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**Mapping the contact points between eukaryotic
translation initiation factor eIF3 and the 40S
ribosomal subunit**

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**Mapování kontaktních míst mezi eukaryotickým
translačním iniciačním faktorem eIF3 a 40S
ribozomální podjednotkou**

Disertační práce

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ABBREVIATIONS

aa	amino acid residue
ASC1	C yp1 A bsence of growth S uppressor
A-site	aminoacyl-tRNA site
CTD	C-terminal domain
CTT	C-terminal tail
DT	doubling time
eIF	eukaryotic initiation factor
ES	expansion segment
FLAG	FLAG octapeptide DYKDDDDK
GAP	GTPase-activating
GST	glutathione S-transferase
HEAT	H untingtin, elongation factor 3 (E F3), protein phosphatase 2A (PP2 A) and the yeast kinase I OR1
IC	initiation complex
MAT	mating type locus
Met-tRNAi	initiator methionyl-tRNA ^{Met}
MFC	multifactor complex
NMR	nuclear magnetic resonance spectroscopy
NTD	N-terminal domain
MS	mass spectroscopy
ORF	open reading frame
P/M	polysome-to-monosome ratio
PABP	poly(A)-binding protein
PCI	26S p roteasome lid, COP9 signalosome and eIF3
Pi	inorganic phosphate
PIC	preinitiation complex
P-site	peptidyl-tRNA site
PTC	peptidyl transferase center
RACK1	r eceptor for a ctivated C -kinase
REI	reinitiation
RPS	small ribosomal subunit protein
RRM	RNA recognition motif
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
Slg-	slow growth phenotype
SNR	small nucleolar RNA
Sui-	s uppressor of initiation codon mutation phenotype
<i>T. thermophila</i>	<i>Tetrahymena thermophila</i>
TC	ternary complex
TREX	transcription-export complex
Ts-	temperature-sensitive phenotype
uORF	short upstream ORF
WCE	whole cell extract
WH	winged helix

ABSTRACT

Translation initiation in eukaryotes is a multistep process requiring the orchestrated interaction of several eukaryotic initiation factors (eIFs) together with the small ribosomal subunit to locate the mRNA's translational start and to properly decode the genetic message that it carries. The largest of these factors, eIF3, forms the scaffold for other initiation factors to promote their spatially coordinated placement on the ribosomal surface. It is our long-standing pursuit to map the 40S-binding site of the yeast multisubunit eIF3 and here we present three new mutual interactions between these two macromolecules (i) The C-terminal region of the eIF3c/NIP1 subunit is comprised of the conserved bipartite PCI domain and we show that a short C-terminal truncation and two clustered mutations directly disturbing the PCI domain produce lethal or slow growth phenotypes and significantly reduce amounts of 40S-bound eIF3 *in vivo*. The extreme C-terminus directly interacts with small subunit ribosomal protein RACK1/ASC1, which is a part of the 40S head, and, consistently, deletion of ASC1 impairs eIF3 association with ribosomes. The PCI domain *per se* shows strong but unspecific binding to RNA, for the first time implicating this protein fold in protein–RNA interactions. We conclude that the c/NIP1 C-terminal region forms an important intermolecular bridge between eIF3 and the 40S head region by contacting RACK1/ASC1 and most probably also 18S rRNA. (ii) The interaction between the N-terminal domain of the eIF3a/TIF32 subunit of eIF3 and the small subunit ribosomal protein RPS0A was discovered previously, but here we analyzed this contact in detail and defined the residues 201-400 of a/TIF32 that specifically interact with a flexible extreme C-terminal tail (CTT) of RPS0A. Indeed, the conditional depletion and a viable CTT-less mutant of RPS0A inflict a marked drop in amounts of eIF3 and other eIFs associated with 40S subunits in the preinitiation complexes. (iii) The eIF3g/TIF35 subunit specifically interacts with RPS3 and RPS20 located near the ribosomal mRNA entry channel. These observations underscore the placement of the major body of the yeast eIF3 to the head, neck, beak and shoulder regions of the solvent-exposed side of the 40S subunit.

1. INTRODUCTION

Translation is the ultimate step of the central dogma of molecular biology providing the flow of genetic information in the cell. The process of translation can be divided into initiation, elongation, termination, and ribosome recycling [1].

Initiation of translation is the process of assembly of elongation-competent 80S ribosomes, in which the initiation codon of mRNA is base-paired with the anticodon loop of initiator tRNA in the ribosomal P-site. Compared to prokaryotes, where initiation relies on the Shine–Dalgarno sequence of the mRNA to locate the start codon and require just three initiation factors, the eukaryotic initiation is much more complex with respect to the number of factors involved and the fact that the start codon is in the majority of cases identified by a scanning mechanism (reviewed in [2]).

Most regulation of the translation process is exerted at the initiation phase [3] and in eukaryotes, this process is a complex stage of gene expression, allowing its rapid, reversible and spatial control.

1.1. Overview of the translation initiation pathway in yeast

The basic outline of the eukaryotic cap-dependent initiation pathway is presented in Figure 1. It requires the action of at least 12 eukaryotic initiation factors (eIFs), and comprises two steps: the formation of 48S initiation complexes with established codon–anticodon base-pairing in the P-site of the small ribosomal 40S subunits (Figure 2), and the joining of 48S complexes with 60S ribosomal subunits.

Identification of the initiation codon begins with binding of the ternary complex (TC) consisting of initiator methionyl-tRNA (Met-tRNA_i) and the GTP-bound form of eukaryotic initiation factor 2 (eIF2) [4,5] to the 40S subunit to form the 43S preinitiation complex (PIC). Binding of the TC to the 40S subunit is promoted by eIFs 1, 1A, 5 and the eIF3 (reviewed in [6]). eIFs 1, 5 and 3 are together with TC physically connected in a so called multifactor complex [7] (MFC, Figure 5), which occurs in yeast, mammals [8] and plants [9]. At least in the budding yeast the MFC enhances the formation and stability of the 43S PIC *in vivo* (reviewed in [6]). The 43S PIC binds to the mRNA near the 5′-7-

methylguanosine cap in a process facilitated by eIF3 [10], the poly(A)-binding protein (PABP), eIF4F and eIF4B [11-14]. The eIF4F cap-binding complex is comprised of the eIF4E, eIF4G, and eIF4A [15]. eIF4E binds to the m⁷GpppG 5' terminal cap structure of mRNA [16], eIF4G is a scaffold protein that harbors binding domains for PABP, eIF4E, eIF4A and binds mRNA [17-19]. eIF4A is an ATP-dependent RNA helicase and generates a single-stranded landing pad in the mRNA on which the 43S PIC can load [10,20]. RNA-binding activity of PABP, eIFs 4E and 4G itself enable eIF4G to promote formation of a stable, circular messenger ribonucleoprotein (mRNP), referred to as a "closed-loop" structure, via the cap and poly(A) tail sequences of mRNA [15]. Upon unwinding of the mRNA's 5' terminal secondary structure by eIF4A, the 43S preinitiation complex attaches to the capped 5' proximal region of mRNAs and thus forming 48S PIC. On most mRNAs, once bound near the cap, the 48S PIC scans [21,22] the mRNA leader for an AUG codon in a suitable (Kozak) sequence context [23]. eIF2 partially hydrolyzes its GTP with the help of the GTPase-activating (GAP) factor eIF5; however, prior to start codon recognition, the eIF1 prevents the release of the resultant phosphate ion (Pi) producing two, GTP and GDP•Pi-bound states of the eIF2 factor, possibly in equilibrium [24].

AUG recognition - base-pairing between the anticodon of Met-tRNA_i and the AUG in the peptidyl-tRNA (P) site of the 40S subunit causes arrest of the scanning PIC [25]. This triggers irreversible hydrolysis of GTP of the TC and formation of its GDP-bound state and gated phosphate (Pi). Subsequent release of the Pi from the 48S PIC is triggered by eIF1 [26] and controlled by eIF1A [27]. The PIC can now join the large ribosomal subunit with the help of GTP-bound eIF5B [28,29], upon which most eIFs are ejected with the exception of eIF1A and eIF3 [30,31]. Finally, GTP-hydrolysis on eIF5B triggers the release of eIF1A and eIF5B itself [29,32] from the ribosome and thus formed 80S initiation complex (IC) containing Met-tRNA_i base-paired to AUG in the P-site is ready to begin the elongation phase of protein synthesis. There is recent evidence that in yeast *S. cerevisiae* eIF3 remains 80S-bound for several rounds of elongation [31,33].

To begin a new translational cycle, separated ribosomal subunits have to be generated from those that have just finished - terminated translation of an mRNA in the „previous“ cycle. Also the GTP in TC is hydrolyzed to GDP during initiation, and thus

eIF2•GDP must be recycled to eIF2•GTP to be able to form TC again. Heteropentameric eIF2B complex catalyzes this guanine nucleotide exchange on eIF2 [34,35].

