# CHARLES UNIVERSITY IN PRAGUE

# FACULTY OF SCIENCE

DEPARTMENT OF GENETICS AND MICROBIOLOGY


MASTER'S THESIS

# Functional analysis of the TIF32-HLD—PRT1-RRM—HCR1 module of the yeast translation initiation factor 3


Anna Herrmannová



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Supervisor: Leoš Valášek, Ph.D.

With this, I declare that I have written this work on my own, appropriately acknowledged citations, and used no other than the listed resources and aids.

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Anna Herrmannová

# Acknowledgement

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# Abstract

The eIF3 is in yeast *S. cerevisiae* composed of five core essential subunits (TIF32, NIP1, PRT1, TIF34 and TIF35) and one nonessential substoichiometric subunit (HCR1), and as such represents the most complex initiation factor among all. Perhaps owing to that, it was shown to stimulate nearly all steps of the initiation pathway culminating in the formation of the 80S initiation complex at the AUG start codon on mRNA. Yeast eIF3 was also demonstrated to assemble together with the ternary complex, eIF1 and eIF5 into so called Multifactor complex that can exist free of ribosomes and whose formation greatly stimulates initiation efficiency. TIF32, the largest eIF3 subunit, was shown to make at least two critical contacts with the 40S ribosomal subunit and its middle domain, designated as the HLD, to share a significant sequence similarity with the HCR1 subunit. Experiments conducted here indicate that the TIF32-HLD and HCR1 share also some functional similarity as the recombinant HLD expressed under control of the HCR1 promoter in a domain-swapping experiment partially suppressed the slow growth phenotype of cells deleted for HCR1. In addition to the HLD, HCR1 also simultaneously interacts with the RRM domain of PRT1, which is considered to be the main scaffolding subunit of eIF3. The group of Dr. P.J. Lukavsky, our collaborators from MRC in Cambridge, recently solved an NMR solution structure of the human eIF3b-RRM and revealed that it creates a hydrophobic pocket to which human HCR1 sticks a critical Trp residue occurring in its N-terminal acidic region. I mutated the key residues of the "hydrophobic pocket" in yeast PRT1-RRM and showed that the mode of its interaction with HCR1 has remained evolutionary conserved. Furthermore, I also demonstrated that this contact is required for HCR1 association with the rest of eIF3 *in vivo* and, most importantly, that the hydrophobic pocket greatly promotes binding of eIF3 itself to the 40S ribosomal subunit. To conclude, my results combined with data of our collaborators from NIH in Bethesda, namely from Dr. A.G. Hinnebusch's group, indicate that the TIF32-HLD, HCR1 and PRT1-RRM together constitute an eIF3 module that significantly stimulates its association with the 40S ribosomal subunit and co-ordinates the process of stringent selection of the AUG start codon.

**Key words:** translation initiation; eIF3; HCR1; TIF32; PRT1

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# List of Abbreviations

| | |
|---|---|
| 5FOA | 5-Fluoroorotic Acid |
| β-ME | β-mercaptoethanol |
| CTD | C-terminal domain |
| $ddH_2O$ | double distilled water |
| $dH_2O$ | distilled water |
| dsDNA | double strand DNA |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| eIF | eukaryotic initiation factor |
| GDP | guanosine diphosphate |
| GST | glutathione S-transferase |
| GTP | guanosine triphosphate |
| HEPES | N-(2-Hydroxyethyl) piperazine-N-2-ethan sulfonic acid |
| HLD | HCR1 like domain |
| IGEPAL | (octylphenoxy)polyethoxyethanol |
| Ni-NTA | nickel-nitrilotriacetic acid |
| NTD | N-terminal domain |
| $m^7GpppN$ | 7-methylguanosine cap |
| Met-$tRNA_i^{Met}$ | methionyl initiator tRNA |
| MFC | multi factor complex |
| miRNA | micro RNA |
| mRNA | messenger RNA |
| $OD_{600}$ | optical density at 600 nm |
| P-site | peptidyl-tRNA binding site |
| PABP | poly A binding protein |
| PBS | Phosphate buffered saline |
| PEG | polyethylene glycol |
| Pi | inorganic phosphate |
| PMSF | phenylmethylsulfonyl fluoride |
| RRM | RNA recognition motif |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SDS | sodium dodecyl sulfate |
| TBE | Tris/Borate/EDTA |
| TBS | Tris-buffered Saline |
| TC | ternary complex |
| TG | Tris-Glycine |
| Tris | Tris(hydroxymethyl)-aminomethane |

# Introduction

Gene expression is a complex process by which the information encoded in individual genes is first transcribed into a nucleotide sequence of a messenger RNA (mRNA) and subsequently translated into a string 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.

Gene expression is regulated at multiple levels. Compared to transcriptional regulation, translational control of existing mRNA's allows for more rapid changes in concentration of the encoded proteins, it is critical for gene regulation during nutrient deprivation and stress, development and differentiation, aging and disease (reviewed in Sonenberg and Hinnebusch, 2009). Recently discovered potential for translational control of the mammalian genome by miRNAs is a fine example of its importance (Carthew and Sontheimer, 2009).

Translation takes place in cytoplasm where the mRNA associates first with small and then with large ribosomal subunits. It can be divided into four steps (initiation, elongation, termination and ribosome recycling) out of which the first step is the most critical as it serves as a target of many regulatory pathways.

Cap-dependent translation initiation requires the participation of numerous proteins and protein complexes that are in eukaryotes called eukaryotic initiation factors (eIFs). Simply speaking, 12 eukaryotic eIFs 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 AUG start codon. There has been enormous progress over the last decade in revealing the molecular mechanisms of eukaryotic translation initiation (reviewed in Sonenberg and Hinnebusch, 2009).

In Laboratory of Regulation of Gene Expression (LRGE), where this thesis was conducted, we are mostly interested in translation initiation factor 3 (eIF3), which represents by far the most complex initiation factor. In yeast *S. cerevisiae*, it is composed of 6 subunits (TIF32, PRT1, NIP1, TIF32, TIF35, HCR1) all of which have corresponding orthologs in mammalian eIF3 that contains additional 7 subunits (d, e, f, h, k, l, m) (reviewed in Hinnebusch, 2006). Given such a complexity, it is not

surprising that eIF3 was demonstrated to promote nearly all initiation steps including Met-tRNA$_i^{Met}$ and mRNA binding to 40S ribosomal subunit, subsequent scanning of mRNA, and the start codon recognition.

