature termination codons causing a plethora of human diseases, translation readthrough has emerged as a potential new therapeutic target. We identified and described a defined group of yeast tRNAs that, when overexpressed, induced translation readthrough in a stop codon tetranucleotide-specific manner. We refer to these tRNAs as readthrough-inducing tRNAs (rti-tRNAs). Rti-tRNAs are the keystones of herein established yeast applied readthrough inducing system (YARIS), a comprehensive reporter-based assay enabling simultaneous detection of readthrough levels at all twelve stop-codon tetranucleotides as a function of individual rti-tRNAs.

We proved the applicability of YARIS for the systematic study of translation readthrough by employing it for the interrogation of the effects of natural modifications of rti-tRNA, as well as various readthrough-inducing drugs (RTIDs). We determined how increased levels of specific rti-tRNAs interact with RTIDs. While some rti-tRNAs are relatively insensitive to RTIDs, others can be specifically enhanced by RTIDs and their readthrough promoting ability increased several fold. These specific effects may be promising for custom-tailored treatments targeted at inducing readthrough of specific premature termination codons. This analysis identified a variety of genetic interactions demonstrating the power of YARIS to characterize existing and identify novel RTIDs.

4.3 Publication III

Mahabub Pasha Mohammad, Vanda Munzarová Pondělíčková, Jakub Zeman, Stanislava Gunišová, Leoš Shivaya Valášek

In vivo evidence that eIF3 stays bound to ribosomes elongating and terminating on short upstream ORFs to promote reinitiation

Some uORFs are able to prevent recycling of the post-termination 40S in order to resume scanning and reinitiate downstream. It was shown that the time needed to translate an uORF is more critical than its length. This led to a hypothesis that some initiation factors needed for reinitiation are preserved on the 80S ribosome during early elongation. Here, we demonstrated that one of these factors is eIF3.

We developed a novel *in vivo* pull-down assay utilizing formaldehyde RNA-protein crosslinking, specific RNase H cleavage, immobilized metal affinity chromatography resin, and RT-qPCR. As a model, we used the yeast *GCN4* mRNA containing four short uORFs that is a textbook example of mRNA regulated by reinitiation. eIF3 but not eIF2 preferentially associated with RNA segments encompassing two *GCN4* reinitiation permissive uORFs (uORF1 and uORF2), containing *cis*-acting 5′ reinitiation-promoting elements (RPEs). We showed that the preferred association of eIF3 with these uORFs is dependent on intact RPEs and the a/Tif32 subunit and sharply declines with the extended length of uORFs. Thus, our data imply that eIF3 travels with early elongating ribosomes and that the RPEs interact with eIF3 in order to stabilize the mRNA-eIF3-40S post-termination complex to stimulate efficient reinitiation downstream.

4.4 Publication IV

Leoš Shivaya Valášek, Jakub Zeman, Susan Wagner, Petra Beznosková, Zuzana Pavlíková, Mahabub Pasha Mohammad, Vladislava Hronová, Anna Herrmannová, Yaser Hashem, Stanislava Gunišová

Embraced by eIF3: structural and functional insights into the roles of eIF3 across the translation cycle

In this comprehensive review, we provided an up-to-date knowledge about eIF3 from different perspectives. First, we described the structure of yeast and mammalian eIF3 complexes, their assembly, and binding sites on 40S. Then, we discussed all known roles of eIF3 in canonical and non-canonical translation initiation, translation termination, ribosomal recycling, stop codon readthrough, NMD pathway, and reinitiation. We also enlisted different human diseases including numerous types of cancer that are associated with deregulation or loss of functionality of eIF3.

Taken all together, this review article is a very valuable source of information about eIF3 from structural, functional, and medical points of view.

4.5 Publication V

Vladislava Hronová, Mahabub Pasha Mohammad, Susan Wagner, Josef Pánek, Stanislava Gunišová, Jakub Zeman, Kristýna Poncová & Leoš Shivaya Valášek

Does eIF3 promote reinitiation after translation of short upstream ORFs also in mammalian cells?