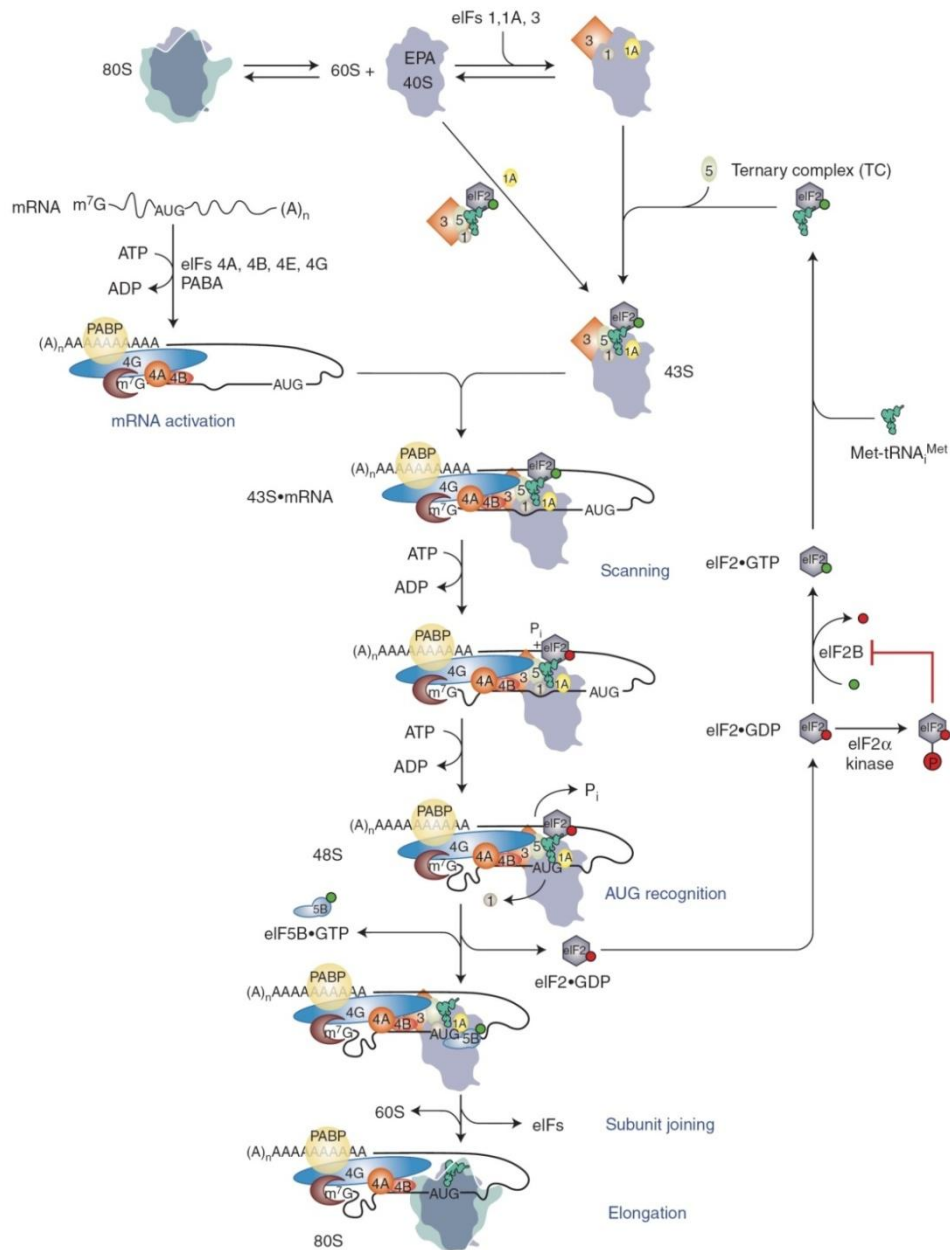


Figure 1: Model of canonical eukaryotic translation initiation pathway. The pathway is shown as a series of discrete steps starting with dissociation of 80S ribosomes into subunits. Binding of factors is depicted both as a single step via the MFC and as two separate steps, with eIFs 1, 1A, and 3 binding first followed by binding of ternary complex and eIF5. The resulting 43S PIC is then loaded onto an activated mRNP near the 5' cap. Subsequent scanning of the mRNA allows recognition of the start codon, which triggers downstream steps in the pathway including eIF1 release from the PIC, P_i release from eIF2, and conversion to the closed, scanning-arrested state of the complex. eIF2•GDP released after subunit joining is recycled back to eIF2•GTP by the exchange factor eIF2B. eIF5B in its GTP-bound form promotes joining of the 60S subunit to the preinitiation complex, which triggers release of eIF5B-GDP and eIF1A to form the final 80S initiation complex, which can begin the elongation phase of protein synthesis. Throughout, GTP is depicted as a green ball and GDP as a red ball. (Adapted from [2])

1.2. Eukaryotic 40S small ribosomal subunit

Recently, several crystal structures of the eukaryotic ribosome (Figure 2) were solved up to the resolution of 3.0 angstroms [36]. Regarding translation initiation, the small ribosomal subunit of *T. thermophila* was crystallized in complex with eIF1 [37]. Structure and position and of all 33 eukaryotic small subunit ribosomal proteins (RPSes) as well as the 18S rRNA and all its expansion segments were defined. The structure of the eukaryotic 40S subunit can be divided into the traditional hallmark features of the small bacterial ribosomal subunit, comprising the head, platform, body, beak, shoulder, right foot, and left foot, which are primarily defined by the fold of the 18S rRNA [38].

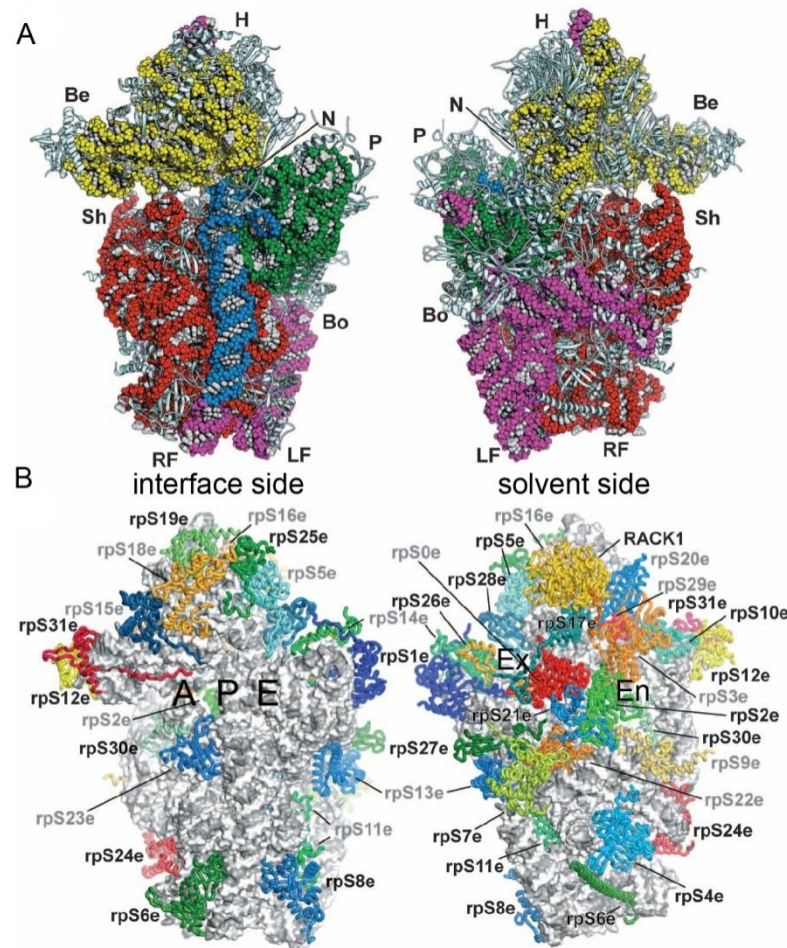


Figure 2: The crystal structure of the 40S subunit. (A) Interface and solvent-exposed views of the tertiary structure of the 40S showing the 18S rRNA as spheres and colored according to each domain (5' domain, red; central domain, green; 3' major domain, yellow; 3' minor domain, blue; ESs, magenta), and the proteins as gray cartoons (abbreviations: H, head; Be, beak; N, neck; P, platform; Sh, shoulder; Bo, body; RF, right foot; LF, left foot). (B) Ribosomal proteins of the 40S are shown as cartoons in individual colors; rRNA is shown as gray surface. (abbreviations: APE, tRNA A P and E site, En mRNA entry channel, Ex mRNA exit channel). The 40S is shown in the same orientation as in (A) (Adapted from [37]).

The increased complexity of eukaryotic protein synthesis is not only reflected in the initiation process, but also in the architecture of the ribosome itself [39]. The core functional regions of the peptidyl transferase center (PTC) at the large ribosomal subunit and the decoding site at the small ribosomal subunit are conserved among all domains of life. However, eukaryotic ribosomes are at least 40% larger than their bacterial counterparts due to additional rRNA elements called expansion segments (ESs), extra proteins and extensions of existing proteins (Figure 3). Moreover, some regions of the eukaryotic ribosome have been extensively remodeled.

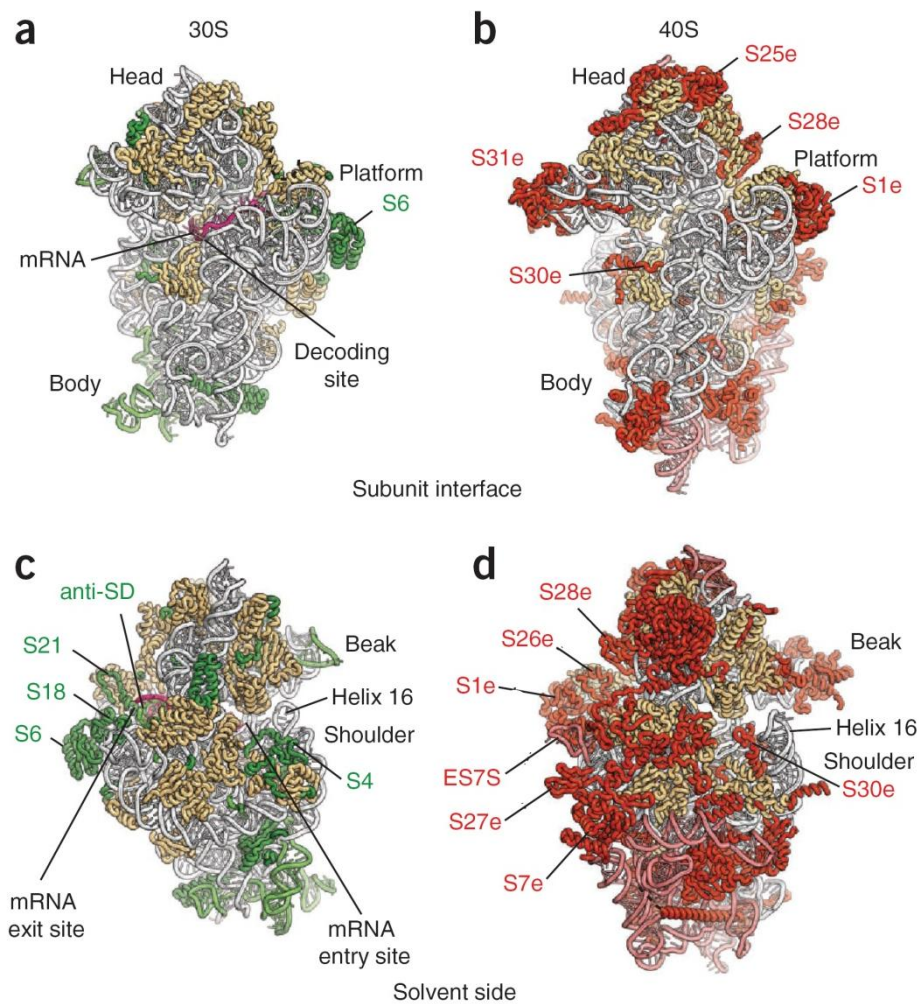


Figure 3: Difference between bacteria- and eukaryote-specific proteins and RNA expansions on the example of the small ribosomal subunit. (a–d) The common core is shown in white (rRNA) and light orange (proteins); bacteria-specific moieties of the 30S subunit (PDB accession numbers 3I8H and 3R8N) are shown in green (a,c), and eukaryote-specific moieties of the 40S subunit (PDB accession numbers 3U5B and 3U5C) are shown in red (b,d). For simplicity, only selected extensions and insertions in conserved proteins are labeled. The position of anti Shine–Dalgarno sequence of 16S rRNA is marked by anti-SD. (Adapted from [39])

Ribosome is not a rigid structure and it undergoes several conformational changes during translation initiation. For example, upon binding of eIF1 and eIF1A, it is thought to make a conformational switch to an “open” state [40,41], when the ribosome is capable of recruitment and scanning of the mRNA (Figure 4, see difference between Apo 40S and 40S-eIF1-eIF1A). In the open state, helix h16, which is situated directly below the beak (Figure 2A), is together with RPS3 involved in forming a connection between the head and the body of the 40S (Figure 4 by asterisk). And vice versa, the release of eIF1 (Figure 4, 40S-eIF1A) upon AUG recognition, results in conversion of the complex to the “closed”, scanning-arrested conformation. This process involves a movement of the ribosome head and formation of a virtual “latch” at the mRNA entry pore.

