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BACHELOR WORK

Analysis of the role of TIF32 and HCR1 subunits of eukaryotic initiation factor 3 (eIF3) in association of the Multifactor complex with the small ribosomal subunit.

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Abstract

Translation initiation in eukaryotes requires participation of numerous initiation factors (eIFs) out of which eIF3 is the most complex. eIF3 is an essential multiprotein assembly in higher eukaryotes composed of 13 subunits that stimulates nearly all reactions of translation initiation pathway including Met-$tRNA_i^{Met}$ and mRNA binding to 40S ribosomal subunit, subsequent scanning of mRNA, and the start codon recognition. Yeast eIF3 forms together with the ternary complex, eIF1 and eIF5 so called multifactor complex that is an important intermediate in translation initiation. In yeast *Saccharomyces cerevisiae* eIF3 consists of five core essential subunits (TIF32, NIP1, PRT1, TIF34 and TIF35) and one nonessential substoichiometric subunit (HCR1). TIF32 is the largest eIF3 subunit and, importantly, an unbalanced expression of its human homologue p170/eIF3a has been associated with various types of cancer possibly by affecting affinity of various mRNAs for the ribosome. Indeed, yeast TIF32 was suggested to make at least two critical contacts with the 40S ribosome via its extreme N and C termini. In addition, the middle domain of TIF32 (TIF32-HLD) shares a significant sequence similarity with the noncore HCR1 subunit that was also implicated in stimulating binding of eIF3 to the 40S ribosome by anchoring it nearby the mRNA entry channel where its human orthologue was localized. And last but not least, HCR1 binds simultaneously with the TIF32-HLD to the RRM domain of PRT1 whose role in 40S-binding by eIF3 was demonstrated only recently. All these facts indicated that the TIF32-HLD is a part of the eIF3 module that promotes, possibly in a highly regulated fashion, association of eIF3 with the small ribosomal subunit. Indeed, we have only recently obtained novel evidence implicating the TIF32-HLD in making a contact with the small ribosome that may be mutually exclusive with HCR1. We hypothesize that it might work as an mRNA-entry-channel-clearing mechanism rendering the ribosome competent for proper mRNA loading.

Keywords: translation initiation, eIF3, TIF32, HCR1, multifactor complex

1. Introduction

Gene expression is a complex process by which the information encoded by individual genes is first transcribed into a nucleotide sequence of a messenger RNA (mRNA) that is subsequently translated into a sequence of amino acids of a new polypeptide chain. This principal - the flow of genetic information from DNA to RNA to protein is called the central dogma of molecular biology.

Translation takes place in cytoplasm where the mRNA associates first with small and then with large ribosomal subunits. It can be divided into three steps (initiation, elongation and termination) out of which the initial one is the most critical as it serves as a target of many regulatory pathways. Translation initiation requires the participation of numerous proteins and protein complexes called initiation factors; in eukaryotes they are called eukaryotic initiation factors (eIFs). These orchestrate binding of methionyl initiator tRNA (Met-$tRNA_i^{Met}$) and mRNA to the small ribosomal subunit, in other words the assembly of 43S and 48S preinitiation complexes followed by scanning for the start AUG codon. There are at least 12 initiation factors in eukaryotes (Hershey and Merrick, 2000).

2. Translation initiation

Translation initiation in eukaryotes is a complex series of reactions leading to the formation of an 80S ribosomal complex containing Met-$tRNA_i^{Met}$ base paired with the initiation codon in mRNA. The main initiation pathway in eukaryotes is cap-dependent; ribosomes bind near the cap structure (m^7GpppN) at the 5‘ end of mRNA and then scan the mRNA in the 5‘ to 3‘ direction until they encounter an initiator AUG codon (Kozak M., 1989).

The conventional view of the translation initiation pathway (Fig. 1) starts with the recruitment of the Met-$tRNA_i^{Met}$ to the 40S ribosome to form 43S preinitiation complex. The Met-$tRNA_i^{Met}$ is transferred to the 40S subunit in a form of the ternary complex (TC) with eIF2 and GTP, and the formation of 43S preinitiation complex is stimulated by the eIF3 complex, eIF1 and eIF1A. The

43S preinitiation complex then binds to the capped 5‘ end of mRNA, with the help of the eIF4 group of factors, poly(A)-binding protein and eIF3 (Hershey and Merrick, 2000; Sachs and Varani, 2000). The resulting 48S complex scans the mRNA until an AUG start codon is recognized, the process that is facilitated by eIF1 and eIF1A (Pestova et al., 1998). When the anticodon of Met-tRNA$_i^{Met}$ base-pairs with the AUG start codon, the eIF5 stimulates GTP hydrolysis by eIF2, the eIFs are ejected and the 40S-Met-tRNA$_i^{Met}$-mRNA initiation complex is joined by the 60S ribosomal subunit in a reaction stimulated by GTP-bound eIF5B (Pestova et al., 2000) to form an 80S initiation complex that becomes poised for elongation. For a new round of initiation, the ejected eIF2-GDP must be recycled to eIF2-GTP by the guanine nucleotide exchange factor eIF2B (Hershey and Merrick, 2000; Hinnebusch, 2000).