In this publication, we unravelled the molecular mechanism of reinitiation in human cells using human *ATF4* mRNA as a model. Atf4 is a functional homologue of yeast Gcn4, which we have previously used in our laboratory to understand the process of reinitiation in yeast cells. Both *GCN4* and *ATF4* mRNA molecules contain the REI-permissive uORF1. By targeted mutagenesis of the sequences flanking the *ATF4* uORF1, we discovered that these sequences are, analogously to yeast, REI-promoting as their mutation decreased the reinitiation efficiency in our reporter assay. These sequences independently enhance the REI capability of this uORF. Thanks to experiments based on bioinformatics predictions, we showed that the 5′ sequence of the *ATF4* uORF1 folds into an evolutionarily conserved structure necessary for its REI-permissive potential. Moreover, we proved that similarly to the yeast *GCN4* mRNA, eIF3 contributes to the reinitiation on human *ATF4* mRNA as well. Due to the higher complexity of the human eIF3, eIF3h and not a/Tif32 is responsible for this role.

Taking together, we proposed that the basics of the molecular mechanism of the reinitiation is well conserved in both yeast and humans.

5 Discussion

5.1 The modified yeast eIF3 purification and reconstitution protocol

There are always two possible basic protocols for the purification of protein complexes: to add a purification tag to at least one subunit and purify the entire complex from the original organism, or to express and isolate individual subunits and reconstitute the complex *in vitro*. As an expression system, either the original organism or different heterologous systems can be used. For the production of eukaryotic proteins, one should always consider the selection of an appropriate expression system based on organismal codon usage, amount of protein needed for downstream application, as well as the requirements for the maintenance of post-translational modifications or disulphide bonds. The selection of optimal expression conditions is critical for proper folding of functional proteins and, at the same time, serves to avoid the potential cross-species toxicity of produced molecules. The directly purified endogenous eIF3 has been usually contaminated with its binding partner or lacked the perfect stoichiometry of its subunits. (Asano et al., 1998; Phan et al., 1998; Sun et al., 2011).

To obtain eIF3 in the highest possible purity and to enable the process of further subunit mutagenesis, we originally decided to deploy a published protocol in which the eIF3 subunits were first individually produced in *E. coli* expression strains and subsequently assembled together *in vitro* (Khoshnevis et al., 2012). The authors shoved that eIF3 reconstituted *in vitro* from subunits expressed in bacteria has the same molecular mass, overall shape, and translation activity in an *in vitro* translation assay as native yeast eIF3. However, the original protocol led to the production of a mixture of full-length (FL) and truncated proteins as well as stable dimers of some subunits.

Our modifications described in Zeman et al. (2019) significantly improved the yields of eIF3 FL subunits, the ratios between FL proteins and partially cleaved subunits, and generally simplified the whole workflow. This protocol is now suitable for further eIF3 structural studies as well as different functional biochemical assays (Zeman et al., 2019).

5.2 Cross-linking versus high-resolution structural methods

Structural flexibility is a fundamental property of eIF3. It is required especially during binding to the ribosome to change the eIF3 conformation adequately in order to enable and stabilise its contacts between eIF3 subunits and ribosomal proteins and other eIFs. Nevertheless, flexibility has always been the biggest concern of all structural biologists, especially crystallographers. For this reasons, only one single particle EM analysis of negatively stained sample is available for free yeast eIF3 (Khoshnevis et al., 2012). Every other scientific group working on the structure of eIF3 has rather chosen to stabilise eIF3 as much as possible by its binding to 40S.

Our original intention was to obtain crystals of free eIF3 or of eIF3 in complex with 80S since, to our knowledge, eIF3 binds more strongly to 80S than to 40S (unpublished observations). We established a collaboration with the laboratory of Marat Yusupov (France) whose work on ribosomal structures is very well known. When our initial attempts with native complexes failed, I prepared modified eIF3 without flexible unstructured parts and eIF3 complexes containing one subunit fused with specifically chosen protein to increase the efficiency of binding to 80S. We designed fusion proteins containing Stm1 protein sequence fused to the N-terminus of b/Prt1, i/Tif34, or j/Hcr1. Stm1 was shown to be a ribosome-associated protein important for protein synthesis (Van Dyke et al., 2006). Therefore, we selected for modification those eIF3 subunits the presence of which was expected in the proximity of the binding site of Stm1 on 80S. The same idea led us to preparation of c/Nip1 C-terminally fused with the ribosomal protein Rack1 as it is already well known that the very C-terminus of c/Nip1 locates in the proximity of Rack1 when eIF3 binds to the ribosome (Kouba et al., 2012b). We then mixed modified eIF3-Rack1 with 80S lacking Rack1 protein to form a more stable complex. In all cases, these modified complexes were successfully assembled and showed high stability during SEC (data not shown). Unfortunately, even despite many attempts and hard work, we were only able to obtain small or needle-shaped crystals not suitable for x-ray crystallography.