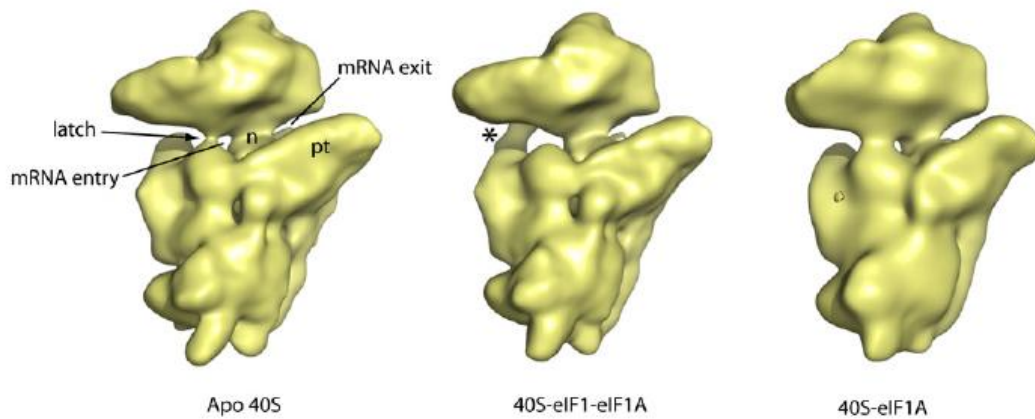


Figure 4: Cryo-EM reconstruction of a yeast interface side of 40S subunit alone (Apo), bound to eIF1 and eIF1A or eIF1A alone modulating the conformation of the “latch” at the mRNA entry channel; pt, platform; n, neck; *, connection between shoulder and head induced by eIF1/eIF1A binding. (Adapted from [40])

Several other protein components of the 40S subunit are probably directly involved in translation initiation. RPS3 and RPS30 are oriented towards the mRNA binding channel and could thus interact with the phosphate backbone of the mRNA [42]. The area corresponding to the bacterial Shine-Dalgarno chamber is partially occupied by the eukaryotic proteins RPS26 and RPS28 [37]. Transduction of some signaling pathway onto the ribosome occurs by RPS6 [37,43] and RPS33/RACK1/ASC1 ([44] and see later).

1.3. Yeast eukaryotic initiation factor 3 (eIF3)

eIF3 is the largest initiation factor (about 360 kD) and has been demonstrated to promote or at least fine tune nearly every single step of translation initiation. In yeast *S.*

cerevisiae eIF3 is composed of five core essential subunits: a/TIF32, b/PRT1, c/NIP1, i/TIF34, and g/TIF35; and one non-core subunit j/HCR1 (Figure 5) all of which have corresponding orthologs in complex of 13 nonidentical subunits (a–m) of mammalian eIF3. One study aimed to probe the subunit interactions within the human eIF3 complex identified three stable modules, one of which, composed of a, b, i, and g subunits [45], closely resembled the yeast eIF3 core.

There is accumulating evidence that eIF3 interacts primarily with the solvent-exposed “backside” of the 40S and that a few of its domains span the entry and exit pores of the mRNA-binding channel [22,30,46-48]. Yeast eIF3 also interacts with the mRNA by itself [10] and very likely at these locations [49].

1.3.1. eIF3 architecture

The network of mutual contacts among the yeast subunits has been mapped in great detail by both *in vivo* and *in vitro* techniques. The b/PRT1 subunit serves as the major scaffolding subunit of eIF3 making contacts with other core subunits [50]. The b/PRT1 N-terminal domain contains a conserved RNA recognition motif (RRM) [51], which provides an interaction surface for the C-terminal half of a/TIF32 called the HCR1-like domain (HLD) and the NTD of j/HCR1 [52,53]. The middle domain is predicted to fold into two WD-40 (Trp-Asp) β -propeller structures [54], the second of which contains binding the site for c/NIP1. The extreme CTD of the b/PRT1 scaffold forms an extended α -helix which is required for association with i/TIF34 and g/TIF35 subunits [50,55]. This interaction was later confirmed by observation that the C-terminal part of PRT1 (lacking its first 181 aa) forms stable subcomplex with TIF34 and TIF35 *in vitro* [56]. The aforementioned domain immediately preceding the i and g binding site provides a binding site for c/NIP1 and all NIP1-PRT1-TIF34-TIF35 can also form a stable subcomplex *in vitro* [56].

The N-terminal domain of c/NIP1 makes direct contacts with eIFs 1 and 5 and via the latter indirectly associates also with TC [7,57], thus serving as a nucleation center for the MFC formation. The following domain interacts with the PCI domain of a/TIF32 and indeed, a complex of N-terminal half of TIF32 and the C-terminal half of NIP1 was observed also *in vitro* [56]. Further down towards the C-terminus, c/NIP1 binds to b/PRT1,

which was also confirmed by existence of the aforementioned NIP1-PRT1-TIF34-TIF35 assembly. The very CTD of c/NIP1 is formed by a canonical PCI domain [58]. Together a, b and c subunits make a triangle-like network of interactions within eIF3 that is crucial for its integrity. In addition, a/TIF32 contributes to the integrity of the MFC by contacting the TC via its extreme C-terminus (which was proposed to protrude out of eIF3 complex [56]) and eIF1 via its HCR1-like domain.

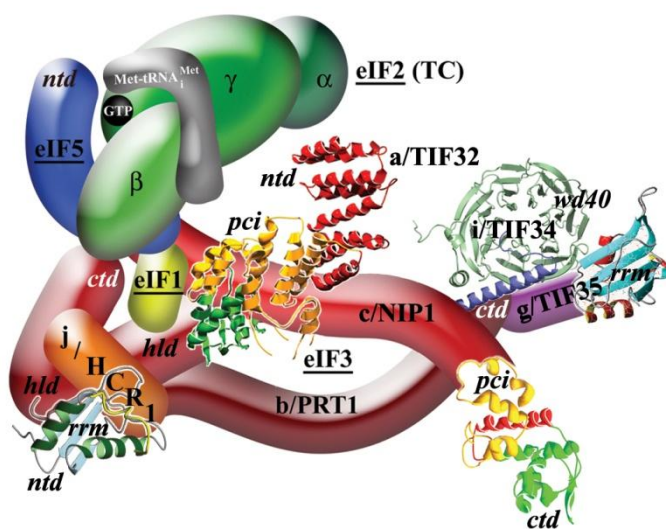


Figure 5: A model of eIF3 and its associated eIFs in the MFC (adapted from [55]). ntd, N-terminal domain; ctd, C-terminal domain; hld, HCR1-like domain; rrm, RNA recognition motif; pci, PCI domain; TC, ternary complex. The NMR structure of the interaction between the RRM of human eIF3b (green and light blue) and the N-terminal peptide of human eIF3j (yellow) [53], the NMR structure of the C-terminal RRM of human eIF3g (red and sky-blue, PDB accession code 2CQ0), the X-ray structure of the yeast i/TIF34 – b/PRT1-CTD complex [55], the 3D homology model of the c/NIP1-CTD and the 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.

i/TIF34 adopts a seven-bladed β -propeller structure made up of seven WD-40 repeats and interacts with the b/PRT1 via two contacts [55] which were identified in cocrystal structure of i/TIF34 and fragment of residues 654–700 of b/PRT1. g/TIF35 interacts strongly with i/TIF34 and weakly with b/PRT1 through its NTD containing a predicted Zn-finger domain by yet to be defined binding sites [50]. Even though many interactions among eIF3 are known and several structural studies of individual eIF3 subunits were published, a high-resolution structure of the entire eIF3 is an important goal to achieve to greatly extend our knowledge on this important topic.

1.3.2. eIF3 molecular function

As a part of a multifactor complex composed also of the TC and eIFs 1 and 5 [7], yeast eIF3 was shown to enhance the efficiency of the 43S and 48S PIC assembly processes and also to stimulate the post-assembly steps such as scanning and AUG recognition [22,46,48,53,57,59-61]. Recent findings also indicate a direct role for yeast eIF3 in 43S binding to capped, native mRNA *in vitro*, even more critical than that of eIF4F and eIF4B [10].

Mutations in a/TIF32, c/NIP1, eIF5 or eIF1 led to reduced 40S binding *in vivo* of not only the mutated factor but also of other MFC constituents [48,57]. Moreover, certain mutations in c/NIP1 [47], eIF5 [62], and eIF1 [26] that destabilize the MFC also reduced TC binding to the 40S subunit. This is in agreement with the fact that both a/TIF32-CTD and c/NIP1-NTD interact either directly or indirectly with eIF1, the CTD of eIF5 [7,57] and with the β -subunit of eIF2, at least off the ribosome. For example, the binding site of eIF1 in the eIF1–40S complex lacking all other eIFs was determined to be close to the ribosomal P-site [37,63] in the interface platform area (Figure 2). Hence, the eIF1 contact with the c/NIP1-NTD and a/TIF32-CTD, which bring eIF1 to the ribosome as a part of the MFC must be broken at a certain point of early initiation to allow eIF1 to relocate to the ribosomal P-site area (the distance between these two locations is expected not to allow physical presence at both positions at the same time). Until recently, it was not known what happens with eIF3 contacts with eIF2 and eIF5 on the ribosome. But recent analysis indicated that eIF2 β most probably faces the ribosomal A-site [64], and considering that the eIF5-CTD is known to interact with the N-terminal lysine-boxes of eIF2 β in the MFC [65], it could imply that the eIF5-CTD also occurs somewhere in this area. These findings could thus imply that the MFC-established contacts between c/NIP1-NTD–eIF5-CTD and a/TIF32-CTD–eIF2 β contacts could be preserved also in the scanning PICs and provide eIF3 with direct means to actively regulate scanning as well as AUG recognition [48,53,55,61].