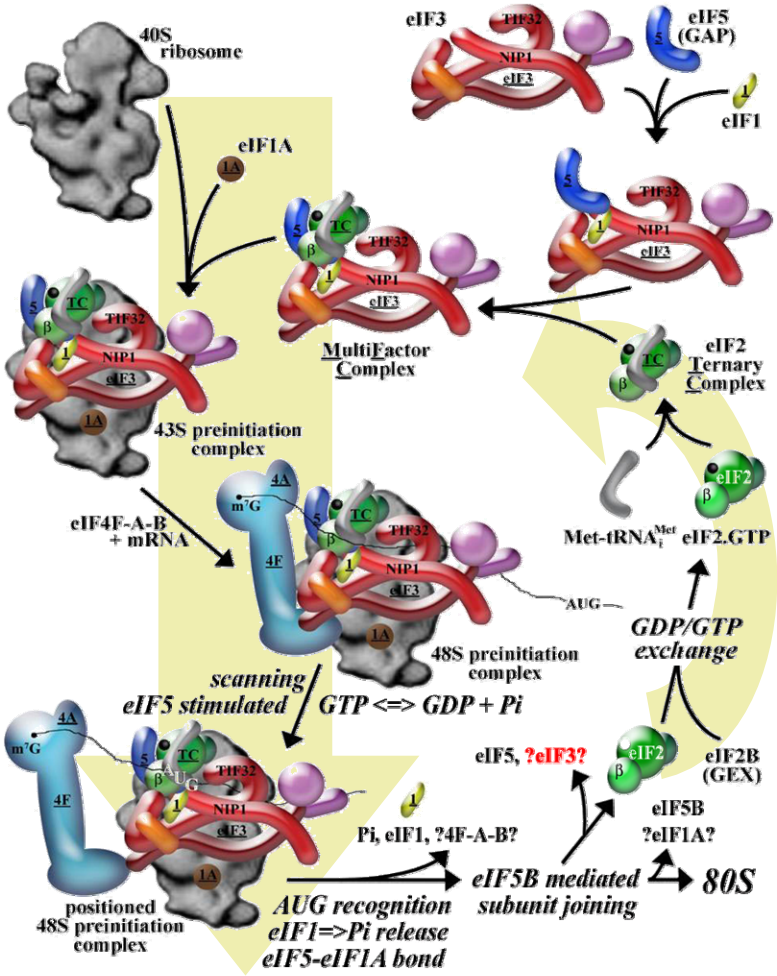

FIGURE 1. Translation initiation pathway. (Valášek et al., unpublished data)

Experiments with the yeast *Saccharomyces cerevisiae* revealed that translation initiation in this lower eukaryote strongly resembles cap-dependent initiation in mammals. This is perhaps most convincingly demonstrated by finding that some mammalian initiation factors can substitute for yeast factors *in vivo* (Schwelberger et al., 1993). Numerous key studies that were conducted with this ideal model organism since early 80s have contributed by a great deal to our understanding of this fundamental process.

3. The eIF3 complex

Eukaryotic translation initiation factor 3 (eIF3) was one of the first initiation factors to be identified in the 1970s, but it is only in the past seven years that much of our knowledge about how eIF3 functions at the molecular level has been gained. Evidence now indicates that most of the reactions in the initiation pathway are stimulated by eIF3 (Hinnebusch 2006).

eIF3 is a large multisubunit protein complex that plays an essential role in the binding of Met-$tRNA_i^{Met}$ and mRNA to 40S ribosomal subunit, and also in the subsequent scanning and AUG recognition. It can bind directly to the 40S ribosome (Hershey and Merrick, 2000; Hinnebusch, 2000), is involved in the dissociation of the 80S ribosome to 40S and 60S subunit (Kolupaeva et al., 2005) and also prevents dissociation of the ternary complex caused by addition of RNA (Gupta et al., 1990).

Consistent with its diverse functions, mammalian eIF3 has the most complex structure of the initiation factors, containing 13 non-identical subunits that are designated eIF3a to eIF3m (Asano et al., 1997; Vornlocher et al., 1999; Browning et al., 2001; Hinnebusch 2006) (Table 1).

By contrast, eIF3 form the yeast *Saccharomyces cerevisiae* is much simpler, the core of eIF3 is thought to be composed of five essential subunits: TIF32 (RPG1), NIP1, PRT1, TIF34 and TIF35 (Asano et al. 1998). All of them have homologues in the mammalian eIF3 complex. Only one additional mammalian eIF3 subunit (eIF3j) has a homolog encoded in the *Saccharomyces*

cerevisiae genome. This protein, called HCR1, is a non-essential substoichiometric component of yeast eIF3 that enhances interactions with other eIFs, promotes binding of eIF3 to the 40S subunit and has an independent function in 40S ribosome biogenesis (Valášek et al., 2001a,b).

Importantly, the 5-subunit complex purified from yeast can restore binding of Met-tRNA$_i^{Met}$ (Danaie et al., 1995; Phan et al., 1998) and mRNA (Phan et al., 2001) to 40S ribosomes in heat-inactivated *prt1-1* mutant extracts. Thus, yeast eIF3 possesses two critical functions ascribed to the more complex mammalian factor.