As a next strategy, we decided to apply XL-MS. This method was previously succesfuly used to study macromolecular complexes like RNA polymerases I (Jennebach et al., 2012) and II (Chen et al., 2010), proteasome (Lasker et al., 2012; Karadzic et al., 2012; Kao et al., 2012) or membrane proteins (Jacobsen et al., 2006). Cross-linking constraints could be even used for solving protein structures and molecular dynamics simulations (Kahraman et al., 2013; Belsom et al., 2016; Brodie et al., 2017). However, potential concerns exist whether chemical cross-linkers could produce an artificial conformational change or oligomerization. A recent

study showed that chemical cross-linking can indeed affect protein function, in this case enzymatic activity, but does not significantly affect protein structure at low cross-linker concentrations (Rozbeský et al., 2018). Having this in mind, we first optimised the protein concentration and molar excess of cross-linkers in our samples to obtain enough cross-links and, at the same time, to avoid any unwanted oligomerization having impact on the structure. However, with the increasing total amount of amino acid residues per sample (adding other factors or 40S into reactions), we were forced to increase the amount of cross-linker as well. Nevertheless, using sucrose gradient centrifugation and dot blot analysis, we were able to prove that our samples were not oligomerized. In samples containing 40S, we also have to deal with the impact of a high amount of rRNA on MS analysis. Therefore, incorporation of treatment with RNase I after cross-linking was necessary. After optimisation of these variables, we were able to obtain high confidence cross-links.

We were also able to obtain good results using cleavable cross-linker DSBU (disuccinimidyl dibutyric urea), as characteristic patterns of the cross-linker as well as backbone fragments of the connected peptides are observable using collision-induced dissociation (CID)-MS/MS workflow (Arlt et al., 2016). This helped us greatly to distinguish more precisely between true-positive and false-positive cross-links. On the other hand, otherwise widely used so-called StageTips (manually prepared pipette tips containing very small disks made of beads with reversed phase, cation-exchange, or anion-exchange surfaces embedded in a Teflon mesh) which are supposed to enrich the number of cross-links in cleaved samples (Rappsilber et al., 2007) did not work in our hands (data not shown).

Our next effort was to use the obtained cross-links as constraints for modelling protein complexes using already known 3D structures of eIF3 subunits and domains with the help of Vojtěch Spiwok group (UCT, Prague). Unfortunately, due to high complexity and flexible/unstructured segments of eIF3 subunits, current protocols (Karaca et al., 2010; Kahraman et al., 2013; Van Zundert et al., 2016) were unable to give us any results. Because of that, we were compelled to use a general 3D modelling software to create simplified ‘sausage’ models (Zeman et al., 2019).

Some structural biologists consider XL-MS being not necessary and outdated in the age of x-ray crystallography and cryo-EM. But taken together, XL-MS is still a useful method for mapping protein complexes, their geometry, stoichiometry, and dynamics, and can be used in combination with other structural methods (reviewed in Urlaub, 2017). In addition, cross-linkers alone are used for stabilisation of flexible proteins and complexes in cryo-EM samples as well (Aylett et al., 2015; Engel et al., 2016).

5.3 Structural and functional implications of yeast eIF3 geometry

First, it is necessary to perform a thought experiment on how amounts of free eIF3 and eIF3 in complex with its binding partners differ in a yeast cell. Based on a meta-analysis of 21 protein abundance analyses utilising MS, GFP-microscopy, and tandem affinity purification (TAP)-immunoblot, median values for *S. cerevisiae* eIF3 subunits abundance are between 12 175 (Tif35) and 28 010 (Tif32) molecules per cell (Table 1). Values for eIF1 and eIF5 are 17 154 and 30 676, respectively (Ho et al., 2018). In theory, there should be enough of eIF1 and eIF5 molecules to saturate all eIF3 complexes in cell (assuming that the total number of eIF3 complexes is defined by the lowest abundant subunit) and those eIF3 not bound on ribosomes should probably exist in the form of (partial) MFC. In light of this these theoretical conclusions, our effort to obtain the structure of free eIF3 could be seen as futile, especially when the general flexibility of eIF3 and its contamination with other factors after purification directly from cells were already mentioned many times before. However, when this project started several years ago, almost nothing was known about the structure of neither free eIF3, MFC, nor the eIF3-40S complex. In that time, crystallisation of eIF3 or obtaining at least some structural information about free eIF3 was the ultimate goal of many groups.