Mutations in the extreme N-terminus of c/NIP1 produce so called Sui- phenotype indicating the importance of its interactions with eIF1 or eIF5 in stringent selection of AUG as the start codon [66]. The Sui- phenotype (suppressor of initiation codon

mutation) allows increased utilization of near-cognate codons (such as UUG or AUU) as start codons. Besides c/NIP1, j/HCR1 was shown to form together with the a/TIF32-CTD and the RRM of b/PRT1 an eIF3 subassembly that ensures stringency of the AUG start codon selection by preventing so called “leaky scanning” - skipping AUGs [46,53]. A robust leaky scanning phenotype was also observed with mutations disrupting the web of interactions among the b/PRT1-CTD and i/TIF34 and g/TIF35 [55]. Hence it appears that the scaffold b/PRT1 subunit serves to connect two eIF3 modules at each of its termini (a/TIF32-CTD–j/HCR1 at the N-terminal RRM, and i/TIF34–g/TIF35 at the C-terminal α -helix) that work together and with c/NIP1 [48] and other eIFs [67] to fine-tune the AUG selection process. Consistently, the very original *prt1-1* point mutation in b/PRT1 [61,68] and singlepoint substitutions in the conserved Lys-Glu-Arg-Arg (KERR) motif of the a/TIF32-HLD [61] as well as mutations in i/TIF34 [55] were shown to affect either the rate or processivity of ribosomal scanning, which may in turn impact the AUG selection process.

Besides playing these canonical roles in general translation initiation, eIF3 is also implicated in the gene-specific translational control mechanism termed reinitiation (REI) in yeast, plant, and mammalian cells [69-71]. REI is utilized to up- or downregulate translation of regulatory proteins, such as transcription factors, in response to various environmental stimuli [72]. In general, it relies on the ability of the small ribosomal subunit to remain attached to the mRNA after termination of translation on a short upstream ORF (uORF) in order to resume scanning on the same mRNA molecule [73]. The next critical step of REI is *de novo* recruitment of the TC, which is required to recognize the next AUG codon. Therefore, REI can be regulated and response to actual cellular levels of eIF2/GTP/Met-tRNAⁱ. The uORFs thus function as context-dependent cis regulators of translation. In particular, the extreme N-terminal 200 residues of a/TIF32 were found crucial for efficient reinitiation after translation of uORFs in yeast by contacting specific mRNA features that precede them [30,31].

1.3.3. eIF3 contacts with 40S ribosome

To better understand the molecular details of the eIF3 role in the 43S and 48S PIC assembly processes, we have begun a systematic mapping of the positions of specific

First, the C-terminal one-third, in particular the last 240 amino acid residues, of eIF3c/NIP1. This domain was shown not to be involved in the interaction network linking together eIF3 subunits and as such has no impact on the overall MFC integrity [57]. Nevertheless, the truncation of the last 240 residues in *nip1-ΔB'* (Figure 1A, Publication #1) is lethal, and, when expressed in the background of the wild type *NIP1* gene, the mutant form of eIF3 containing c/*nip1-ΔB'* could not compete with the wild-type eIF3 for binding to 40S ribosomes *in vivo* [47]. To eliminate the effect of competition and examine the true importance of the c/NIP1-CTD for the 43–48S PIC assembly we characterized this region more deeply and showed that it forms an important intermolecular bridge between eIF3 and the 40S by contacting small ribosomal protein RACK1/ASC1/RPS33 (designated as ASC1 henceforth) and probably also 18S rRNA segments of the solvent-exposed head region.

Second, it was previously found that the N-terminal domain (NTD) of a/TIF32 forms a crucial intermolecular bridge between eIF3 and the 40S by interacting with small ribosomal protein RPS0 [30,47], which occurs in the vicinity of the mRNA exit pore. However, the true physiological importance of this interaction remained to be corroborated by *in vivo* approaches. Here we bring this type of analysis for the a/TIF32-NTD–RPS0A interaction by analyzing the RPS0A contribution to the stable 40S-binding of eIF3.

Third, the interaction between the g/TIF35 subunit of eIF3 and the 40S beak proteins RPS3 and RPS20 is described here.

1.4. Selected eIF3 and 40S components described in this study

1.4.1. The C-terminus of the eIF3c/NIP1 subunit contains PCI domain

The c/NIP1-CTD is formed by the bipartite PCI domain that is known to serve as the principal scaffold for the 26S proteasome lid, COP9 signalosome (CSN) and eIF3 and this is also where the name PCI comes from (reviewed in [75,76]). The canonical version of these multisubunit assemblies, as found in multicellular organisms, comprises six subunits bearing the hallmark PCI domain and two subunits carrying an MPN (Mpr1-Pad1-N-

terminal) domain. Typical example is mammalian eIF3 which is composed of a canonical number of 6 PCI subunits a, c, e, k, l, m and two subunits f and h containing MPN domain.

The PCI domain is defined by a conserved arrangement of curved bihelical repeats, terminating in a globular winged helix (WH) subdomain (Figure 7) [76,77]. The WH domain is strongly conserved among PCI-proteins and has helix-turn-helix fold with an α - β - α - β - β topology, in which the β -strands form a short anti-parallel β -sheet on the protein surface. The N-terminal HD domains have a common structural theme, based on a right-handed superhelical arrangement of α -helices that share characteristics of both tetratricopeptide (TPR) and HEAT repeats and supports the WH.

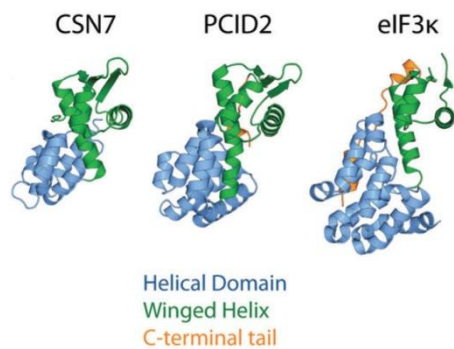


Figure 7: Crystal structure of PCI proteins COP9 signalosome subunit 7 (CSN7), PCID2 and mammalian eIF3 k subunit (eIF3k). PCI-proteins contain a bipartite fold consisting of a N-terminal helical domain (blue) and a C-terminal winged-helix domain (green). The C-terminal winged-helix domain often leads to a C-terminal tail (orange) that interacts with the surface of the helical domain. (Adapted from [76])

In the higher eukaryotic cells PCI subunits probably serve as structural scaffolds of the three parent complexes, thereby regulating proper complex assembly via interactions between each other as well as with other protein partners. The recent structure of transcription-export complex (TREX-2) heterodimer of PCI domains provides the first high-resolution view of the molecular nature of the interactions formed between PCI-proteins [78]. The interaction is based on insertion of an “interaction” helix of one WH domain into a cleft formed by the second WH domain and the C-terminal helical bundle of the HD domain. Moreover, this interaction pattern is compatible with recent progress on the electron microscopy structure of the 26S proteasome [79,80], where 6 PCI domains align laterally by WH domains in the same manner.

However, besides c/NIP1, yeast eIF3 contains only one additional PCI subunit in a/TIF32 and so yeast eIF3 does not manifest the typical PCI complex feature. Moreover, it was shown that deletion of the PCI domain in *nip1-ΔB'* did not influence the eIF3 integrity

and the direct interaction between PCI domains of a/TIF32 and c/NIP1 was tested but not observed [56]. Thus it seems that here the PCI domains do not contact each other and in this fashion do not provide a critical scaffolding interaction. This implicates that PCI domain in yeast might serve for a different function and/or could be considered as the evolutionary ancestor of the canonical PCI complexes.

WH motifs are well-documented mediators of DNA and RNA binding (reviewed in [81]), with multiple identified modes of nucleic acid interaction, and so it has been hypothesized that PCI-proteins or PCI-complexes could modulate functionally important interactions with nucleic acids. Here we present that PCI domain of c/NIP1 shows strong but unspecific binding to RNA as the first report ever implicating a PCI domain in direct RNA binding.

1.4.2. Ribosomal protein ASC1

The yeast ribosomal protein ASC1 and its mammalian ortholog RACK1 are both members of the WD-40 repeat scaffold protein family [82] that adopt a seven-bladed β -propeller structure (Figure 8). Originally, RACK1 was discovered independent of ribosome as a receptor for activated C-kinase (reviewed in [83]), and indeed it has homology to the β -subunit of heterotrimeric G proteins. ASC1 (**C**yp1 **A**bsence of growth **S**uppressor) was firstly identified as recessive extragenic suppressors of gene first identified as *CYP1* [84], which has as an essential function in anaerobic or heme-deficient growth. Subsequently, ASC1/RACK1 has been identified as a core ribosomal protein of the small ribosomal subunit [85] that is located on the head of the 40S ribosomal subunit above the mRNA exit tunnel, making contacts with helices h39 and h40 of 18S rRNA and ribosomal proteins RPS16, 17, and 3 [37,86]. This double face behavior suggested that ASC1/RACK1 might serve as a link between signaling or other regulatory pathways and translation [87-89]. This was indeed confirmed by the fact that mammalian RACK1 recruits activated protein kinase C to the ribosome, which leads to the stimulation of translation through the phosphorylation of eIF6 [90].

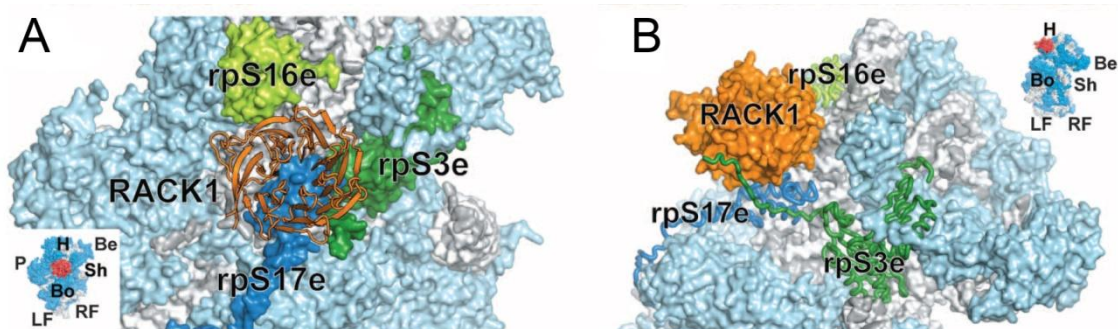


Figure 8: (A) Top view of ASC1/RACK1 at the ribosome. 18S rRNA is shown in surface representation in gray; proteins not involved in RACK1 binding are shown in surface representation in light blue. Proteins RPS3e, RPS16, and RPS17, which were found to interact with ASC1/RACK1, are shown in dark green, light green, and blue, respectively. **(B)** Side view of ASC1/RACK1 at the ribosome shows binding of the C terminus of RPS3 to ASC1/RACK1. Proteins RPS3, RPS16, and RPS17 are shown in dark green, light green, and blue tube representation, respectively; ASC1/RACK1 is shown as orange surface. Insets in **A** and **B** show the overall view of the 40S in the same orientation as the magnified images. (Adapted from [37])

Yeast ASC1 was also shown to interact with a number of signaling molecules on and off the ribosome [91-93], but it is important to note that given the extent of the contacts between ASC1 and the ribosome, it is likely that it associates very tightly with the ribosome and so that the pool of free ASC1, that exists in the cytoplasm, comes from an extra amount of ASC1 that is made, rather than from its mass released off from the ribosomes. Notably, rRNA and ribosomal proteins obscure a large part of the ASC1 surface in the 40S structure [37], thus inclusion into the 40S subunit may alter the properties of ASC1 in signaling. In any case, ribosome bound ASC1 is without a doubt instrumental for docking interacting partners to the ribosomes or docking ribosome itself to sites where local translation is required [94].