TABLE 1. Summary of eIF3 subunits in selected eukaryotes (Hinnebusch 2006)

Unified nomenclature	Homo sapiens	Schizosaccharomyces pombe	Saccharomyces cerevisiae	Functions
eIF3a	p170	p107	TIF32	40S binding; eIF4B binding; MFC assembly; TC and mRNA recruitment
eIF3b	p116	p84	PRT1	40S binding; MFC assembly; TC and mRNA recruitment; scanning
eIF3c	p110	p104	NIP1	40S binding; MFC assembly; TC and mRNA recruitment; AUG recognition
eIF3d	p66	Moel	–	
eIF3e	p48	Int6	–	
eIF3f	p47	Csn6	–	
eIF3g	p44	TIF35	TIF35	Binding eIF4B
eIF3h	p40	p40	–	
eIF3i	p36	Sum1	TIF34	
eIF3j	p35	–	HCR1	40S binding; MFC assembly
eIF3k	p28	–	–	
eIF3l	p67	–	–	
eIF3m	GA17	Csn7B	–	

3.1. Interactions of eIF3 subunits

Studies using deletion mutations in individual eIF3 subunits and yeast two hybrid analysis showed that the C-terminus of the *S. cerevisiae* PRT1 interacted

with TIF35 and TIF34 and that the N-terminal RNA recognition motif (RRM) in PRT1 interacted simultaneously with TIF32 and HCR1 (Asano et al., 1998; Valášek et al., 2001a). Removal of the RRM domain in PRT1 resulted in dissociation of TIF32, NIP1 and HCR1 from the eIF3 complex and destroyed the 40S ribosomal subunit binding by the residual PRT1-TIF35-TIF34 subcomplex. Hence, the RRM of PRT1 is crucial for the integrity of the eIF3 complex in *S. cerevisiae* (Valášek et al., 2001a).

NIP1 interacts with the N-terminal half of TIF32 and with the C-terminus of PRT1. Residues 371-570 of NIP1 contain a binding site for PRT1 that is required for tight association of the PRT1-TIF34-TIF35 subcomplex with other eIF3 subunits. The binding site for NIP1 is located just upstream from the TIF34-TIF35 binding site at the extreme C-terminus of PRT1 (Valášek et al., 2002).

The interactions between the mammalian eIF3 subunits are less understood due to its higher complexity.

3.2. Interactions of eIF3 with other eIFs and with 40S subunit

eIF3 is physically associated with other eIFs in yeast. It interacts directly with eIF5, eIF2, and eIF1 and these interactions may contribute to the stimulatory effect of eIF3 in biding of eIF1 and TC to the 40S subunit observed *in vitro* (Hinnebusch, 2000; Kolupaeva et al., 2005; Jivotovskaya et al., 2006; Nielsen et al., 2006).

Two interactions between yeast eIF3 and eIF2 have been described so far; a direct interaction between eIF2β and TIF32 and an indirect interaction between eIF2β and NIP1 that is bridged by eIF5. In addition to binding eIF5, NIP1 also interacts with eIF1, which additionally interacts with eIF5, TIF32 and eIF2β. Interestingly, both eIF5 and eIF1 were implicated in selecting AUG as the start codon, and their related functions in scanning may be coordinated by mutual association with NIP1 (Asano et al., 2000; Algire et al., 2002; Valášek et al., 1999, 2002; Nielsen et al., 2006), (see Fig. 2).

Studies in budding yeast have shown that deleting the N and C termini of NIP1 or the TIF32 N-terminal domain (TIF32-NTD) impairs 40S binding by otherwise intact eIF3 complexes, suggesting that these segments contact the 40S subunit. Indeed, a subcomplex consisting of NIP1, the N-terminal half of TIF32, and eIF5 can bind the 40S subunit *in vitro* and *in vivo*. The TIF32 C-terminal domain (TIF32-CTD) is also required for 40S binding when the connection between eIF3 and eIF5 is disrupted by mutation. In addition, a putative RNP1 element in the RNA recognition motif (RRM) present in PRT1-NTD mediates a protein-protein interaction with HCR1, and both the RNP1 element and HCR1 are required for wild-type 40S binding by eIF3. Thus there are probably many contacts between the 40S subunit and the different eIF3 subunits (Nielsen et al., 2006; Hinnebusch 2006).

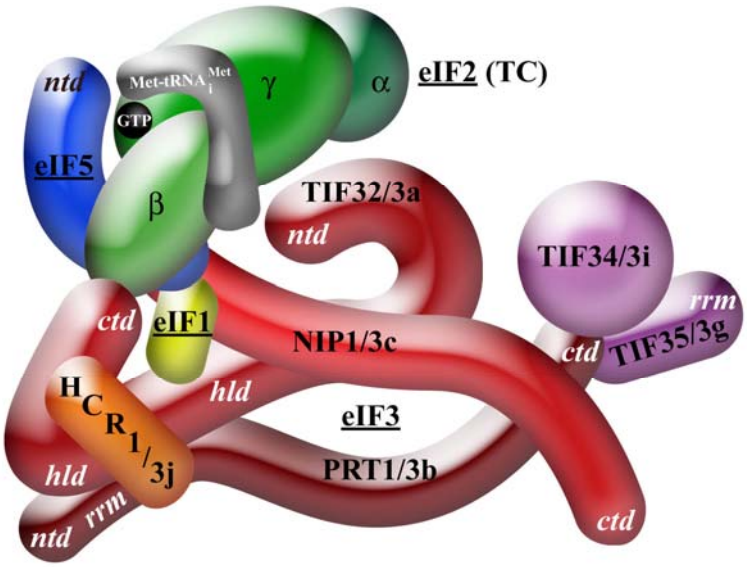

FIGURE 2. A 3D model of the MFC (Valášek et al. 2002)

3.3. Yeast Multifactor complex

In budding yeast, eIF3 and eIF1 reside with the TC and with eIF5 in a ribosome-free multifactor complex (MFC) (Fig. 2.) (Asano et al., 2000), that is thought to exist as a preformed unit capable of binding to 40S ribosomes at once

and was shown to represent an important intermediate in translation initiation (Valášek et al., 2002, 2003, 2004; Asano et al., 2001; Nielsen et al., 2006; Jivotovskaya et al., 2006; Yamamoto et al., 2005; Singh et al., 2004). The integrity of the MFC is required for optimal rates of translation initiation *in vivo* as destabilizing mutations in its individual components affect TC and mRNA binding to 40S subunits as well as the post-assembly reactions such as scanning and AUG recognition (Valášek et al., 2002; Nielsen et al., 2004). The importance of MFC integrity for 43S assembly in yeast is evident from experiments in which mutations in eIF5, TIF32, NIP1, or eIF1 led to reduced 40S binding in vivo of not only the mutated factor but also other MFC constituents (Asano et al., 2001; Nielsen et. al., 2004; Singh et. al., 2004)

The stable association of eIFs 1, 3, and 5 and TC raises the possibility that assembly of these factors in MFC facilitates their cooperative binding to the 40S subunit as a preformed unit (Hinnebusch 2006).