Table 1: The abundance of selected proteins in *S. cerevisiae* cells according to (Ho et al., 2018).

Protein	Mean molecules per cell	Median molecules per cell
3a/Tif32	30 782	28 010
3b/Prt1	26 807	26 937
3c/Nip1	29 536	27 447
3i/Tif34	18 518	17 836
3g/Tif35	13 037	12 175
eIF1/Sui1	23 223	17 154
eIF5/Tif5	31 136	30 676

As our study as well as cryo-EM studies showed, there is a dramatic structural rearrangement when eIF3 binds to the 40S (Erzberger et al., 2014; Aylett et al., 2015; Llácer et al., 2015, 2018b; Zeman et al., 2019), but only small structural changes occur when eIF1 and eIF5 bind to eIF3 (Zeman et al., 2019). Because of the lack of indication of any steric clashes, there is apparently no need for a huge rearrangement of the eIF3 geometry prior to the binding of eIF1 and. In view of this fact, we concluded that the contact between eIF3 and 40S is the ultimate trigger of those dramatic structural changes. Moreover, our hypothesis that all important parts of eIF3 are in this conformation perfectly accessible for the initial contact with

ribosomal proteins corresponds with already proposed most probable mediators of this initial interactions (a/Tif32-NTD–uS2 and c/Nip1-CTD–RACK1) (Fraser et al., 2007; Mitchell et al., 2010) as well as with a subset of our cross-links of the eIF3-40S complex (Zeman et al., 2019).

Our models of eIF3 either free or in complex with eIF1 and eIF5 are in striking contrast with the structures of eIF3 embracing 40S published in recent years (Erzberger et al., 2014; Aylett et al., 2015; Llácer et al., 2015, 2018b). However, even from low-resolution EM images it is obvious that yeast eIF3 adopts roughly globular shape stretched in one plane (Khoshnevis et al., 2012). In order to further confirm this globular shape conformation, small-angle X-ray scattering (SAXS) or combination of size exclusion chromatography with multi-angle light scattering (SEC-MALS) analysis could be used.

Besides this just described globular geometry of eIF3, there is another important result of our cross-linking study. Many years ago, Leoš Valášek proposed in his articles that there is another a/Tif32 binding site on c/Nip1 roughly between amino acid residues 157 and 370 (Valášek et al., 2002, 2003) in addition to the PCI-PCI contact between these two subunits (Khoshnevis et al., 2012; Erzberger et al., 2014). This binding site was mapped using *in vitro* and *in vivo* pull-down assays with mutated variants of c/Nip1 subunit. Since then, structures of PCI-PCI heterodimers or whole eIF3 bound to 40S were published and all these studies only captured a/Tif32 binding to c/Nip1 via their PCI domains (Erzberger et al., 2014; Aylett et al., 2015; Llácer et al., 2015). This is in perfect agreement with our results showing eIF3 rearrangement after binding on 40S, however, our cross-links also support the existence of the second a/Tif32 binding site in the first third of c/Nip1. This binding site is most probably used in free eIF3 where three main subunits a/Tif32-b/Prt1-c/Nip1 forms a triangle and possibly for some regulatory reasons keep both PCI domains apart. We propose that the rearrangement of a/Tif32 and c/Nip1 subunits after the initial contact between eIF3 and 40S enables anchoring of eIF3 to 40S via the PCI-PCI heterodimer, movement of eIF3b-g-i module and binding and wrapping of c/Nip1-NTD around the 40S platform to reach the P-site. To test this hypothesis, methods like single-molecule fluorescence resonance energy transfer (smFRET) could be employed.