It should be stressed that ASC1 gene contains an intron encoding a small nucleolar RNA U24 (*SNR24*) [84] that plays a critical role in biogenesis of the large ribosomal subunit [95]. And although there are numerous reports ascribing various phenotypes to the null allele of non-essential yeast ASC1, many of these reports worked not only with the deletion of the ASC1 coding sequence, but also with the deletion of the *SNR24*, thus it is unclear what phenotypes derive from the lack of what functional molecule. In our study we clearly show the separate functions of these two molecules in terms of translation initiation.

1.4.3. The N-terminus of the subunit eIF3a/TIF32 and ribosomal protein RPS0A

The N-terminal half of α /TIF32 is a stable protein domain [56], predicted to harbor the second PCI domain of yeast eIF3, preceded by several alpha helices. The α /TIF32-NTD was previously shown to interact with the small ribosomal protein RPS0A *in vitro* [47], and subsequently it was found that the N-terminal truncation of 200 amino acid residues in α /TIF32- Δ 8 severely reduced association of eIF3 and its associated eIFs with the small ribosomal subunit *in vivo* [30]. Detailed genetic analysis also showed that besides contacting RPS0A, the entire α /TIF32-NTD also contains two regions that promote efficient REI by interacting with distinct elements within the 5' sequences of uORFs of specific mRNAs [31]. The genetic studies revealed that residues 51–60 together with 71–80 and 161–170 impact REI specifically by impairing the α /TIF32-NTD interaction with these 5' sequences. The group of Ralf Ficner has recently managed to solve the crystal structure of the PCI domain of α /TIF32, which spans residues 276–494 (Figure 10) and showed that the entire N-terminal half of α /TIF32 interacts with RNA (unpublished data). Based on these and other finding [30,31], the α /TIF32-NTD was proposed to create an extension of the mRNA exit channel. In this study we analyzed the RPS0A side of the α /TIF32-NTD interaction in more detail by various *in vivo* and *in vitro* techniques.

RPS0A is a non-essential protein, but yeast genome also contains the *RPS0B* gene of which protein product is about 95% identical to RPS0A. Deletion of both genes is lethal, but as they are almost identical, one can substitute for the other [93]. RPS0A is positioned near the mRNA exit pore on the solvent side of the 40S subunit (Figure 2), next to the RPS17 of which extensions wrap around the RPS0A surface [36]. It also makes extensive contacts with RPS21 and RPS2. The last 45 C-terminal residues of RPS0A, (residues 208–252) are missing in the crystal structure [36], suggesting that this C-terminal tail (CTT) is flexible, exposed to the solvent (Figure 9, CTT in red). So far, RPS0A was characterized functionally and shown to contribute to the cytoplasmic maturation of the 40S ribosome, during the final processing of the 20S rRNA precursor to 18S rRNA, the so called cleavage D. RPS0A belongs to a group of ribosomal proteins, which when depleted, the 20S rRNA is made and exported from the nucleus but not converted into its final 18S rRNA form

[96,97]. Here we demonstrate that RPS0A participate in recruitment and/or stable association of eIF3 and other MFC components with 40S ribosome by interacting with the N-terminal half of a/TIF32.

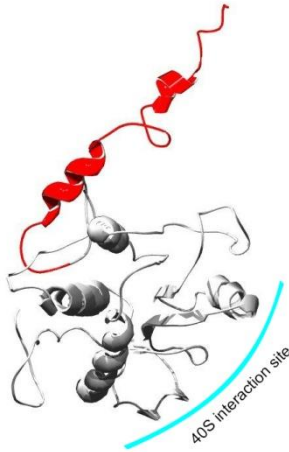


Figure 9: The 3D threading model of the RPS0A protein computed by Phyre2 prediction server [98] on the bases of crystal structure of 80S ribosome (PDB accession number 3U5C). The arrangement of residues 1-207 (grey) correspond to the crystal structure and the interaction site with 40S ribosome is marked by sky blue. The arrangement of residues 208-252 (red) is predicted to contain two α -helices and to stick out from the protein on a flexible loop making a C-terminal tail of RPS0A.

1.4.4. Small eIF3 subunit g/TIF35

The g/TIF35 subunit of eIF3 is a small, essential protein of 30,5 kDa that was previously shown to be required *in vivo* for initiation of protein synthesis [99]. It binds to both mRNA and rRNA fragments thanks to its a RNA recognition motif occurring at the C-terminus, but even though the RRM is functionally important, it is not essential [100]. The human eIF3/g RRM fold was solved by NMR (Figure 5, K. Tsuda et al., unpublished; PDB accession code 2CQ0) and as both yeast and human protein sequences of this region are homologous (Figure 1D, Publication #3), we can presume that they also share the structural homology. The eIF3/g RRM contains two typical RNA binding motives in β -sheets 1 and 3 [101], but the yeast protein seem to differ in several residues specific for the RNA interaction.

As mentioned above, g/TIF34 and i/TIF35 form a stable binary complex, and it was shown that the NTD of g/TIF35 (predicted to contain a Zn-finger domain) is responsible for this contact [50]. g/TIF35 alone interacts with b/PRT1 very weakly, but the i/TIF34-g/TIF35 mini-module forms with b/PRT1 a very stable complex, indicating that binding of i/TIF34 to b/PRT1 (i/TIF34 binds strongly to both g/TIF35 and b/PRT1) markedly stabilizes the g/TIF35 association with b/PRT1 [50,56,57]. In agreement with this observation,

mutations of residues mediating the b/PRT1—i/TIF34 contact results in loss of the entire i/TIF34-g/TIF35 mini-module from the rest of eIF3 and from preinitiation complexes, accompanied with severe growth phenotypes [55].

Here we identified interaction partner of g/TIF35 subunit among ribosomal proteins and performed functional analysis of this small essential eIF3 subunit, which contribution to general translation initiation was virtually unknown.

2. AIMS OF THE STUDY

This study is a part of long-term pursuit to define the location of yeast eukaryotic initiation factor eIF3 on the 40S ribosomal subunit and to explore its specific functions in the initiation of translation. The aim of the presented study was to determine the interaction sites of selected parts or domains of eIF3 subunits with 40S ribosomal subunit components *in vitro* and to verify the identified contact sites *in vivo*. We also addressed how the disruption of the identified contacts affects the process of translation initiation. Several specific objectives were designed in order to explore these biochemical and biological issues:

- To identify the 40S interaction partner of the C-terminal domain (CTD) of the c/NIP1 subunit (CTD contains a PCI domain).
- To identify the amino acids or protein subdomains of C-terminal part of c/NIP1 specific for the binding of c/NIP1 to 40S and to explore their effect on overall eIF3 40S binding *in vivo*.
- To determine the effect of ribosomal protein RACK/ASC1/RPS33 deletion on eIF3 binding to 40S subunit *in vivo*.
- To characterize the interaction between a/TIF32 subunit and small ribosomal protein RPS0A *in vitro*.
- To explore the impact of RPS0 conditional depletion and deletion mutants on efficiency and progress of the translation initiation process *in vivo*.
- To identify an interaction partner(s) of g/TIF35 subunit among small ribosomal proteins and functionally characterize its RRM domain.

3. MATERIALS AND METHODS

3.1. Yeast and bacterial strains

Yeast *Saccharomyces cerevisiae* was used as a reference eukaryotic cell model for *in vivo* studies. We took advantage of the current knowledge of molecular biology, cellular biology, and genetics of the yeast *S. cerevisiae* and used it as a robust experimental platform for our investigations. Yeast strains were constructed using standard techniques of yeast transformation [102,103], gene replacement [104] and plasmid shuffling [105]. Preparation of media was done essentially as described by Sherman [106]. H2880 MATa *trp1Δ leu2-3,112 ura3-52* was used as a parental strain [68]

Bacterial *Escherichia coli* strain DH5alpha (Invitrogen) was used for all recombinant DNA methods. Bacterial *Escherichia coli* strain BL21 (DE3, Invitrogen) was used for protein expressions and purifications.

3.2. Yeast genetics

Translation factors and ribosomal proteins are, in general, essential for yeast growth. Thus, their mutations, deletions, overexpressions or combinations of aforementioned can be screened by growth phenotypes [107]. Growth curve or quantitative growth assay (spot assay) is measured at 30°C or elevated temperatures (34-37°C) to observe slow growth (Slg-) and temperature-sensitive (Ts-) phenotypes.

3.2.1. Translational control specific yeast phenotypic assays

Decreased stringency in start codon selection – Sui- phenotype is determined using HIS4 enzyme as a reporter [107]. In *his4-303* background histidine is required in the growth medium, due to alteration of the HIS4 start codon to AUU. Thus growth in the absence of histidine indicates a Sui- phenotype as the AUU codon is used instead canonical AUG start codon.

Proper amino acid response of yeast to amino acid availability is mediated by controlled derepression of *GCN4* translation [107]. Decreased assembly of TC (independent of eIF2α phosphorylation) or the rate of TC loading on scanning 40S

subunits is indicated by general control derepressed (Gcd-) phenotype. Gcd- phenotype is tested by 3-amino-1,2,4-triazole (3AT, an inhibitor of histidine biosynthetic genes) resistance in *gcn2Δ* background. Defects in AUG start codon recognition (leaky scanning), slow rate or abortive scanning, or defect in subunit joining may lead to the Gcn- phenotype. Gcn- phenotype is tested by 3AT resistance.

3.2.2. Yeast two-hybrid screening

Two-hybrid analysis [108] of protein-protein interactions between subunits of eIF3 and all 33 small subunit ribosomal proteins was conducted using the Matchmaker Two-Hybrid System 3 (Clontech) according to the vendor's instructions using strain AH109.