The aim of this work is to bring a closer insight into the functions of three subunits of eIF3, namely TIF32, PRT1 and HCR1, and molecular determinants of their interactions with each other and with the remaining eIF3 subunits. To achieve these goals I used the budding yeast *S. cerevisiae* as a model organism and a whole variety of molecular biology, genetic and biochemical techniques.

# Review of previous research

## 1. 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 to the AUG start codon in the ribosomal P-site. The main initiation pathway in eukaryotes is cap-dependent; ribosomes bind near the cap structure (m$^7$GpppN) 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 bound by eIF2 in its GTP form to produce the ternary complex (TC), and the formation of 43S preinitiation complex is stimulated by the eIF3 complex, eIF5, eIF1 and eIF1A. The 43S preinitiation complex then binds to the capped 5‘ end of mRNA, with the help of the eIF4F, eIF4B, PABP, and eIF3 (Hershey and Merrick, 2000; Sachs and Varani, 2000; Marintchev and Wagner 2005). The resulting 48S complex scans the mRNA until the AUG start codon is recognized. Scanning is promoted by eIF1, eIF1A, and eIF4F in a mammalian reconstituted systems (Pestova and Kolupaeva 2002), whereas yeast genetic data indicate that eIF3 and eIF5 also participate in vivo (Nielsen et al. 2004; Yamamoto et al. 2005). eIF5 stimulates partial GTP hydrolysis by eIF2 to GDP and Pi, but the Pi is not released from the scanning complex until the anticodon of Met-tRNA$_i^{Met}$ base-pairs with the AUG start codon, which induces dissociation or displacement of eIF1 (Algire et al. 2005; Cheung et al. 2007). Met-tRNA$_i^{Met}$ is then released into the P-site 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. Subunit joining is thought to facilitate ejection of all eIFs but eIF1A (Unbehaun et al. 2004) and eIF3 (Szamecz et al. 2008). 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).

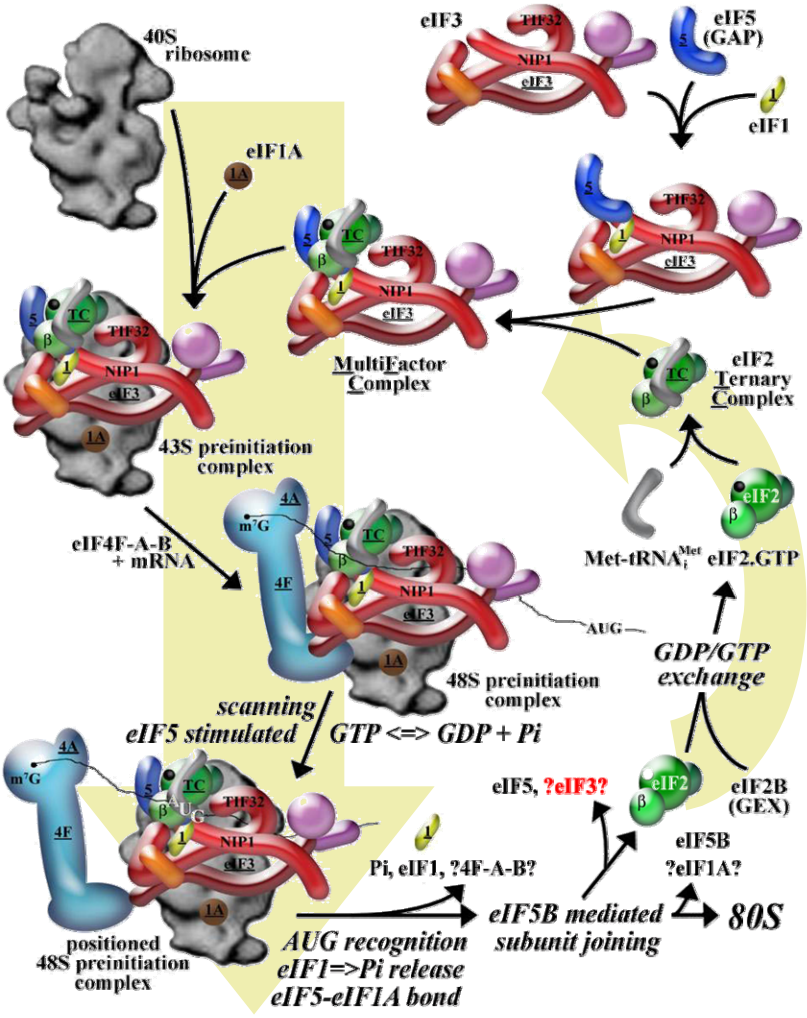


**FIGURE 1**. Translation initiation pathway. (Valášek et al., unpublished data)

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.

# 2. 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 decade 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 (Kolupaeva et. al., 2005), is involved in the dissociation of the 80S ribosome to 40S and 60S subunit (Kolupaeva et al., 2005) and is also required for the process of reinitiation (Szamecz et al. 2008).

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 ) (Tab. 1).

By contrast, eIF3 from the yeast *Saccharomyces cerevisiae* is much simpler, the core of eIF3 is thought to be composed of five essential subunits: eIF3a/TIF32 (RPG1), eIF3b/PRT1, eIF3c/NIP1, eIF3i/TIF34 and eIF3g/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 subunit 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; Nielsen et al., 2006).

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 | – | |

## 2.1 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 binding 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).