3.4. eIF3 and translational regulation

In addition to their important roles in initiation of general mRNA translation, the subunits of eIF3 have been suggested to play regulatory roles in initiating translation of a subpopulation of mRNAs (Dong and Zhang 2006).

The strong evidence for the regulatory role of eIF3 subunits in translation initiation came from a recent study of eIF3 in *Schizosaccharomyces pombe* (Zhou et al., 2005). As discussed above, the yeast eIF3 has a core complex consisting of five subunits. In *S. pombe*, there are several non-core subunits, eIF3d, eIF3e, eIF3f, eIF3h and eIF3m. Zhou et al. found that two eIF3 subcomplexes exist in *S. pombe*: one subcomplex containing eIF3f, eIF3h and eIF3m (named as eIF3m subcomplex) and the other containing eIF3d, eIF3e and eIF3f, but lack eIF3h and eIF3m (named eIF3e subcomplex) in addition to the five core subunits. The eIF3m subcomplex appeared to associate with the bulk of cellular mRNAs whereas the eIF3e subcomplex associated with a far more restricted set of mRNAs. These findings suggest that different subunits of eIF3 may confer the

core complex of eIF3 different functions and, consequently, effect on the initiation of translation of different mRNA species (Dong and Zhang 2006). This hypothesis is most convincingly confirmed by studies of mammalian IF3a.

The mammalian eIF3a, unlike the yeast homologue TIF32, is not essential for general translation. But interestingly, it has been identified to be down-regulating the synthesis of ribonucleotide reductase M2 (RRM2) (Dong et al., 2004). Over-expression of ectopic eIF3a increased the protein level of RRM2 whereas down-regulating endogenous eIF3a by antisence cDNA decreased its protein level. This suggests that mammalian eIF3a may play role in regulating translation of specific mRNAs. Analogous to the eIF3e and eIF3m complexes in *S. Pombe*, it is possible that there are two eIF3 sub-complexes in mammalian cells with or without eIF3a that may be responsible for initiation of translation of different mRNA species (Dong and Zhang 2006).

3.5. eIF3 and cancer

The initiation factors have been recognized to play important roles in oncogenesis (Dong and Zhang 2006). It was thought that the unbalanced expression of eIFs might cause changes in efficiency of translation of specific mRNAs such as enabling the translation of limited pool of mRNAs that are normally translated at low efficiency and encode key proteins involved in cellular growth, angiogenesis, survival and malignancy (De Benedetti et al., 1999, 2004). Recently, various subunits of eIF3 have been found to have altered expression in malignant tumors. The altered expression of some of these eIF3 subunits have been demonstrated to play important roles in oncogenesis.

For example, the expression of eIF3a has been found increased in various cancer cell lines and tissues. Bachmann et al. first identified that the expression level of eIF3a was elevated in breast cancer tissues compared with paired normal mammary tissues (Bachmann et al., 1997). The increased eIF3a expression has also been found in cancers of lung, cervix, esophagus, stomach and colon (Dong and Zhang 2006). The association of higher expression level of eIF3a with tumors

compared with normal tissues suggests that it may be involved in oncogenesis. Furthermore, the altered expression of eIF3a in cancers has been linked to prognosis. Breast and stomach cancer patients with higher expression level of eIF3a had a better overall survival rate than the ones with lower eIF3a expression (Chen et al., 1999, 2004).

Another good example is eIF3e; over-expression of truncated C-terminaly deleted eIF3e causes malignant transformation of mammary ephitelial cells (Mayeur and Hershey 2002).

Clearly, more works are needed to further investigate the role of eIF3a and eIF3e in oncogenesis, because how the altered expression of these proteins causes cancer and affect prognosis remain to be determined (Dong and Zhang 2006).

4. The TIF32 subunit of eIF3

TIF32, the largest subunit of eIF3, is an essential protein with a calculated molecular mass of approximately 110kDa. The *Saccharomyces cerevisiae* TIF32 is the sequential and functional homologue of the mammalian p170 protein with 29% identity and 54% similarity between their amino acid sequences (Valášek et al., 1998).

It was originally described as an essential protein being required for passage through the G1 phase of the cell cycle. Elutriated cells of the temperature sensitive *rpg1-1* mutant arrested in the early G1 phase of the first cell cycle and ceased cell growth when shifted to restrictive conditions (Kovarik et al., 1998). There are three points in the yeast cell cycle where the further progression depends on active protein synthesis (G1 phase, medial nuclear division and late nuclear division), of which the G1 phase is the most important (Burke et al., 1991). Since mating of haploid *Saccharomyces cerevisiae* cells of opposite mating types also requires a functional translational mechanism, the *rpg1-1* mutant was accordingly found to severely impair the mating ability at the restrictive temperature (Valášek et al., 1998).