3.3. Biochemical methods

3.3.1. Protein purifications

Protein purifications were conducted as glutathione S-transferase (GTS) fusions [109,110]. Appropriate cDNAs were cloned to either pGEX-5X-3 vector containing N-terminal GST tag (GE Healthcare) or pET42b (Novagen) modified vector containing N-terminal GST tag, His tag and AcTEV protease (Invitrogen) cleavage site right before the protein coding region. The cells were lysed by sonication into phosphate or HEPES buffered saline. Proteins were either immobilized or purified using a Glutathione Sepharose 4B resin (GE Healthcare) and then eluted by 30 mM reduced glutathione. If needed, the GST tag was removed using AcTEV Protease while dialyzing against storage buffer (usually based on 200-500 mM NaCl or KCl, 30-50 mM HEPES/NaOH (pH 7.5), 5-10 % glycerol and 2 mM β -mercaptoethanol) overnight. GST, non-cleaved protein and the protease were removed by binding to Ni sepharose 6 Fast Flow (GE Healthcare) resin while the targeted protein was collected as the flow-through. The protein was concentrated using Amicon Ultra centrifugal concentrator (Millipore) and the final concentration was measured by absorbance assay at 280 nm or by Bradford method.

The *in vitro* protein synthesis was conducted using TNT T7 Transcription/Translation Coupled Reticulocyte Lysate System (Promega) which contains all cellular components necessary for RNA transcription and protein synthesis (RNA polymerase, NTPs, tRNAs,

ribosomes, amino acids, initiation, elongation and termination factors). To label the protein, [³⁵S]-methionine was added to the reaction to incorporate into the synthesized proteins. Plasmid DNA containing appropriate protein coding sequences under T7 RNA polymerase promoter were used as templates.

3.3.2. Protein-protein, -RNA or -40S subunit interaction assays

Protein interactions were explored by GST-pulldown assays [111]. Individual GST-fusion proteins were expressed in *Escherichia coli*, immobilized on glutathione-Sepharose beads, and incubated with *in vitro*-synthesized [³⁵S]-labeled (using reticulocyte lysate system) potential binding partners. The beads were washed three times with phosphate-buffered saline, and bound proteins were separated by SDS-PAGE. Gels were first stained with Gelcode Blue stain reagent (Pierce) and then subjected to autoradiography.

RNA-protein interactions were explored either by RNA binding assay or RNA electrophoretic mobility shift assay [112]. RNA binding assay: the [³²P]-labeled RNA species were prepared *in vitro* using the MAXIscript™ SP6 or T7 transcription kit (Ambion) and enzymatically linearized RNA-encoding vectors. The transcripts were purified using a size exclusion column. GST-fusion proteins were incubated with [³²P]-labeled RNA species and bound RNAs were separated by electrophoresis on 5.5% denaturing (8M urea) polyacrylamide gel and subjected to autoradiography. RNA electrophoretic mobility shift assay (REMSA): GST-fusion or pure proteins were incubated with [³²P]-labeled RNA species and RNA-protein complexes were resolved on native 3.5% polyacrylamide mini-gel and subjected to autoradiography.

40S ribosomal subunit-protein interactions were explored by 40S-binding assay [113]. The 40S ribosomal subunits were purified according to Acker et al [114]. GST-fusion proteins were incubated with purified ribosomal 40S subunits and the resulting 40S-protein complexes were subjected to SDS-PAGE electrophoresis followed by immunoblotting with anti-RPS0, antiRPS22 or anti-ASC1 antibodies.

3.3.3. Polysome profile analysis

The most widely used method to separate ribosomal species is sucrose density centrifugation [107,115]. Under normal growth conditions, a typical polysome profile

(Figure 3B, Publication #1) shows peaks corresponding to free 40S and 60S ribosomal species and mRNAs containing different numbers of 80S elongating ribosomes appearing as monosomes, disomes, trisomes and polysomes. Translation initiation is considered to be the rate-limiting step and a reduction in the polysome-to-monomosome (P/M) ratio in a mutant strain serves as a hallmark of its defect. Analyzed yeast cells are grown into exponential and cycloheximide is added before harvesting. Cycloheximide is a translation elongation inhibitor which blocks the translocation step in is used in order to preserve ribosomes on mRNAs. Thus pretreated cells are lysed and the whole-cell extracts (WCEs) are resolved by velocity sedimentation through 5–45% sucrose gradient. The resulting gradients are scanned at 254nm to visualize the ribosomal species.

3.3.4. Analysis of preinitiation complexes

This method is used to analyze the 43S/48S preinitiation complexes composition [115], in other words, which eIFs and in what quantity are associated with the 40S ribosomal subunit. Yeast cells are treated with 2% formaldehyde before harvesting; this treatment cross-links eIFs to 40S ribosomes *in vivo*. Thus pretreated cells are lysed and the whole-cell extracts are resolved by velocity sedimentation through 7.5–30% sucrose gradients. Proteins from the collected fractions are subjected to western blot analysis using antibodies against subunits of eIF3, other eIFs and ribosomal proteins. If needed, the resedimentation protocol is applied, which circumvent trailing of eIFs from the immediately preceding fractions. In this case, fractions containing the 43–48S PICs are collected, pooled, concentrated and resolved on a second 7.5–30% gradient, the resulting 43–48S fractions are then subjected to Western blotting.

3.3.5. β -galactosidase reporter assay

In vivo expression of *GCN4* gene was measured by β -galactosidase reporter assays [116]. Plasmids containing the corresponding GCN4-lacZ fusions were introduced into yeast strains and B-galactosidase activity was assayed. B-galactosidase activity is expressed as nanomoles of o-nitrophenyl-,D-galactopyranoside (ONPG) hydrolyzed per minute per microgram of total protein (U).

3.4. Bioinformatic methods and statistical analysis

Protein sequence alignments were performed by ClustalX version 2.0.12 [117]. Protein secondary structure was predicted by PSIPRED secondary structure prediction method [118]. Computation of protein 3D models was conducted by 3D threading engine on the Phyre2 protein fold recognition server [119,120]. Structural homology was measured by DaliLite online structure alignment server [121]. Protein visualization and electrostatic potential rendering was calculated using Swiss-PDBViewer 4.0.1 [122].

All data was measured from at least three replicate experiments, standard deviations are given.

4. RESULTS AND DISCUSSION

In this thesis, four major topics are presented; (i) the PCI domain of the c/NIP1-CTD is capable of strong RNA binding and the extreme CTD interacts with the small subunit ribosomal protein RACK1/ASC1; two specific substitution mutants in the PCI domain and a truncation of the extreme CTD have a significant impact on formation of the PICs and cellular growth; (ii) deletion of *ASC1* only, without its *SNR24*-coding intron, also disturbs the PICs assembly; (iii) another intermolecular bridge spanning between the C-terminal tail (CTT) of RPS0A and the α /TIF32-NTD anchors the MFC to the small ribosomal subunit; and (iv) γ /TIF35 specifically interacts with RPS20 and mainly with RPS3, which is directly involved in transition between the closed/scanning-incompetent and open/scanning-conductive conformations of the 40S ribosome during initiation.

4.1. The extreme CTD of c/NIP1 interacts with the small ribosomal protein ASC1 and the preceding PCI domain binds RNA

First we wanted to identify potential interaction partner(s) of c/NIP1-CTD among the constituents of the small ribosomal subunit. We began by testing yeast two-hybrid interactions among two C-terminal fragments of c/NIP1 (residues 571–812 and 701–812) and all 33 small ribosomal proteins and from all pairwise combinations both c/NIP1 fragments interacted only with ASC1. To confirm these two-hybrid interactions and determine what part of the ASC1 protein mediates them, the c/NIP1 fragments were fused with the GST moiety and tested for binding to [³⁵S]-labeled segments of ASC1 synthesized *in vitro*. This experiment revealed that even only the very extreme CTD of c/NIP1 is capable of strong and specific interaction with blades 1–3 of ASC1 (Figure 6B, Publication #1). It is worth noting that we did not observe *in vitro* interaction of GST-c/NIP1-CTD fusions with any other ribosomal protein, including RPS0A, RPS2, RPS3 and RPS20 that interact with other eIF3 segments.

Next, as it was proposed but not examined experimentally that the molecular function of a PCI domain, known to mediate protein-protein interaction, might be also nucleic acid binding. Since a potential interaction between PCI and RNA could serve as an additional anchor point for eIF3 on the 40S ribosomal surface, we investigated whether

the c/NIP1-PCI domain specifically interacts with ribosomal 18S rRNA *in vitro*. We incubated the aforementioned GST-c/NIP1-CTD fusions with yeast radiolabeled 18S rRNA (either full length or subdivided into three distinct domains), 25S rRNA and *Xenopus laevis* b-globin mRNA, and the longer but not the shorter GST-c/NIP1 fusion protein strongly interacted with all mentioned RNA moieties. This indicates that the PCI domain of c/NIP1 only as a whole is capable of stable but unspecific binding to RNA (Figure 6B, Publication #1). We also performed an RNA electrophoretic mobility shift assay with selected RNAs and the PCI-containing c/NIP1-571–812 fragment fused to GST, but demobilized from the beads, and observed an upshift of the majority of the b-globin mRNA as well as 18S rRNA species in the native gel demonstrating indisputable RNA-binding affinity of the c/NIP1-PCI domain (Figure 6C, Publication #1).

Finally, we examined whether the c/NIP1-CTD, carrying two potential anchor sites for the small subunit—in ASC1 and 18S rRNA, interacts with purified 40S ribosomes *in vitro* as a whole. We did observe a weak but specific interaction between 40S subunits and both GST-c/NIP1 constructs when compared to the GST alone control (Figure 6B, bottom panel, Publication #1). In accord with the presence of 40S binding sites, more ribosomes (by ~2–3-fold) were pulled down when the entire c/NIP1-CTD, including both the ASC1 and RNA binding sites was used in the assay.

On the basis of all these results, we proposed a linkage map between the C-terminal of the c/NIP1 subunit of eIF3 and the 40S components, where the determined binding sites of the c/NIP-PCI to RNA and the extreme CTD to ASC1 are marked in green and red, respectively (Figure 7A, Publication #1). To our knowledge, this is the first report unambiguously showing the RNA-binding capability of the canonical PCI domain. In support of our observation, three subunits Sac3-Thp1-Sem1 of the TREX complex, two of which also contain a PCI domain, were shortly after the release of our report also implicated in binding to various types of the RNA species [78].

4.2. The c/NIP1-PCI serves as an important intermolecular bridge between eIF3 and the 40S subunit *in vivo*

To characterize the role of the c/NIP1-CTD in translation, we generated a battery of progressive C-terminal truncations. The shortest c/NIP1-CTD truncation of the last 60 amino acid residues in *nip1-Δ60* produced a desired Slg⁻ phenotype (Figure 3A, Publication #1) with a doubling time (DT) of 2.8 h (compare to 1.5 h of the wt strain). All other larger truncations were lethal. Next, we introduced Ala substitutions in six consecutive blocks of 10 residues between residues 693 and 752 just upstream of the *nip1-Δ60* truncation. The most C-terminal substitution of residues 743 through 752 in *nip1-743A752* produced a temperature-dependent Slg⁻ with DT of 3.4 h at 30°C and 6.6 at 37°C, and the more N-terminal substitution between residues 723 and 732 in *nip1-723A732* was lethal. Notably, the severity of the phenotype nicely correlated with the severity of the loss of *in vitro* RNA binding of the corresponding mutant proteins (Figure 6B, Publication #1).