5.4 Flexibility of yeast eIF3 bound on 40S

As was already described in chapter 1.4, the eIF3b-g-i module moves dramatically during translation initiation thanks to the CTD of a/Tif32 (Llácer et al., 2018a, 2018b). However, the part of a/Tif32 between the end of the PCI domain towards the C-terminus (amino acid residue

496) and the α-helix that is in contact with the β-propeller of b/Prt1 (from amino acid residue 814) was not visible in any structure to date. This problem is most probably caused by the movement of this a/Tif32 part on or above the surface of 40S. Using secondary structure prediction tools like PSIPRED (Figure 9) (Jones, 1999) or Jpred4 (data not shown) (Drozdetskiy et al., 2015), this whole part of a/Tif32 seems to be folded into individual α-helices. More interestingly, protein structure prediction tools Phyre2 (Kelley et al., 2015) and SWISS-MODEL (Waterhouse et al., 2018) predict that these α-helices are folded together in a coiled coil structural motif (Figure 8) (reviewed in Mason and Arndt, 2004). This could possibly implicate that the a/Tif32-CTD is folded in the coiled coil motif in 40S-free eIF3 and just after the rearrangement during the contact with 40S, this whole domain's fold is loosened to fulfil its role of a flexible mechanical arm.

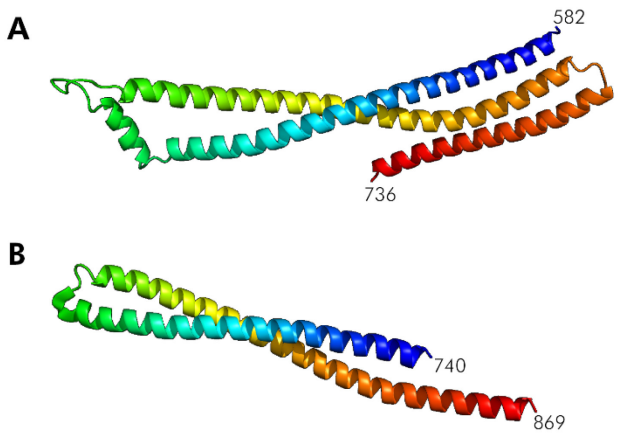

Figure 8: Predicted 3D structures of the a/Tif32-CTD
(A, B) Predicted coiled coil helices using Phyre2 (A) and SWISS-MODEL (B) protein structure prediction tools. Amino acid residues are numbered according to the sequence of a/Tif32.

Similarly, the c/Nip1-NTD is usually not resolved in cryo-EM structures of PICs (Aylett et al., 2015; Llácer et al., 2015, 2018b) with some exceptions of the very N-terminus. According to our cross-linking data, this part of yeast eIF3 is also very flexible and can move all the way between the head and the right foot of 40S. This could be in agreement with recently published structure, where the eIF3b-g-i module relocates in the 48S PIC to the area where the c/Nip1-NTD should otherwise bind (Llácer et al., 2018b). In theory, the c/Nip1-NTD could free the space for the eIF3b-g-i module, or is just possibly covered by the module.