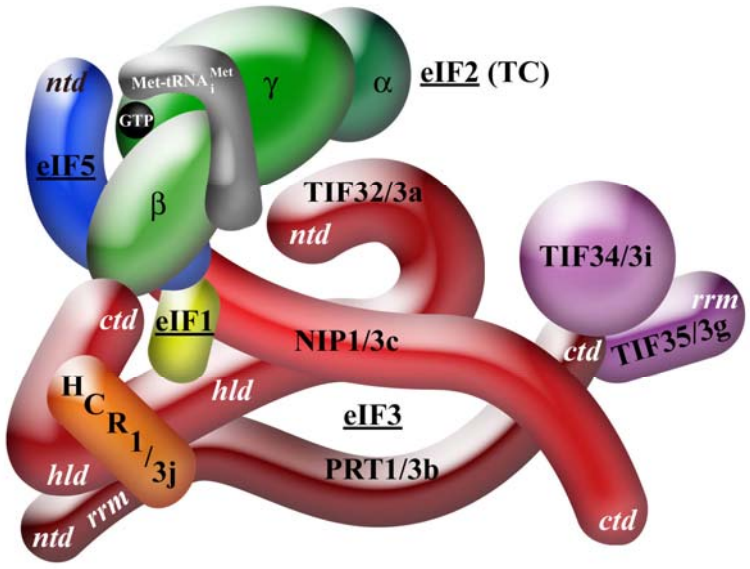


**FIGURE 2.** A 3D model of the MFC (Valášek et al. 2002)

## 3. Yeast Multifactor complex

As aforementioned, 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 in cytoplasm and be capable of cooperative binding to 40S ribosomes at once (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). As such it was proposed to represent an important intermediate in translation initiation (Hinnebusch, 2006). Indeed, the integrity of the MFC was shown to be required for optimal rates of translation initiation *in vivo* as destabilizing mutations in its individual components affected 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 also 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).

# 4. The PRT1 subunit of eIF3

PRT1 is a 90kDa subunit of eIF3 homologous to the mammalian p116 subunit. Yeast PRT1 was identified by complementation of one of the original Hartwell's temperature sensitive CDC mutants, *cdc63/prt1-1*, and it was the first gene encoding a protein involved in the initiation of protein synthesis in any eukaryotic organism to be isolated (Keierleber et al. 1986).

Among the components of eIF3, PRT1 is considered to be the major scaffolding subunit within the eIF3 complex, interacting with all other eIF3 subunits such as TIF32, TIF35, TIF34 and HCR1. PRT1 is an essential subunit and contains an N-terminal RNA recognition motif (RRM), which is crucial for the integrity of the eIF3 complex and important for 40S subunit binding (Valášek et. al., 2001a; Nielsen et. al., 2006) (see Figure 3. for schematic interaction map). Studies using deletion mutations in individual eIF3 subunits and yeast two hybrid analysis showed that the 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 (Valášek et. al., 2001a).

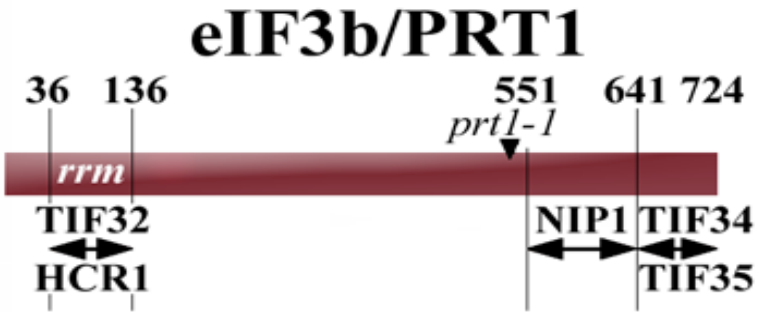


**FIGURE 3.** Schematic of PRT1 with arrows delimiting minimal binding domains for indicated proteins. RRM domain and position of prt1-1 mutation are indicated (Valášek, unpublished data).

The solution structure of the RRM of human homolog of PRT1 (eIF3b-RRM) was determined by NMR spectroscopy. The structure reveals a noncanonical RRM with a negatively charged surface in the beta-sheet area contradictory with potential RNA binding activity (Fig. 4.) (ElAntak et. al., 2007).

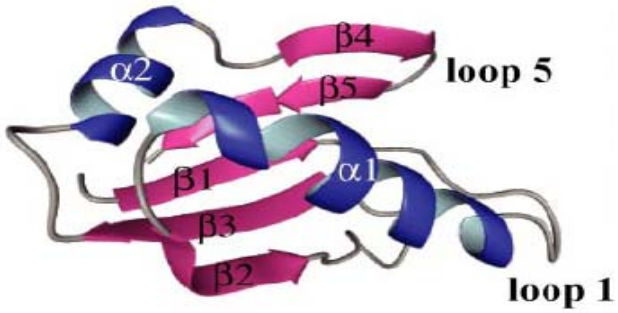


**FIGURE 4.** Solution structure of the human eIF3b-RRM (residues 185-264 are displayed). Ribbon diagram of the eIF3b-RRM structure. The secondary structure elements are numbered. Adapted from (ElAntak et. al., 2007)

C-terminal domain of PRT1 does not contain any recognizable domain or motif, nevertheless, it is critical for the PRT1 function because it anchors two other essential subunits of unknown function, TIF35 and TIF34 (Asano et al. 1998; Valášek et al. 2001a). The binding site for NIP1 is located just upstream from the TIF34-TIF35 binding site at the extreme 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 (Valášek et al., 2002).

# 5. 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 subunit 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).

It was shown that the TIF32 protein is required for translation initiation *in vivo* and *in vitro* and interacts with the 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) (see Figure 5. for schematic interaction map).

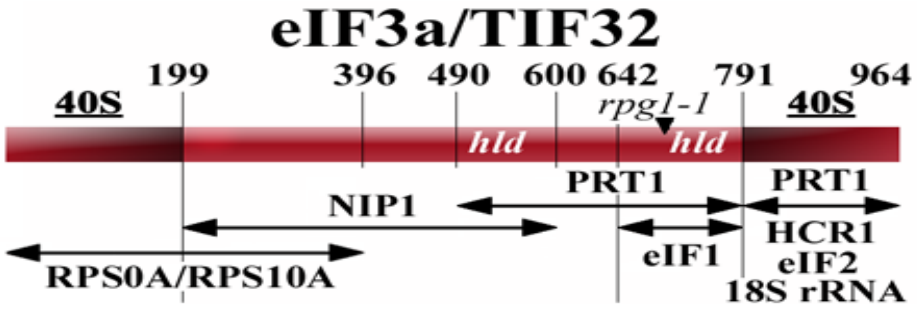


**FIGURE 5.** Schematic of TIF32 with arrows delimiting minimal binding domains for indicated proteins. HCR1-like domain (HLD) and position of rpg1-1 mutation are indicated (Valášek, unpublished data).