We next analyzed the polysome content (Figure 3B, Publication #1) of the *nip1-743A752* and *nip1-Δ60* cells and demonstrated that the observed growth phenotypes are associated with a defect in translation initiation. In both WCEs an increased amount of 80S monosomes was observed leading to a similar ~2.6-fold decrease in the polysome to monosome (P/M) ratio indicating a reduced rate of general translation initiation.

Finally, both *nip1-743A752* and *nip1-Δ60* significantly impaired composition of the PICs (Figure 4, Publication #1). The 40S-associated amounts of eIF3 and its direct interacting partner eIF5 were markedly reduced in both mutants; ~30% reductions in the 40S-bound amounts of selected eIF3 subunits in *nip1-Δ60* (the drop was even more pronounced (by ~70%) when the resedimentation protocol was used), ~70% reductions were observed in *nip1-743A752* directly without resedimentation. The more deleterious impact in *nip1-743A752* is consistent with the severity of growth phenotypes at 37°C. Binding of eIF1A, which associates with the 40S ribosomes in the MFC-independent manner, was affected only very modestly suggesting that this c/NIP1 mutation selectively impacts 40S-binding of the eIF3-associated factors. Unexpectedly, the amounts of eIF2

were found to be increased in *nip1-743A752* and to a certain extent also in the *nip1-Δ60* mutant.

Taken together, we propose that the initiation defect in both of these mutants is by a large degree caused by a reduced rate of the 43S PIC formation and/or by its decreased stability.

4.3. ASC1 deletion destabilizes formation of the 43S PICs

We next wished to show that the deletion of the non-essential *ASC1* gene has an impact on formation of the 43S PICs. Previously, *asc1Δ* was suggested to impair growth, produce halfmer polysomes at 37°C and affect translational rates [84,85]. Halfmers are formed by mRNAs containing elongating 80S ribosomes and the 48S PICs stuck on the mRNA leader awaiting the joining of the 60S ribosomal subunit. The *ASC1* gene also contains an intron carrying *SNR24* encoding the C/D box U24 snoRNA that is required to guide 2'-O-methylation of the large subunit rRNA during 60S biogenesis [123]. The previous studies worked with strains deleted for both *ASC1* and *SNR24* and overlooking this fact, ascribed the aforementioned phenotypes solely to the loss of the *ASC1* gene.

We started by testing the effects of the *ASC1* or *SNR24* single deletions on formation of halfmer polysomes (Figure 8, Publication #1). In contrast to what was previously published, halfmer polysomes are formed in *snr24Δ* to the same extent as in the double deletion strain but were absent in *asc1Δ*. We clearly show that deletion of the *ASC1* intron encoding small nucleolar RNA U24, but not of the coding region of *ASC1 per se*, is the primary cause of the halfmer phenotype. The U24 is crucial for 60S subunit maturation [95] and so it is highly likely that the halfmer phenotype arises from defective subunit joining due to insufficient amounts of mature 60S subunits [124]. This had implied that the true *ASC1* role in translation initiation needed to be revised to account for these novel facts.

Hence we analyzed the effect of *asc1Δ* on PICs formation and observed ~50% reductions in the 40S-associated amounts of all selected eIF3 subunits and MFC-associated factor eIF5 (Figure 9, Publication #1). This reduction was similar to that displayed by the *nip1-Δ60* mutant, which is in accordance with the fact that the latter

c/NIP1 mutant lacks precisely the ASC1-binding site. However, eIF2 was found reduced not increased in *asc1Δ*, in contrast to what we observed for *nip1-Δ60*. This could be explained by proposing that the ASC1 protein is a dominant component of the 40S head region and its loss might thus change the overall conformation of the 40S head relative to its body in such a way that indirectly weakens the eIF2/TC binding affinity for the ribosome. In support of this, *asc1Δ* but not *nip1-Δ60* cells impart a severe Gcd- phenotype suppressible by overexpression of all three eIF2 subunits (Gcd- phenotype is indicator of a defect in the TC recruitment as it is specific for mutants that reduce 40S-binding affinity of eIF2 [69]).

Recent crystal structure analysis revealed that although a part of the ASC1 protein is obscured when bound to ribosome [37], a large portion of it is still accessible and, indeed, we found that the extreme CTD of c/NIP1 specifically interacted only with the half corresponding to blades 1–3 (Figure 6, Publication #1). Taking into consideration that ASC1 interacts with a number of signaling and regulatory molecules [125,126], and that it was suggested to serve as independent docking platform accommodating several of its interacting partners at the same time [44], we hypothesize that the signaling molecules bound to ASC1 next to eIF3 could directly influence the translational rates by acting upon eIF3.

4.4. The C-terminal tail of RPS0A makes a direct contact with the α/TIF32-NTD

It was previously demonstrated that the C-terminal 117 amino acid residues of RPS0A (at positions 135–252) were sufficient for an *in vitro* interaction with the N-terminal half (residues 1–490) of the α/TIF32 subunit of yeast eIF3 [47]. The yeast two hybrid analysis also suggested that the N-terminal 396 residues of α/TIF32 suffice for binding to full length RPS0A, and since the deletion of the extreme N-terminal 200 residues of α/TIF32 in the mutant reduced 40S-binding affinity of eIF3 *in vivo* [30], it was assumed that the RPS0A binding site resides most likely in the first 200 residues of α/TIF32.

Here we wanted to characterize this molecular contact more precisely and towards this goal we fused full length RPS0A with the GST moiety and tested the resulting

recombinant protein for binding to three N-terminal segments (residues 1-200, 201-400 and 1-400) of α /TIF32 synthesized and radiolabeled in rabbit reticulocyte lysates (Figure 2A, Publication #2). Notably, the region 1-200 was predicted to contain helical repeats preceding the PCI domain of α /TIF32 and the region 201-400 corresponds to canonical helix-bundle of the PCI domain, of which the structure spanning residues 276-494 was recently solved by X-ray crystallography (Figure 10, S. Khoshnevis, unpublished data). In accord with the two hybrid data, the α /TIF32-NTD segment comprising the residues 1–400 shows binding to GST-RPS0A, but in contrary to the effect of the deletion of the extreme N-terminal 200 residues *in vivo*, the removal of these residues did not abolish but perhaps even improved the quality of the observed interaction. Moreover, the segment comprising the residues 1–200 did not show any binding to RPS0A at all. This suggests that not the N-terminal stretch of 200 residues but the immediately following one is not only sufficient but also necessary for α /TIF32 binding to RPS0A *in vitro*. In support, the α /TIF32 segment of residues 201-400 fused to GST but not the preceding one between residues 1 and 200 interacted with the isolated 40S species *in vitro* (Figure 2B, Publication #2).

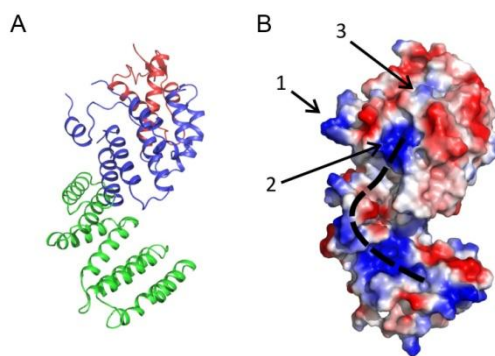


Figure 10. Model of the entire α /TIF32-NTD. The crystal structure (residues 276-494) was extended by a homology-based prediction of residues 1-275. **(A)** The ribbon representation of the chimeric molecule. The WH and HB sub-domains of the crystal structure and the predicted helical extension are depicted in red, blue and green, respectively. **(B)** Surface charge distribution of the chimeric molecule shows the dominance of the basic side chains over the surface of the N-terminal helical extension (the predicted model). Two major patches of positive charges on opposite sides of the molecule are marked by dark blue. Three major positive patches on the surface of the crystal structure are shown (1, 2 and 3) which collectively form a basic area that aligns with one of the continuous basic patches on the N-terminal extension (dashed line). (Adapted from S. Khoshnevis, unpublished data)

Having previously shown that the C-terminal 117 residues of RPS0A are sufficient for binding to α /TIF32 [47], we next wished to examine what part of the RPS0A-CTT is

necessary for this contact. We generated three C-terminal RPS0A truncations fused to GST, examined their binding affinities towards full-length α /TIF32 *in vitro* and observed that already the shortest truncation of 42 residues markedly reduced this binding and no further decrease was observed with more progressive truncations (Figure 2C, Publication #2). Given that these RPS0A terminal residues are suggested to be exposed to the solvent and thus accessible for any potential interactions, and that their removal disturbs the α /TIF32-NTD–RPS0A interaction *in vitro*, we propose that this short RPS0A sequence bears important binding determinants for the attachment of eIF3 to the 40S ribosome via the NTD of its α /TIF32 subunit. In support, RPS0A-CTT is highly acidic (data not shown) and thus it could interact with some of the predicted basic patches on the surface of the helical part of the α /TIF32-NTD PCI domain (Figure 10). Actually, it is quite common that a PCI domain interacts with its binding partners via this helical fold [76].

4.5. The CTT of RPS0A contributes to the RPS0A role in anchoring eIF3 to the small ribosomal subunit.

To address the role of RPS0A in mediating the binding of eIF3 to the 40S *in vivo*, we constructed an *rps0AΔ rps0BΔ* double deletion strain harboring the *RPS0A* allele on a high copy vector under control of the *MET3* promoter, which can be turned off by supplementing the medium with methionine. As expected, after addition of methionine to the media, RPS0A was promptly depleted from the cell accompanied by ceased cell growth (Figure 3A and 3B, Publication #2). To demonstrate that the observed impaired cell growth is associated with a defect in translation initiation, we analyzed the polysome content in the cell. We observed that the rapid depletion of RPS0A reduced the P/M ratio 3.8-fold (Figure 3C, Publication #2) and caused so called polysome run-off with a concomitant increase in the amount of free 80S ribosomes, which is typical for decreased initiation rates. But it should be stressed here, that some portion of the observed effect might be caused by a misbalanced ribosome biogenesis, owing to the RPS0A role in the final maturation of 20S rRNA.