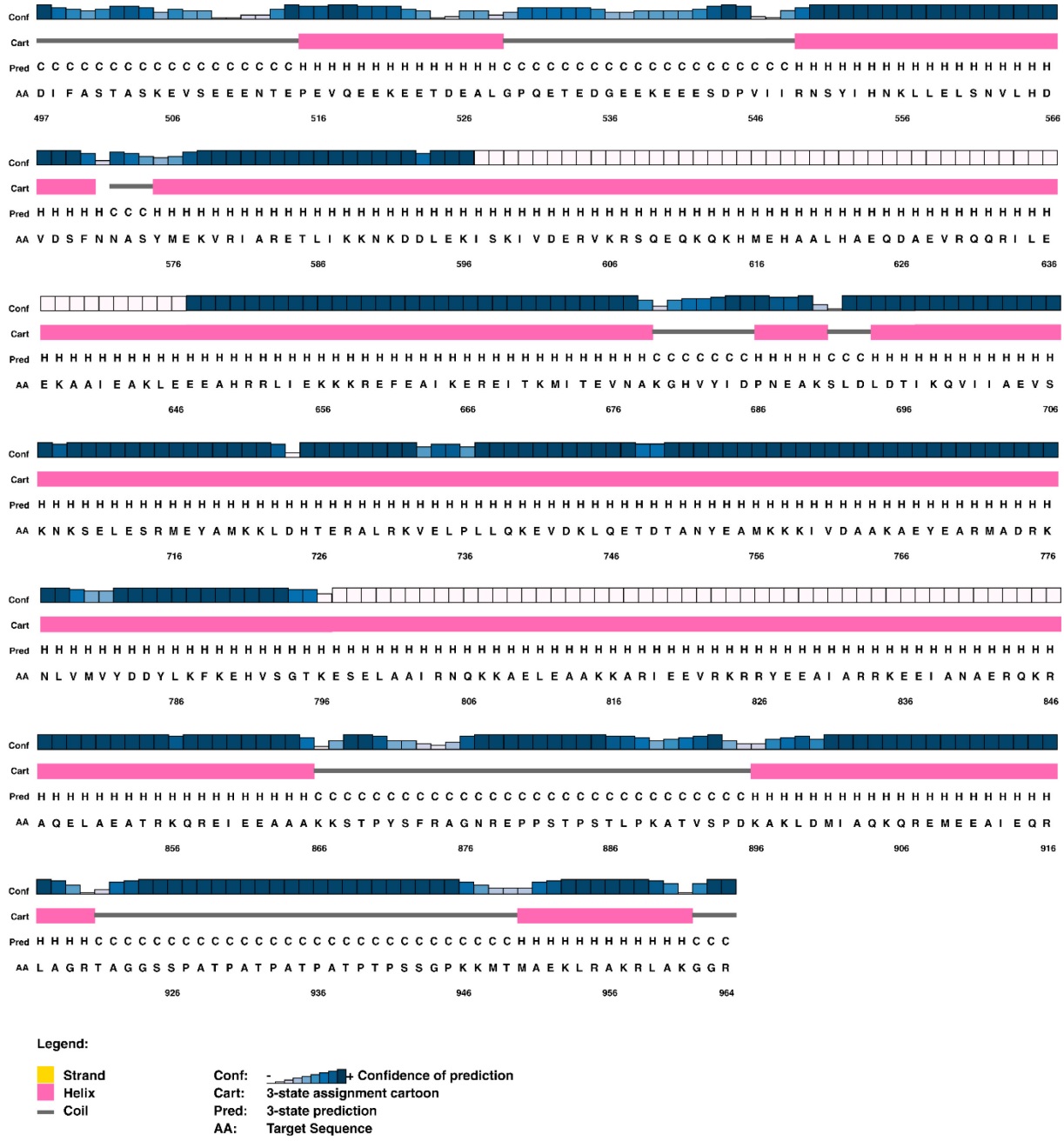

Figure 9: Secondary structure prediction of the a/Tif32-CTD
Cartoon representing the result of PSIPRED prediction tool for the CTD of a/Tif32 from the amino acid residue D497 to residue R964 (C-terminus) of the sequence of a/Tif32. Colour coding according to the legend.

5.5 The binding site of yeast eIF5 on 40S is still not fully known

Roles of eIF5 as a GTPase-activating protein and as a GDP dissociation inhibitor to prevent recycling of eIF2 were described years ago (Asano et al., 2000; Das et al., 2001; Singh et al., 2006) as well as structures of both of its domains (Conte et al., 2006; Wei et al., 2006).

However, its precise position on 40S remained a mystery for a long time and even nowadays is not completely known. Just recently, partial yeast 48S PIC cryo-EM maps containing clear density for eIF5-NTD were obtained (Llácer et al., 2018b) and to my best knowledge, structures containing also density for eIF5-CTD should be published soon.

Our effort to describe the binding site of eIF5 on 40S using our cross-linking methodology has brought some partial success. Our data are in agreement with the only recently published study which placed the eIF5-NTD near the P-site where eIF1 binds in the open state (Llácer et al., 2018b). According to our results, the eIF5-CTD seems to reside near the E-site. However, since the existence of sole eIF5-40S complex was not observed in cells and eIF5 is very flexible and, we obtained more than one binding site for each domain (Zeman et al., 2019). This could be probably also caused by the absence of other eIFs since their presence on 40S may possibly limit the eIF5 flexibility and restrict its binding area on ribosome.

5.6 The presence of eIF3 on 80S during early elongation

Our novel *in vivo* Rap-NiP assay brought more direct evidence that yeast eIF3 stays bound on 80S during first few elongation cycles after initiation on uORFs, as was already proposed earlier (Szamecz et al., 2008; Beznosková et al., 2013; Mohammad et al., 2017). Even before it was described that the cauliflower mosaic virus transactivator (TAV) is associated with polysomes on viral polycistronic RNA and recruits eIF3 to 80S to promote reinitiation on the downstream cistron (Park et al., 2001).