## 5.1 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).

## 5.2 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 the TIF32-NTD strongly interacts with the C-terminal domain of 40S ribosomal protein RPS0A and with RPS10A (Valášek et al., 2003, Szamecz et al., 2008).

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.6C).

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 6D). Thus eIF3 binds to the solvent side but has access to the 60S-interface side of the 40S ribosome (Valášek et al., 2003).

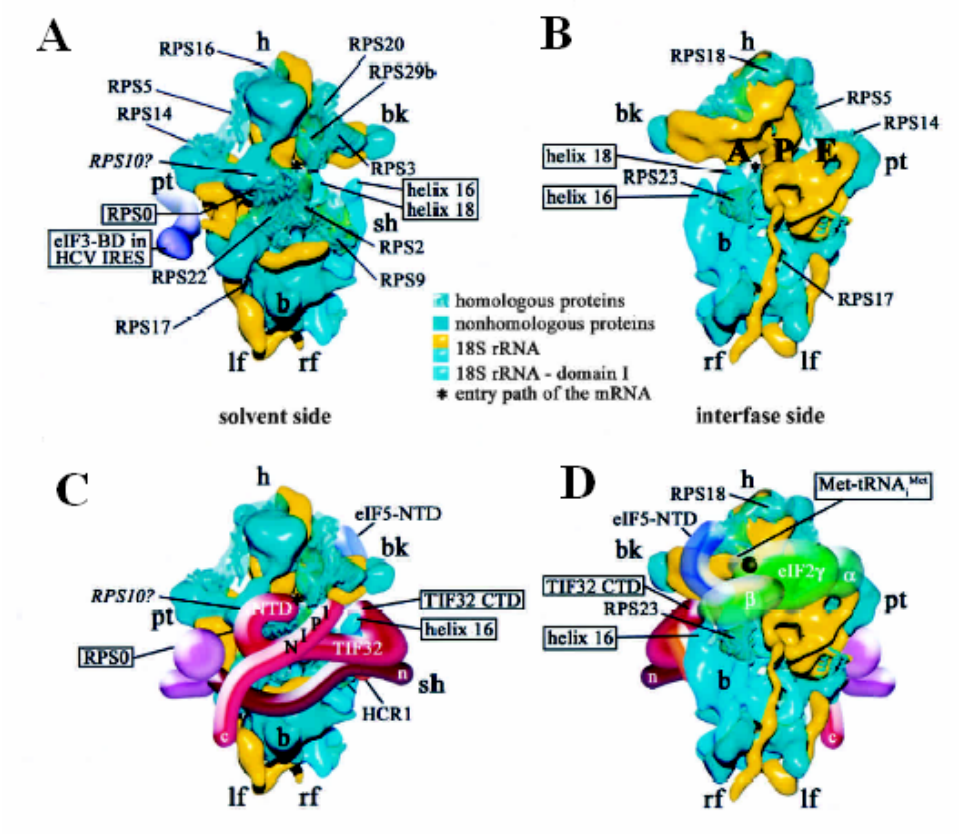


**FIGURE 6.** Model predicting the interaction of eIF3 with the *Saccharomyces cerevisiae* 40S ribosomal subunit. (*A, B*) Cryo-EM reconstruction of the *S. cerevisiae* 40S subunit docked with modified atomic models of 18S rRNA and 40S ribosomal proteins, adapted from Spahn et al. (2001). The 40S subunit 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).

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).

## 6. 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 7.) (Valášek et al., 1999).


**FIGURE 7.** Sequence comparison between HCR1 and the p35 subunit of human eIF3. (Valášek et al., 1999)

Deletion of *HCR1* led to a slow growth ($Slg^-$) 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). Deletion of *HCR1* also reduced the 40S association of eIF3 core subunits, suggesting that HCR1 contributes to 40S binding by yeast eIF3 *in vivo* (Nielsen et al., 2006).

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).

## 6.1 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).

## 6.2 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. HCR1 and TIF32 can bind simultaneously to the PRT1 RRM (Valášek et al. 2001b).

Based on unpublished observations from the group of our collaborator P. J. Lukavsky, a human homolog of HCR1, eIF3j, specifically binds to the hydrophobic pocket formed by helix α1 (α1) and loop 5 (L5) of the human eIF3b-RRM (Fig. 8) by its N-terminal acidic motif centered by the conserved tryptophan (Trp52) residue (L. ElAntak and P.J. Lukavsky, unpublished observations).

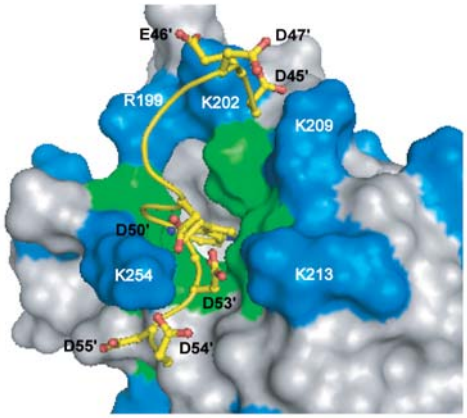


**FIGURE 8.** Surface representation of the contacts between human eIF3j peptide and human eIF3b-RRM. Green and blue surfaces indicate hydrophobic and basic eIF3b-RRM residues, respectively. Basic residues are labelled. eIF3j peptide is shown as a ribbon ball-and-stick representation, and most of its residues are numbered with primed numbers. The lowest-energy structure of eIF3b-RRM bound to eIF3j peptide is shown. (L. ElAntak and P.J. Lukavsky, unpublished observations).

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).

## 6.3 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. 9.). 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. 9.) and, moreover, corresponds to the most C-terminal residue of a K-$x_5$-ER-$x_2$-R (KERR) motif that is completely conserved among *Saccharomyces cerevisiae* HCR1 and all known TIF32 orthologs (Fig. 10.). A similar motif K-$x_3$-EK-$x_2$-K (KEKK) also occurs in human eIF3-p35 (Valášek et al., 2001a).