It was shown that TIF32 protein is required for translation initiation *in vivo* and *in vitro* and interacts with PRT1 subunit of eIF3 (Valášek et al., 1998). Involvement of TIF32 in the process of translation initiation was clearly demonstrated in a cell-free system dependent on exogenous eIF3 (Altmann et al., 1997): the sucrose gradient fraction containing TIF32 possessed the biochemical activity ascribed to eIF3, *e.g.* the restoration of translation in an extract in which an endogenous eIF3 subunit had been inactivated. In addition to PRT1, TIF32 has been also shown to interact with NIP1 (Asano et. al., 1998), with HCR1 (Valášek et al., 1999) and, as described in detail thereinafter, with 40S ribosomal subunits (Valášek et al., 2003).

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4.1. Interactions of TIF32 with PRT1 RRM and HCR1

The HCR1-like domain (HLD) of TIF32 (see below) is sufficient, but not absolutely required for TIF32 binding to the RRM domain in PRT1. TIF32 can bind to the RRM domain of PRT1 through its HLD or C-terminus (Valášek et al. 2001a). The interaction between TIF32 and the RRM domain of PRT1 is conserved in the human homologues of TIF32 (p170) and PRT1 (p116) (Methot et al., 1997). In addition, HCR1 makes multiple contacts with TIF32-CTD indicating a network of physical interactions involving TIF32, HCR1 and PRT1 RRM where HCR1 probably serves to stabilize or modulate the interaction between TIF32 and PRT1 RMM which is crucial for integrity of the MFC and its association with 40S ribosomes (Valášek et al. 2001a).

4.2. Interactions of TIF32 with 40S ribosomes

As already mentioned above, deletion of the extreme N-terminus of TIF32 led to nearly complete abolishment of 40S binding by eIF3, suggesting that the TIF32-NTD plays a crucial role in association of the MFC with the 40S ribosomes. Since its removal had a minimal impact on MFC composition, it seems

likely, that the TIF32-NTD may interact directly with the 40S ribosomes. Consistent with this idea, it was discovered, that TIF32-NTD strongly interacts with the C-terminal domain of 40S ribosomal protein RPS0A and with RPS10A (Valášek et al., 2003).

RPS0A is expected to reside on the solvent side of the 40S subunit (opposite to the 60S-interface side), between the protuberance (pt) and beak (bk). Hence, binding of TIF32-NTD to RPS0A would place this portion of eIF3 on the solvent side of the 40S ribosome (Fig.3C).

In addition, it was shown that the extreme CTD of TIF32 is also critical for 40S binding in the presence of a mutant form of eIF5 that interferes with integrity of the MFC. Further studies have shown that the TIF32-CTD specifically interacts with 18S rRNA. The yeast 18S rRNA can be divided into three domains based on its tertiary structure, with domain I forming the body (b) and shoulder (sh) of the 40S ribosome, domain II forming the protuberance (pt), and domain III forming the head (h), beak (bk), and common core (helix 44) of the 40S subunit (Spahn et al. 2001). To investigate which domain mediates the interaction between 18S rRNA and the TIF32-CTD, three RNA transcripts corresponding to isolated domains I, II, and III were tested for TIF32-CTD binding. Only domain I, in particular, a short segment spanning helices 16-18 showed specific interaction with TIF32-CTD (Valášek et al., 2003).

Whereas the TIF32-NTD – RPS0A interactions places the main body of eIF3 on the solvent head side of the 40S ribosome, the interaction observed between the TIF32-CTD and 18S rRNA would provide eIF3 with access to the 60S-interface side. Helices 16 and 18 are accessible from both sides of 40S subunit, and helix 16 protrudes into the solvent. If the TIF32-CTD wraps around helix 16, it would be exposed on the interface side of the 40S subunit (Fig 3D). Thus eIF3 binds to the solvent side but has access to the 60S-interface side of the 40S ribosome (Valášek et al., 2003).

The location of eIF3 on the solvent side of the 40S makes it an ideal landing pad for regulatory factors that must be targeted to the initiation complex without interfering with the mechanics of initiation on the interface surface. It

might also permit interactions with mRNA that wraps around the back of the 40S subunit (Hinnebusch 2006).