From a structural point of view, both position and mechanism of action of eIF3 bound on 80S during early elongation steps are very interesting. Because in the elongation ribosomes 60S is bound to the intersubunit side of 40S, the steric clashes between 60S and the very N-terminal parts of c/Nip1 and b/Prt1 (RRM) protruding into the interphase area might disrupt or at least weakens the interactions of these subunits with 40S as well as with other eIF3 subunits (possibly helical region of c/Nip1 and a/Tif32-NTD). This weakened interaction could then lead to gradual dissociation of eIF3 from the 80S during prolonged elongation, which was confirmed by our results using constructs with systematically extended coding regions of tested uORFs (Mohammad et al., 2017). However, because eIF3 was shown to stay bound on 80S during early elongation even without the presence of stabilising RPEs (Mohammad et al., 2017), this shortly persisting interaction could be possibly supported via its contact with other factor(s) present on early elongating 80S, possibly eIF4F or eIF4G that were previously reported to be required for efficient reinitiation (Pöyry et al., 2004). For a deeper look into behaviour of eIF3 on 80S,

methods like smFRET or cry-EM could be used. With the latter method, a proper way how to stabilise this complex would need to be found.

5.7 Limitations and directions for the future research of eIF3

In years after its discovery in the 1970s, eIF3 was shown to participate not only as a key player in translation initiation but also in termination, ribosomal recycling, stop codon readthrough, reinitiation and NMD pathway. Moreover, this list is most probably far from being complete, especially considering the potentially distinctive roles of individual eIF3 subunits or its subcomplexes in higher eukaryotes (reviewed in Valášek et al., 2017). Despite their importance, the structures of both yeast as well as mammalian eIF3 were for a long time a mystery. And even though the cryo-EM instrumentation and data analysis are improving rapidly, there is still a lot to learn about the structure of eIF3. Other methods like XL-MS, smFRET, pull-down assays, NMR, and x-ray crystallography also greatly helped with elucidating of particular steps of how eIF3 works. However, it will be for sure the cryo-EM that will further help us to understand the particular structures and mechanisms of eIF3 in different complexes and situations. Although we already know a lot about 43S and 48S PICs, there are still other equally important areas to explore: position of eIF4F on PIC during initiation and its possible contact with eIF3 or other factors, the structure of eIF3 on early elongating 80S, during reinitiation, as well as during readthrough, recycling etc. Nevertheless, all of those tasks are certainly highly challenging not only from the technical but also from the functional point of view – how to stabilise the required complex just in the right moment.

In recent years, almost all the attention in the field of translation has been naturally drawn to structural biology almost constantly providing us with new and more detailed structures. Still, we have to be very careful with drawing any conclusions and every new case of structural study that tries to convince us about some new ways of molecule functioning has to be taken under thorough investigation with the ultimate effort for a deeper understanding of the biology hidden behind that process.

6 Conclusions

This thesis brings novel information about yeast eIF3, its structural changes and its diverse roles not only in translation initiation.

We substantially optimised the yeast eIF3 purification and reconstitution protocol in order to produce higher yields of full-length proteins for further structural applications.

Even despite many attempts and modifications, our effort to obtain some crystallographic data of yeast eIF3 or eIF3 bound on 80S was not successful.

We succeeded in optimising the protocol for chemical cross-linking and sample preparation for mass spectrometry to acquire structural information about large protein complexes. We discovered that free yeast eIF3 adopts a globular architecture that is further compacted by the binding of eIF1 and eIF5.

Our results show that eIF3 undergoes a dramatic structural rearrangement during its binding to 40S from the compactly packed globular geometry with separated PCI domains to the stretched form almost completely embracing 40S with PCI-PCI heterodimer serving as an anchor.

Using to our newly developed *in vivo* assay, we demonstrated that eIF3 travels with early elongating ribosomes and that the RPEs interact with eIF3 in order to stabilize the mRNA-eIF3-40S post-termination complex to stimulate efficient reinitiation. Comparing our knowledge of reinitiation on uORFs of yeast *GCN4* mRNA with mammalian *ATF4* mRNA, we concluded that the molecular mechanism of the reinitiation is well conserved both in yeast and in humans.

Finally, we proved the applicability of the newly developed *in vivo* assay YARIS for the systematic study of translation readthrough. This process is specifically influenced by readthrough-inducing tRNAs and drugs. Further studies could identify new and more specific ways how to treat diseases caused by the presence of premature stop codons.

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