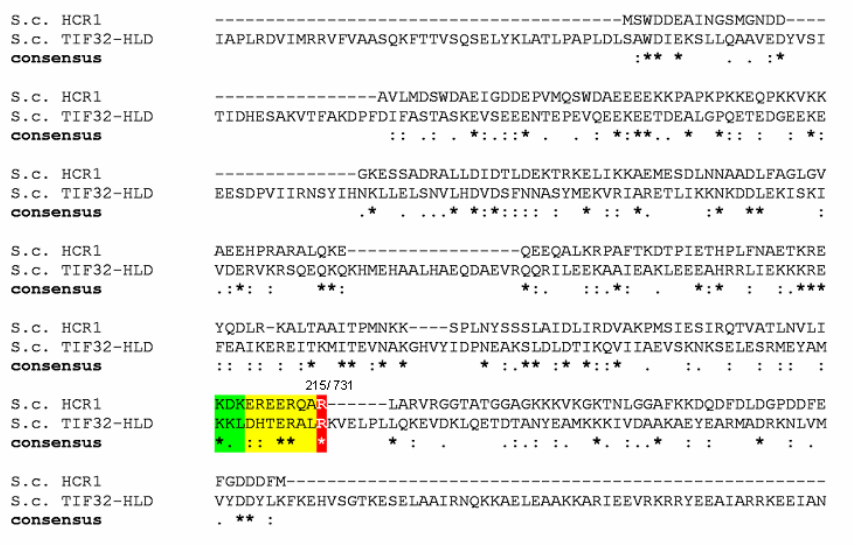


**FIGURE 9.** Sequence comparison between full-length HCR1 and the HLD of TIF32. (Valášek et al., unpublished data)

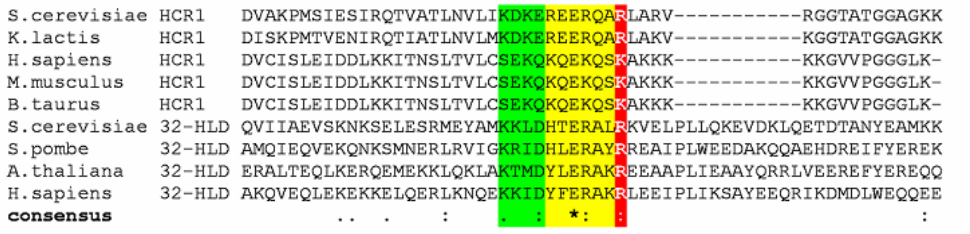


**FIGURE 10.** Multiple sequence alignment. The K-$x_5$-ER-$x_2$-R motif is conserved among TIF32 and HCR1 orthologs in various species. (Valášek et al., unpublished data)

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 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).

# Material and methods

## 7. Laboratory equipment

### 7.1 Centrifuges

Beckman Coulter Allegra® X-15R Centrifuge, rotor SX 4750A

Beckman Coulter optima$^{TM}$L-90K Ultracentrifuge, rotor SW 41

Biosan Centrifuge/vortex Multi Spin MSC-300

Eppendorf Centrifuge 5414D

### 7.2 Electrophoresis

Bio-Rad Criterion Cell

Bio-Rad Mini-Sub Cell GT Cell

Bio-Rad Wide-Sub Cell GT Cell

Bio-Rad PowerPac Basic power supply

Bio-Rad PowerPac HC power supply

Bio-Rad PowerPac Universal power supply

### 7.3 Other equipment

Beckman Coulter DU®530 Life Science UV/VIS Spectrophotometer

BioComp Gradient Master

Bio-Rad Gene Pulser Xcell Electroporation System

Biosan Bio RS-24 Rotator

Biosan Mini Rocker MR-1

Biosan Multi RS-60 Rotator

Brandel BR-188 Density Gradient Fractionation System

Eppendorf Mastercycler ep gradient S

Eppendorf Thermomixer Comfort

Syngene G:BOX iChemi – gel documentation and analysis system

Multitron INFORS HT shaker

Scientific Industries Vortex genie 2

Thermo Scientific SGD2000 Slab Gel Dryer

# 8. Chemicals

10x TBE (Bio-Rad)

10x TBS (Bio-Rad)

10x TG (Bio-Rad)

10x PBS (Bio-Rad)

Agar (Serva)

Bacto Peptone (Becton, Dickinson and Company)

Bacto Tryptone (Becton, Dickinson and Company)

Bacto Yeast Extract (Becton, Dickinson and Company)

Bio-Rad Protein Assay Kit (Bio-Rad)

Bromophenol Blue (Sigma)

β-Mercaptoethanol (Sigma)

Complete Protease Inhibitor Mix tablets (Roche)

Criterion Precast Gels, 4-20% Tris-HCl, 1mm, 12+2 Well Comb, 45 µl (Bio-Rad)

D-glucose (Lachner)

ECL™ Anti Rabbit IgG, HRP-linked whole antibody (from donkey) (GE Healthcare)

EDTA (Sigma)

EDTA-free Complete Protease Inhibitor Mix tablets (Roche)

Electroporation cuvettes (Eppendorf)

EP-MAX™ 10B Competent Cells (Bio-Rad)

Ethidium bromide (Serva)

Gel Code® Blue Stain Reagent (Thermo Scientific)

Glutathione Sepharose™ 4B (GE Healthcare)

HEPES (Serva)

Imidazole (Fluka)

Igepal CA-630 (Sigma)

Lithium acetate dihydrate (Sigma)

Ni-NTA silica (Qiagen)

PEG (Fluka)

PMSF (Serva)

SDS solution 20% (Sigma)

SeaKem® LE Agarose (Lonza)

Sodium Floride (Fluka)

Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen)

Sucrose (Fluka)

SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific)

Tris (Serva)

Triton X-100 (Sigma)

Tween 20 (Sigma)

# 9. Solutions

**B buffer for GST pull-downs:** 20mM HEPES (pH 7,5); 75mM KCl; 0,1mM EDTA; 2,5mM $MgCl_2$; 0,05% IGEPAL

**BB buffer for Nickel affinity purification:** 20 mM Tris-HCl (pH 7,5); 100 mM KCl; 5 mM $MgCl_2$; 0,5 mM β-ME; 1mM PMSF; 20 mM imidazole; 10% (v/v) glycerol, 1 x EDTA-free Complete Protease Inhibitor Mix tablets