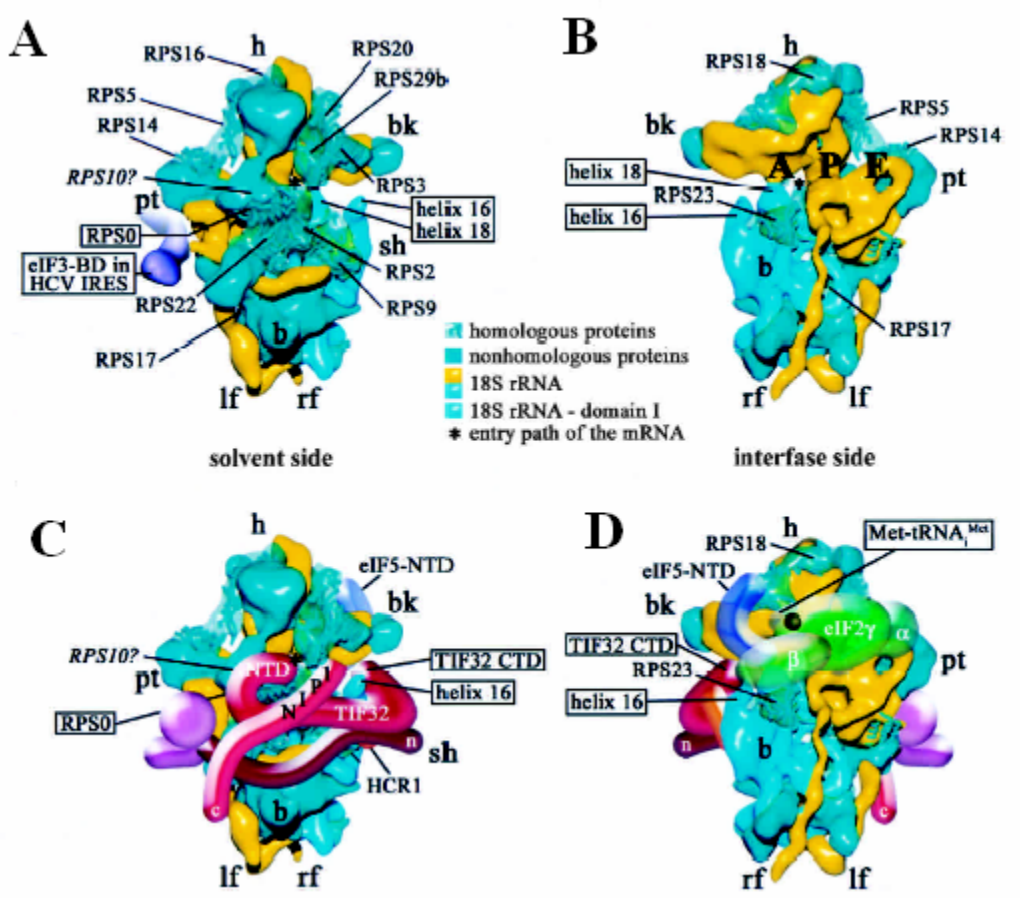

FIGURE 3. Model predicting the interaction of eIF3 with the *Saccharomyces cerevisiae* 40S ribosomal subunit. (*A*, *B*) Cryo-EM reconstruction of the *S. cerevisiae* 40S subunit docked with modified atomic models of 18S rRNA and 40S ribosomal proteins, adapted from Spahn et al. (2001a). The 40S subunit is shown from the solvent (A) or interface (B) sides, with RNA segments in yellow or turquoise and proteins in green. (C, D) Hypothetical location of the eIF3 complex on the 3D model of the 40S subunit based on the results of (Valášek et al., 2003).

5. HCR1 (high copy suppressor of RPG1)

HCR1 protein was originally isolated as a high copy suppressor of temperature sensitive (Ts$^-$) phenotype of the *rpg1-1* allele of *TIF32*, encoding the largest subunit of yeast eIF3. This high dosage HCR1-driven complementation is *rpg1-1* allele specific; HCR1 is not able to functionally replace TIF32. Functional relationship between the latter proteins was, however, further illustrated by the

fact that combining the *hcr1Δ* allele with *rpg1-1* exacerbated the growth defect conferred by *rpg1-1* (Valášek et al., 1998, 1999).

HCR1 is a non-essential substoichiometric subunit of eIF3, no more than a quarter of the total eIF3 complexes contain HCR1 and it is less tightly associated with the eIF3 than are the five core subunits (Valášek et al., 2001a). It represents the *S. cerevisiae* homologue of the p35 subunit of human eIF3, sharing 26% identity and 42% similarity with its human orthologue (Fig 5.) (Valášek et al., 1999).

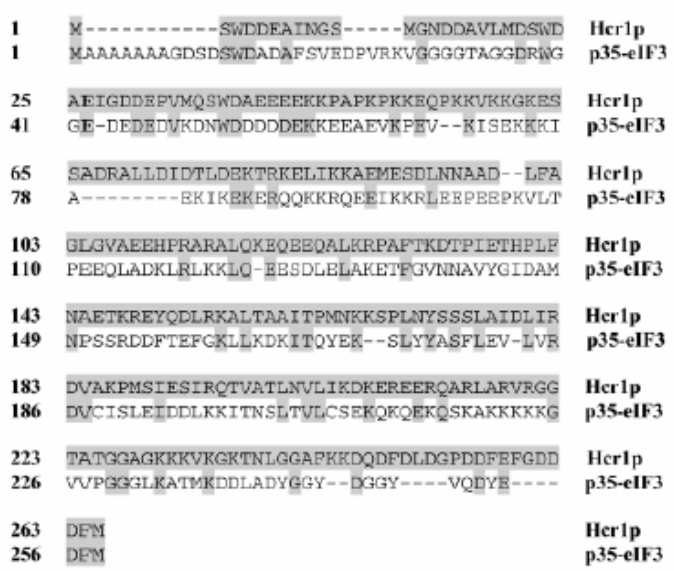

FIGURE 4. Sequence comparison between HCR1 and the p35 subunit of human eIF3. (Valášek et al., 1999)

Deletion of *HCR1* led to a slow growth phenotype and produced abnormalities in the polysome profile – an obvious decrease in the portion of large polysomes, indicating a reduced rate of translation initiation. It also reduced the amounts of eIF2 and eIF5 (by ~ 30%) and the amount of eIF1 (by ~ 75%) that coimmunoprecipitated with eIF3, suggesting that HCR1 has a significant effect on the formation and stability of the MFC (Valášek et al., 2001a).

Mammalian homolog of HCR1, p35/eIF3j, is the most important eIF3 subunit for forming a stable eIF3-40S complex, it promotes the stable association of eIF3 subcomplexes to the 40S ribosomal subunit and binds specifically to the 40S ribosomal subunit *in vitro* (Fraser et al., 2004).