To answer the key question, whether the loss of RPS0A influences the eIF3 association with the 40S ribosomal subunit *in vivo*, we analyzed the composition of native

43S/48S PICs derived from cells grown in Met[±] SD medium (Figure 4, Publication #2). Upon RPS0A depletion after 8 hours, all ribosomes found in cells lacked the 0A ribosomal protein and we also observed a clear shift in the overall distribution of the amounts of selected eIF3 subunits and to a little less pronounced extent also of eIF5 and the TC from the 40S-containing fractions to the free cytoplasmatic fractions. This result directly indicates that RPS0A mediates association of eIF3 and its associated eIFs with the 40S ribosomal subunit *in vivo*.

We next took advantage of the fact that the CTT truncation of RPS0A is viable, has no effect on ribosome biogenesis (Andreas Neueder and Philipp Milkereit, unpublished data) but impairs the RPS0A contact with the α /TIF32-NTD *in vitro*, and examined its contribution to the role of RPS0A in translation initiation *in vivo*. We introduced the FLAG-tagged truncation of RPS0A (residues 1–197) into the *rps0aΔ rps0bΔ* double deletion strain and analyzed the phenotype of the resulting mutant in comparison with the strain expressing wt RPS0A-FLAG; a mild slow growth phenotype was observed at 37°C. The analysis of the PIC composition in mutant cells then showed a modest but reproducible redistribution of eIF3, eIF2 and eIF5 from the 40S-containing fractions into the lighter fractions (Figure 5A, Publication #2). Importantly, the distribution of eIF1A, which is not a part of the MFC, did not change at all. This result suggests the RPS0A-CTT is specifically required for *in vivo* binding of eIF3 (and its associated eIFs such as 5 and 2) to 40S ribosomes. Further support for the role of the RPS0A-CTT in anchoring eIF3 to the 40S ribosome comes from our observation that ribosomes containing *rps0a-ΔCTT*-FLAG could not compete well with native ribosomes for recruitment of eIFs in otherwise wt cells, when compared to the wt RPS0A-FLAG ribosomes (Figure 5B, Publication #2).

Hence, this work identified specific residues of the RPS0A-CTT that stick out from the ribosomal surface and interact with the α /TIF32-NTD to promote binding of the entire MFC to the small ribosomal subunit.

4.6. g/TIF35 specifically interacts with the 40S beak proteins

RPS3 and RPS20

To gain more insight into where eIF3 interacts with the 40S ribosome, we wanted to determine the ribosomal position of its g/TIF35 subunit. To do that we tested the GST-g/TIF35 fusion for interactions against all 33 small ribosomal proteins, which were synthesized and ³⁵S-labeled in rabbit reticulocyte lysates. Among all RPSes, only RPS3 and RPS20 interacted with GST-g/TIF35 (Figure 6A, Publication #3). Consistently, RPS3 and RPS20 also interact tightly with each other and form a part of the beak on the solvent-exposed side of the 40S subunit near the mRNA entry channel (Figure 2). The fact that g/TIF35 interacted only with two RPSes occurring next to each (Figure 6A, Publication #3; as a control we tested binding to RPS2 that lies near both RPS3 and RPS20), supports the specificity of this interaction. Based on this result and on earlier observations that g/TIF35 forms with other eIF3 subunits a specific b/PRT1-CTD/i/TIF34/g/TIF35 submodule, we placed this part of the eIF3 moiety near the beak of the 40S ribosome in the proximity of the mRNA entry channel (Figure 6).

4.7. The RRM of g/TIF35 is required for efficient translation initiation.

In effort to elucidate the role of g/TIF35-RRM in translation initiation, we first mutated conserved residues in either one or both of its RNA binding motives (RNPs) (Figure 1, Publication #3), tested the resulting mutants for growth phenotypes and observed that only the combination of both RNP1 and RNP2 mutations in *g/tif35-K194A-L235A-F237A* (henceforth termed *g/tif35-KLF*) resulted in the Ts- phenotype. The *g/tif35-KLF* mutation was also found to provoke approximately 3-fold reductions in the P/M ratio at 34°C (Figure 1D, Publication #3), indicating a decrease in the rate of translation initiation *in vivo*. As expected, the *g/tif35-KLF* mutant did not affect the integrity of eIF3 in the MFC, as the RRM domain was previously shown to be dispensable for the g/TIF35 binding to both i/TIF34 and b/PRT1 [50]. However, somewhat unexpectedly, the KLF mutation neither showed a significant effect on RNA binding, at least in our *in vitro* assay (Figure 2, Publication #3).

Nevertheless, taking into account that the *g/tif35-KLF* mutation did reduce rates of translation initiation that *g/TIF35* occurs near the RNA entry channel, we explored whether it contributes to mRNA scanning and/or proper AUG start codon selection. Indeed, we observed that this mutation decreased the processivity of scanning through a stable secondary structure inserted into the 5'-UTR of the reporter *GCN4-lacZ* mRNA construct (Figure 3, Publication #3). No other effects such as abortive, slow, or leaky scanning, or a defect in proper AUG selection were observed.

Given the fact that *g/TIF35* interacts with RPS20 and with RPS3 (Figure 6), the latter of which is directly involved in transition between the closed/scanning-incompetent and open/scanning-conductive conformations of the 40S ribosome (Figure 4) [40], we speculate that the *g/TIF35*-RPS3 contact places its *i/TIF34* interacting partner in a favorable position to influence the latter conformational transitions; specific mutations of *i/TIF34* were shown to slow the rate of ribosomal scanning [55]. At the same time, the predicted position of *g/TIF35* above the mRNA entry channel may enable its RRM to stimulate processivity of scanning by presenting mRNAs to the decoding center and/or by promoting the action of helicases.

5. CONCLUSIONS

This work contributes to our understanding of the location-structure-function relationship between the eIF3 and 40S ribosome. We identified several key contacts between specific domains of eIF3 subunits and either protein or RNA components of the 40S ribosome, the high resolution structure of which is now available (Figure 2). All identified interactions are summarized in Figure 6 placing the major body of eIF3 to solvent-exposed head, beak and shoulder regions of the 40S ribosome. Importantly, the spatial arrangement of eIF3 on 40S also determines how eIF3 functions during 43S and 48S assembly and how it acts at different stages of translation initiation. Indeed we were able to reveal a part of this sophisticated interplay between the ribosome and several of eIF3 subunits in the presented study.

The eIF3 occurrence in the vicinity of both the mRNA entry and exit channels predestines eIF3 to control mRNA recruitment and scanning as well as to respond to signals encrypted in the cis-acting features of mRNAs 5' UTRs. For example, the eIF3 interaction with RPS3 and helices 16-18 of 18S rRNA, both representing the components of the 40S participating in the formation of “latch” at the mRNA entry pore, might govern the proper selection of the AUG start codon or the proper response of the initiating PIC to this event. High affinity of eIF3 towards the 40S ribosome greatly stimulates recruitment of all MFC components to the 40 ribosome and their stabilization thereon, nevertheless, their final “functional” destinations on the surface of the 40S subunit certainly differ from their original placement most likely due to specific conformational changes of the ribosome during the initiation phase. And last but not least, the eIF3 interaction with the ribosomal protein ASC1 might serve as a connection between translation initiation and signaling pathways.

In this and previous studies several regions or even distinct amino acids of eIF3 were described to serve as contact points with the 40S ribosome and their molecular functions were characterized in great detail. However, until now, the true physiological importance of the most of the 40S ribosomal counterparts of these eIF3 functional interactions has not been corroborated by the appropriate *in vivo* approaches. Here we shed light on the function of ASC1 and RPS0A in the translation initiation. In the near future, we will

continue in this direction aiming to explore the contribution of individual ribosomal proteins to translation initiation, especially of those identified to directly contact eIF3 (such as RPS3 and RPS20) or those that appear to lie in or near the predicted position of eIF3 on the 40S (such as RPSes 26, 28, 17, 10, 12 and others). We will take advantage of the structural knowledge of individual RPSes and carefully select those residues that could be participating in interactions with eIF3 and its associated eIFs or bear important functional features required for optimal progress of translation initiation. These residues will be mutated and characterized by variety of *in vitro* and *in vivo* tools mentioned throughout this work. We hope that this approach will uncover important residues of several RPSes, which will show direct effects specifically in translation initiation but not in ribosome biogenesis, the latter of which always dominate in cells deleted for ribosomal genes as a whole [96,97].

Although there has been a tremendous progress in our knowledge of the eIF3 architecture, we still lack the atomic structure and the precise molecular details of amino acid contacts among its individual subunits. Thanks to the fact that we are now able to purify and reconstitute yeast eIF3 in great stoichiometry and stability *in vitro* [56], our future plan is to analyze the contacts among eIF3 subunits by chemical cross-linking coupled with high resolution mass spectrometry [127,128], which is based on identification of specific masses of dipeptides connected by juxtaposed lysine residues in a complex under study. The next step would be to use the same approach or perhaps even include the hydrogen–deuterium exchange protocol [127,128] for the *in vitro* established complex between eIF3 and the 40S subunit to verify and expand our knowledge on their mutual interaction sites.

5.1. List of publications

Publication #1: **Tomáš Kouba**, Edit Rutkai, Martina Karásková and Leoš Shivaya Valášek; The eIF3c/NIP1 PCI domain interacts with RNA and RACK1/ASC1 and promotes assembly of translation preinitiation complexes. **Nucleic Acids Research**, 2012, Vol. 40, No. 6 2683–2699

Publication #2: **Tomáš Kouba***, István Dányi*, Stanislava Gunišova, Vanda Munzarová, Vladislava Vlčková, Lucie Cuchalová, Andreas Neueder, Philipp Milkereit and Leoš Shivaya Valášek; Small ribosomal protein RPS0 stimulates translation initiation by mediating 40S-binding of eIF3 *via* its direct contact with the eIF3a/TIF32 subunit. **PLoS ONE**, 2012, Volume 7, Issue 7, e40464 *These authors contributed equally to this work.

Publication #3: Lucie Cuchalová, **Tomáš Kouba**, Anna Herrmannová, István Dányi, Wen-ling Chiu and Leoš Valášek; The RNA recognition motif of eukaryotic translation initiation factor 3g (eIF3g) is required for resumption of scanning of posttermination ribosomes for reinitiation on GCN4 and together with eIF3i stimulates linear scanning. **Molecular and Cellular Biology**, 2010, Volume 30, No. 19, p. 4671–4686

Sohail Khoshnevis, Stanislava Gunišová, Vladislava Vlčková, **Tomáš Kouba**, Piotr Neumann, Ralf Ficner and Leoš Shivaya Valášek, Structural integrity of the PCI domain of eIF3a/TIF32 is critically required for mRNA recruitment to the 43S pre-initiation complexes. *in preparation*

5.2. Declaration of authors on contribution on individual publications

This work was conducted with contribution of members of laboratories as follows:

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The individual authors all agree on that the contribution of Tomáš Kouba on
publication #1 was 85 %, on publication #2 was 30 % and on the publication #3 was 15 %.

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Publication #1

Publication #2

Publication #3