**Blotting buffer (1000 ml for 1 blotting):** 700 ml $dH_2O$; 200 ml methanol; 100 ml 10xTG

**E buffer:** 20mM Tris-HCl (pH 7,5); 100 mM KCl; 5mM $MgCl_2$; 0,5 mM β-ME; 1mM PMSF; 250 mM imidazole; 10% (v/v) glycerol, 1 x Complete Protease Inhibitor Mix tablets

**GA solution:** 20mM Tris-HCl (pH 7,5); 50 mM KCl; 10 mM $MgCl_2$

**GB solution:** 20mM Tris-HCl (pH 7,5); 50 mM KCl; 10 mM $MgCl_2$; 45% sucrose

**GF buffer:** 25 ml GA; 5mM NaF; 1mM DTT; 1mM PMSF; 1 μg/ml Aprotinin; 1 μg/ml Leupeptin; 1 μg/ml Pepstatin; 1 tablet of Complete EDTA-free Protease Inhibitor Mix

**Sample buffer for agarose gel electrophoresis 6x:** 30% glycerol; 0,25% bromophenol blue

**Sample loading buffer for SDS-PAGE 4x:** 1M Tris-HCl (pH 6,8); 40% glycerol; 8% SDS; 0,06% bromophenol blue; 1,47% β-ME

**SDS-Loading Buffer 6x**: 3,3 ml 1 M Tris (pH 7,5); 6 ml glycerol; 1,2 g SDS; heat up and add 150 µl β-ME and a little bit bromophenol blue

**SDS-PAGE running buffer:** 1xTG; 0.1% SDS

**TBS-T buffer:** 1x TBS; 0,1% Tween 20

## 10. *Saccharomyces cerevisiae* strains

| Strain | Genotype | Plasmid | Source |
|---|---|---|---|
| H423 | MATa, leu2-3, -112, ura3-52, trp1, gcn2-del', tif32-del' (TIF32, URA3) | TIF32, AmpR, URA3 | Klaus Nielsen |
| H425 | MATa, leu2-3, -112, ura3-52, trp1, gcn2-del', prt1 (PRT1, URA3) | PRT1, AmpR, URA3 | Klaus Nielsen |
| H428 | MATa, PRT1, leu2-3, -112, ura3-52, hcr1Δ | NONE | Klaus Nielsen |

## 11. *Escherichia coli* strains

| Strain | Genotype | Source |
|---|---|---|
| DH5α | fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | Invitrogen |
| BL21 | B F- dcm ompT hsdS($r_B$- $m_B$-) galλ(DE3) | Stratagene |

## 12. Plasmids

| Plasmid | Description | Source or reference |
|---|---|---|
| YEpLVHCR1 | Wt HCR1 on high copy LEU2 plasmid from YEplac181; AmpR | Valášek et al. 1999 |
| YEpHCR1-DS | HCR1 with artificial BamHI and NcoI sites introduced before AUG and the stop codon, respectively on high copy LEU2 plasmid from YEplac181; AmpR | This study |

| | | |
|---|---|---|
| pRSTIF32-His | C-term $His_8$ tagged TIF32 on low copy LEU2 plasmid from pRS315; AmpR | Valášek et al. 2002 |
| pRSTIF32-His-DS | C-term $His_8$ tagged TIF32 with artificial BamHI and NcoI site introduced before and after the HLD domain on low copy LEU2 plasmid from pRS315; AmpR | This study |
| YEpHLD | TIF32's HLD placed under the HCR1 promoter using BamHI-NcoI artificial sites on high copy LEU2 plasmid from YEplac181; AmpR | This study |
| pRSTIF32-HCR1-His | TIF32 with HLD replaced by HCR1 using BHI-NcoI artificial sites on low copy LEU2 plasmid from pRS315; AmpR | This study |
| YCpTIF32-His | C-term $His_8$ tagged TIF32 on single copy URA3 plasmid from YCplac33; AmpR | Valášek et al. 2003 |
| pRS315 | low copy cloning vector, LEU2 ; AmpR | Sikorski and Hieter 1989 |
| YEplac181 | high copy cloning vector, LEU2; AmpR | Gietz and Sugino 1988 |
| pRSPRT1-His-XS | C-term $His_8$ tagged PRT1 on low copy LEU2 plasmid from pRS315; AmpR | This study |
| pKHN45 (= pRSPRT1-His) | C-term $His_8$ tagged PRT1 on low copy LEU2 plasmid from pRS315; AmpR | Nielsen et al. 2006 |
| pRSPRT1-AALA-His | C-term $His_8$ tagged PRT1 containing AALA mutation on low copy LEU2 plasmid from pRS315; AmpR | This study |
| pRSPRT1-LFSK-His | C-term $His_8$ tagged PRT1 containing LFSK mutation on low copy LEU2 plasmid from pRS315; AmpR | This study |
| pRSPRT1-α1L5-His | C-term $His_8$ tagged PRT1 containing α1L5 mutation on low copy LEU2 plasmid from pRS315; AmpR | This study |
| pT7-7 | Contains the T7 RNA polymerase promoter for in vitro transcription and translation; AmpR | Tabor and Richardson 1987 |
| pT7-prt1-rrm (ΔA) | PRT1[1-136]ORF cloned under T7 promoter | Valášek et al. 2001a |
| pT7-prt1-rrm-AALA | PRT1[1-136]ORF containing AALA mutation cloned under T7 promoter | This study |
| pT7-prt1-rrm-LFSK | PRT1[1-136]ORF containing LFSK mutation cloned under T7 promoter | This study |
| pT7-prt1-rrm-α1L5 | PRT1[1-136]ORF containing α1L5 mutation cloned under T7 promoter | This study |
| pGEX-5x-3 | GST fusion expression vector; AmpR | Smith and Johnson 1988 |
| pGEX-HCR1 | GST-HCR1 fusion plasmid from pGEX-5x-3; AmpR | Valášek et al. 2001a |
| CR52 | wt PRT1 on single copy URA3 plasmid | Cigan et al. 1991 |