5.1. Function of HCR1 in ribosome biogenesis

Deletion of HCR1 also led to a striking reduction in the abundance of free 40S subunits and a corresponding increase in free 60S subunits, suggesting that HCR1 is required for the biogenesis or stability of 40S ribosomes (Valášek et al., 2001a). There is also evidence that HCR1 can interact with 40S ribosomes independently of eIF3 and other components of the MFC (Phan et al., 2001).

HCR1 is localized predominantly in the cytoplasm, consistent with a role in the final steps of 40S biogenesis occurring in this compartment. It is required for a wild-type rate of processing of 20S pre-rRNA to mature 18S rRNA - it is an RNA binding protein, it binds to 20S pre-rRNA in the nascent 40S subunit and directly influences the efficiency of cleavage at the D processing site (Valášek et al., 2001b).

5.2. Interactions of HCR1

A network of interactions physically links HCR1 to the two largest subunits of eIF3, TIF32 and PRT1. HCR1 binds to an RNA recognition motif (RRM) in PRT1 N-terminal domain and makes multiple contacts with TIF32 C-terminal domain (Valášek et al. 2001b).

The RRM in PRT1 is necessary and sufficient for binding to HCR1, whereas TIF32 binding requires additional PRT1 residues immediately flanking the RRM. HCR1 and TIF32 can bind simultaneously to the PRT1 RRM (Valášek et al. 2001b). HCR1 specifically binds to the rear α-helices of the PRT1 RRM. Moreover, an N-terminal 69-amino acid peptide of HCR1 is sufficient for binding to PRT1 RRM and this interaction is essential for PRT1 RRM recruitment to the 40 S ribosomal subunits (ElAntak et al., 2007).

As mentioned above, HCR1 and its human homologue eIF3j can interact with 40S ribosome on its own. Through its CTD, eIF3j binds directly in the mRNA entry channel and aminoacyl (A) site, placing eIF3j on the 40S subunit interface (Fraser et al., 2007). Notably, the C-terminal 16 amino acids of eIF3j are

required for its high affinity for the 40S subunit, as cleavage with caspase-3 reduces the association of eIF3j with the 40S subunit in vitro (Fraser et al., 2004).

5.3. Sequence similarity between TIF32 and HCR1

The fact that both TIF32 and HCR1 interacted with the RNA recognition motif (RRM) in PRT1 prompted an idea that both protein could share a similar binding motif. Thus TIF32 and HCR1 sequences were aligned and it was discovered that residues 490-790 in TIF32 are 25% identical to the entire sequence of HCR1 (Fig. 5.). This internal segment of TIF32 was thus named HLD (the **H**CR1-**l**ike **d**omain) (Valášek et al., 2001a). Interestingly, the Ts$^-$ phenotype of the *rpg1-1* allele of TIF32 is conferred by a single aminoacid substitution of arginine to isoleucine at position 731 (Valášek et al., 1998) that falls into the C-terminal segment of the HLD of TIF32. Strikingly, this arginine residue is conserved in the HCR1 sequence at position 215 (Fig. 5.) and, moreover, corresponds to the most C-terminal residue of a K-x_5-ER-x_2-R motif that is completely conserved among *Saccharomyces cerevisiae* HCR1 and all known TIF32 orthologs (Fig. 6.). A similar motif (K-x_3-EK-x_2-K) also occurs in human eIF3-p35 (Valášek et al., 2001a).

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S.c. HCR1          ---------------------------------------MSWDDEAINGSMGNDD----
S.c. TIF32-HLD     IAPLRDVIMRRVFVAASQKFTTVSQSELYKLATLPAPLDLSAWDIEKSLLQAAVEDYVSI
consensus                                                  :** *    . . :*

S.c. HCR1          ----------------AVLMDSWDAEIGDDEPVMQSWDAEEEEKKPAPKPKKEQPKKVKK
S.c. TIF32-HLD     TIDHESAKVTFAKDPFDIFASTASKEVSEEENTEPEVQEEKEETDEALGPQETEDGEEKE
consensus                            :: .: . *:.::* .  . : *:**.. *  *:: :  : *:

S.c. HCR1          --------------GKESSADRALLDIDTLDEKTRKELIKKAEMESDLNNAADLFAGLGV
S.c. TIF32-HLD     EESDPVIIRNSYIHNKLLELSNVLHDVDSFNNASYMEKVRIARETLIKKNKDDLEKISKI
consensus                         .*  . ...* *:*:::: :  * :: *.     :*  **     :

S.c. HCR1          AEEHPRARALQKE-----------------QEEQALKRPAFTKDTPIETHPLFNAETKRE
S.c. TIF32-HLD     VDERVKRSQEQKQKHMEHAALHAEQDAEVRQQRILEEKAAIEAKLEEEAHRRLIEKKKRE
consensus          .:*: :    **:                 *:.   ::.*:  .   *:*  :  :.***

S.c. HCR1          YQDLR-KALTAAITPMNKK----SPLNYSSSLAIDLIRDVAKPMSIESIRQTVATLNVLI
S.c. TIF32-HLD     FEAIKEREITKMITEVNAKGHVYIDPNEAKSLDLDTIKQVIIAEVSKNKSELESRMEYAM
consensus          :: :: : :*  ** :* *       * :.** :* *::*  .   :.  :  : ::  :
                              215/731
S.c. HCR1          KDKEREERQAR------LARVRGGTATGGAGKKKVKGKTNLGGAFKKDQDFDLDGPDDFE
S.c. TIF32-HLD     KKLDHTERALRKVELPLLQKEVDKLQETDTANYEAMKKKIVDAAKAEYEARMADRKNLVM
consensus          *. :: **  *       * :  .     .:.: :.  *. :..*  : :    *  : .