# 13. Oligonucleotides

| Name | Sequence (5´ to 3´) |
|---|---|
| DS HCR1-BHI | CAAAAAAAAAAAAGTAATAAAAGGATCCATGTCTTGGGACGA CGAAGC |
| DS HCR1-NcoI | TTCGGTGATGACGACTTTATGCCATGGTAATCTGCTTATTGTTTC TTTGC |
| DS TIF32-BHI | GATTATGTTTCAATTACCATCGGATCCATGGATCATGAATCTGCT AAGGTTACA |
| DS TIF32-NcoI | ATGGTCTATGATGATTATTTGCCATGGAAGTTCAAGGAGCATGTT TCG |
| AH-PRT1-BamHI | TCACTTGGGATCCATCTGGTA |
| AH-PRT1-NotI-R | ATATGCGGCCGCGGATCTTTAGTGGTGGTGGTGGTGGTGGTGGT |
| AH-PRT1-ApaI | GGTACCGGGCCCCCCCTC |
| LV-RRM-AALA-R | TGCTTTTAAATCCAGTCTTTTACC |
| LV-RRM-AALA | GGTAAAAGACTGGATTTAAAAGCAGCTTTGGCACTTTATACTATG AAAGATGTT |
| AH-PRT1-XbaI-R | AGTTCTCTCTAGATTCCACCA |
| AH-PRT1-A1B-R | AGAAGTCAAAGCCTTTTTCAAAAC |
| AH-PRT1-A1B | GTTTTGAAAAAGGCTTTGACTTCTGCAGCAGCTGAAGCTGGTAAA GTTGTTAACATGGAA |
| LVPNDEI-724 | CCACGCATATGACTACCGAGACTTTCGAA |
| LVPC136-724 | CCTAAAAGCTTGAATTCGGTGTCAAAGTCGTC |

# 14. Cultivation media

## 14.1 Bacterial cultivation media and plates

**LB medium :** 10 g/l Bacto tryptone**;** 5 g/l Bacto yeast extract**;** 5 g/l NaCl (if needed add Ampicilin to final concentration 150 μg/ml).

**LB/Amp plates:** 10 g/l Bacto tryptone; 5 g/l Bacto yeast extract**;** 5 g/l NaCl; 25 g/l agar; Ampicilin to final concentration 50 mg/ml.

**SOC medium:** 20 g/l Bacto tryptone; 5 g/l Bacto yeast extract; 0,6 g/l NaCl; 0,2 g/l KCl; 3,5 g/l glucose.

## 14.2 Yeast cultivation media and plates

**YPD medium:** 20g/l Bacto peptone, 10g/l Bacto yeast extract, 20g/l glucose

**YPD plates:** 20g/l Bacto peptone, 10g/l Bacto yeast extract, 20g/l glucose, 25g/l agar

**SD medium:** 1,45g/l YNB (yeast nitrogen base without AA and ammonium sulfate), 5g/l ammonium sulfate, 20g glucose

**SD plates:** 1,45g/l YNB (yeast nitrogen base without AA and ammonium sulfate), 5g/l ammonium sulfate, 20g glucose, 25g/l agar

**5-FOA plates:** 1,44g/l YNB (yeast nitrogen base without AA and ammonium sulfate), 5g/l ammonium sulfate, 20g glucose, 25g/l agar, 20mM uracil, 1g/l 5-FOA

# 15. Bacterial and yeast cultivation

## 15.1 Bacterial cultivation

Bacteria were grown in liquid media at 37°C while shaking or on agar plates at 37°C in incubator.

## 15.2 Yeast cultivation

Yeast were grown in liquid media at 30°C while shaking or on agar plates at 30°C in incubator. Temperature sensitivity was tested at 37°C.

## 15.3 Strain storage

Bacterial strains were stored for short terms on agar plates at 4°C, for long terms at -80°C in 40% glycerol. Yeast strain were stored for short terms on agar plates at 4°C, for long terms at -80°C in 20% glycerol.

## 15.4 Doubling time calculation

Doubling time was calculated for specific strains in this study according to formula: $[\ln(2)/\ln(OD_{END}/OD_{BEGIN})]$*time of growing

# 16. DNA manipulation

## 16.1 Plasmid DNA isolation - QIAprep Spin Miniprep Kit (Qiagen)

The miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane in the presence of high salt. Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions. DNA is adsorbed onto silica membrane while RNA, cellular proteins and metabolites are not retained on the membrane but are found in the flow-through.

**Kit contains:** QIAprep Spin Columns, Buffer P1, Buffer P2, Buffer N3, Buffer PB, Buffer PE, RNase A

**Protocol:**

- inoculate *E. coli* cells bearing plasmid of your interest into 2 ml of LB/Amp medium and grow them over night
- pellet the cells by centrifugation at 13000 rpm for 30 seconds
- resuspend the pellet in 250 µl Buffer P1 (containing RNase A) and transfer to a microcentrifuge tube
- add 250 µl Buffer P2 (lysis buffer) and mix thoroughly by inverting the tube 4-6 times
- add 350 µl Buffer N3 (neutralization buffer) and mix immediately and thoroughly by inverting the tube 4-6 times
- centrifuge at 13000 rpm for 10 minutes
- apply the supernatant to the spin column by pipetting
- centrifuge at 13000 rpm for 30 seconds, discard the flow-through
- wash the spin column by adding 0,75 ml of Buffer PE and centrifuging at 13000 for 30 seconds
- discard the flow-through and spin for an additional 1 minute to remove residual wash buffer
- place the spin column in a clean microcentrifuge tube

- to elute DNA add 50 ml water to the center of spin column, let stand for 1 minute and centrifuge at 13000 rpm for 1 minute, usual concentration of DNA isolated by this method is 0,5 μg/μl

## 16.2 DNA modifications

Digestion by restriction endonucleases was done according to instructions provided by Roche and NEB companies and supplied reaction buffers were used.

For ligation reactions was used T4 DNA Ligase (Roche) and supplied reaction buffer. T4 DNA ligase catalyzes the formation of phosphodiester bonds between neighbouring 3´-hydroxyl and 5´-phosphate ends in dsDNA. This enzyme will join blunt end and cohesive end termini as well as repair single stranded nicks in ds DNA.

## 16.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field. For estimating size and concentration of DNA on the gel was used 1 Kb Plus DNA Ladder (Invitrogen).