S.c. HCR1          FGDDDFM-----------------------------------------------------
S.c. TIF32-HLD     VYDDYLKFKEHVSGTKESELAAIRNQKKAELEAAKKARIEEVRKRRYEEAIARRKEEIAN
consensus          . ** :
```

FIGURE 5. Sequence comparison between full-length HCR1 and the HLD of TIF32. (Valášek et al., unpublished data)

```
S.cerevisiae HCR1   DVAKPMSIESIRQTVATLNVLIKDKEREERQARLARV-----------RGGTATGGAGKK
K.lactis     HCR1   DISKPMTVENIRQTIATLNVLMKDKEREERQARLAKV-----------KGGTATGGAGKK
H.sapiens    HCR1   DVCISLEIDDLKKITNSLTVLCSEKQKQEKQSKAKKK-----------KKGVVPGGGLK-
M.musculus   HCR1   DVCISLEIDDLKKITNSLTVLCSEKQKQEKQSKAKKK-----------KKGVVPGGGLK-
B.taurus     HCR1   DVCISLEIDDLKKITNSLTVLCSEKQKQEKQSKAKKK-----------KKGVVPGGGLK-
S.cerevisiae 32-HLD QVIIAEVSKNKSELESRMEYAMKKLDHTERALRKVELPLLQKEVDKLQETDTANYEAMKK
S.pombe      32-HLD AMQIEQVEKQNKSMNERLRVIGKRIDHLERAYRREAIPLWEEDAKQQAEHDREIFYEREK
A.thaliana   32-HLD ERALTEQLKERQEMEKKLQKLAKTMDYLERAKREEAAPLIEAAYQRRLVEEREFYEREQQ
H.sapiens    32-HLD AKQVEQLEKEKKELQERLKNQEKKIDYFERAKRLEEIPLIKSAYEEQRIKDMDLWEQQEE
consensus                   ..  .    :    .  :  *:  :                        :
```

FIGURE 6. Multiple sequence alignment. The K-x_5-ER-x_2-R motif is conserved among TIF32 and HCR1 orthologs in various species. (Valášek et al., unpublished data)

As mentioned above, TIF32 HLD and HCR1 share significant sequential homology. To explore the physiological significance of the sequence similarity between HCR1 and TIF32, the *hcr1-R215I* mutant was made by substitution of arginine in position 215 with isoleucine. Strikingly, this mutation eliminated suppression of *rpg1-1* mutant, which is suppressible by wild-type HCR1 in high copy. Since the *hcr1-R215I* allele fully complemented the slow growth phenotype of *hcr1Δ* strain when expressed on a single copy number plasmid, it is can not be classified as a null allele. Thus the *R215I* substitution alters the function of HCR1 in a manner that influences its ability to compensate for defects in TIF32 when overexpressed. The fact that mutating the equivalent amino acids in HCR1 and the HLD of TIF32 altered the functions of both proteins *in vivo* supports the physiological relevance of sequence similarity between HCR1 and TIF32 (Valášek et al., 2001a).

7. Conclusion

Eukaryotic initiation factor 3 is an essential protein complex that plays role almost in all reactions of translation initiation pathway, which makes it an important goal for many studies. eIF3 probably serves as a large scaffold anchored to the back of the 40S subunit with appendages reaching round to the interface surface that bear attachment sites for the eIFs involved directly in decoding the AUG start codon. The independent interactions of eIF3 with eIF2, eIF5 and eIF1, which stabilize the yeast MFC, probably enable cooperative associations of these factors with their independent binding sites on the 40S, thereby stabilizing the 43S preinitiation complex (Hinnebusch 2006).

The largest subunit of eIF3, TIF32, is an essential protein that was shown to make direct contacts with 40S ribosomal subunit. Its human homologue, eIF3a has altered expression in cancers and has been linked to prognosis, hence it is not surprising that many studies focused their scopes on this protein (Valášek et al., 1998, 2001a; Vornlocher et al., 1999). Thus it was discovered that another eIF3 subunit, HCR1, shares a significant homology with TIF32 HLD and that this two proteins bind to the same domain (RRM) of PRT1 (Valášek et al., 2001a) and possibly also to the same site on the 40S ribosome (Rutkai and Valášek, unpublished data). To conclude all our current knowledge, the following model of their function was proposed.

As HCR1 and eIF3j were both shown to be able to interact with 40S independently of eIF3 (Phan et al., 2001; Fraser et al., 2004), it may indicate that binding of HCR1 may precede binding of eIF3. Then the role of HCR1 could be to promote association of eIF3/MFC to its initial binding site on the ribosome by interacting simultaneously with 40S (via its CTD) (Fraser et al., 2007), and PRT1-RRM and TIF32 (through its NTD) (ElAntak et. al., 2007) Actually, eIF3j-CTD (Fraser et al., 2007) was mapped to interact with the inner part of the mRNA entry channel from the ribosomal interface site and it was proposed that it regulates access to the mRNA-binding cleft. Thus HCR1 could serve to promote eIF3/MFC binding and at the same time to block premature mRNA recruitment. Upon the eIF3/MFC association, the TIF32-HLD could replace HCR1 from the mRNA-entry

channel by competing for the same binding sites and thus enable proper mRNA threading. While the extreme CTD of TIF32 was predicted to bind helices 16-18 that occur nearby the mRNA-entry channel and are accessible from both solvent and interface sides, the preceding HLD domain can be easily viewed as directly contacting the latter channel on both sides without actually protruding it. This intriguing possibility is currently under investigation in our laboratory (A. Herrmannová and L. Valášek, unpublished data).

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