**Protocol:**

- dissolve 1% of agarose in 1x TBE by heating in microwave oven
- after cooling down to approximately 60°C add ethidium bromide to final concentration 0,5 μg/ml
- stir the solution to disperse the ethidium bromide, then pour it into the gel rack and insert the comb
- when the gel has cooled down and become solid, remove the comb and put the gel with the rack into a tank with 1xTBE
- load the samples mixed with Sample buffer for agarose gel electrophoresis
- apply current, voltage 5 V/cm for approximately 1 hour

## 16.4 Isolation of DNA from the gel - QIAEX®II Gel extraction Kit (Qiagen)

Extraction and purification of DNA fragments are based on solubilization of agarose and selective adsorption of DNA to the silica-gel particles in the presence of high salt. All impurities such as agarose, proteins, salts and ethidium bromide are removed during washing steps. Elution of the DNA is accomplished with a low-salt solution such as water.

**Kit contains:** QIAEX II Suspension, Buffer QX1 and Buffer PE

**Protocol:**

- excise the DNA band from agarose gel with a clean scalpel
- weigh the gel slice in a microcentrifuge tube and add 3 volumes of Buffer QX1 to 1 volume of gel
- add 15 µl QIAEX II Suspension to the sample and mix
- incubate at 50°C for 10 minutes, mix every 2 minutes to keep QIAEX II in suspension
- centrifuge at 13000 rpm for 30 seconds, remove supernatant
- wash the pellet with 500 µl of Buffer QX1
- wash the pellet twice with 500 µl of Buffer PE
- air-dry the pellet for 10 – 15 minutes until the pellet becomes white
- to elute DNA add 20 µl of water, resuspend the pellet by vortexing and incubate in 50°C for 10 minutes
- centrifuge at 13000 rpm for 30 seconds and save the supernatant

## 16.5 QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene)

The QuikChange® Multi site-directed mutagenesis kit offers a method for site-directed mutagenesis of plasmid DNA at several different sites simultaneously. A single mutagenic oligonucleotide is required to mutagenize each site, using a dsDNA template and following three-step procedure. Step 1 uses a thermal cycling procedure to achieve multiple rounds of mutant strand synthesis. First the mutagenic primers

are annealed to denaturated template DNA. DNA polymerase then extends the mutagenic primers generating dsDNA molecules with one strand bearing multiple mutations and containing nicks. The nicks are sealed by components in the enzyme blend. In step 2, the thermal cycling reaction products are treated with *DnpI* restriction endonuclease, which is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to digestion. In Step 3, the reaction mixture, enriched for mutated single stranded DNA, is transformed into XL10-Gold® ultracompetent cells, where the mutant ssDNA is converted into dsDNA *in vivo*. Plasmid DNA may then be isolated from the transformants and analyzed.

**Kit contains:** QuikChange® Multi enzyme blend, 10x QuikChange® Multi reaction buffer, QuikSolution, dNTP Mix, *DpnI* restriction enzyme, XL10-Gold® ultracompetent cells, XL10-Gold® β-mercaptoethanol mix

**Protocol:**

**Step 1**

- prepare the dsDNA template by standard miniprep protocol
- prepare the mutant strand synthesis reactions for thermal cycling as indicated below

| | |
|---|---|
| 10x QuikChange® Multi reaction buffer | 2,5 µl |
| QuikSolution | 0,5 µl |
| Template ds DNA | 100 ng |
| Primer 1 | 100 ng |
| Primer 2 | 100 ng |
| dNTP Mix | 1 µl |
| QuikChange® Multi enzyme blend | 1 µl |
| dd$H_2O$ to a final volume of | 25 µl |

- cycle the reactions using the parameters below

| | | |
|---|---|---|
| 95°C | 1 minute | |
| 95°C | 1 minute | 30x |
| 55°C | 1 minute | |
| 65°C | 2 minutes/kb of plasmid length | |
| 4°C | hold | |

**Step 2**

- add 1,5 μl of *DpnI* to each amplification reaction, mix by pipetting and incubate at 37°C for 90 minutes

**Step 3**

- thaw the XL10-Gold® ultracompetent cells on ice
- for each mutagenesis reaction to be transformed aliquot 45 μl of the ultracompetent cells to a prechilled microcentrifuge tube
- add 2 μl of the β-ME mix to the cells
- swirl the contents of the tube, incubate 10 minutes on ice swirling every 2 minutes
- add 5 μl of the *DpnI* – treated DNA from the mutagenesis reaction
- swirl the transformation reaction and incubate on ice for 30 minutes
- preheat SOC medium to 42°C
- heat-pulse the tubes in a 42°C water bath for 30 seconds and then incubate on ice for 2 minutes
- add 0,5 ml of preheated SOC medium and incubate at 37°C for 1 hour with shaking
- plate the transformation reaction on LB/Amp plates

## 16.6 Polymerase chain reaction (PCR)

Polymerase chain reaction was used to construct several recombinant vectors in this study. Typical PCR amplification program is shown below. Annealing temperature was adjusted according to the melting temperature of primers used in the reaction. Elongation time was adjusted according to the length of a fragment being amplified (1 minute/1kb length).

| | | | |
|---|---|---|---|
| Initial denaturation | 95°C | 5 minutes | |
| Denaturation | 95°C | 1 minute | |
| Annealing | 55°C | 1 minute | 25x |
| Elongation | 72°C | 1 minute | |
| Final elongation | 72°C | 5 minutes | |
| | 4°C | hold | |

Composition of PCR reactions: 1x ThermoPol Reaction Buffer (NEB), 800 μM dNTP mix (200 μM of each), ~20 ng template DNA, 2 μM primers, 1 Unit VentR® DNA Polymerase (NEB), dd$H_2O$ to a final volume of 50 μl.

## 16.7 Sequencing

All sequencing analysis were conducted at Centre Of DNA Sequencing at Institute of Microbiology, Academy of Science of Czech Republic. For sequencing reaction was used template DNA prepared by QIAprep Spin Miniprep Kit and specific primers in concentration 10 pmol/μl. The sequencing data were analyzed with BioEdit and Clone Manager programmes.

## 16.8 Introducing DNA into target cells

### 16.8.1 Transformation of *E. coli* by electroporation method

This method was used for introducing newly constructed vectors directly from the ligation reaction.

**Protocol:**

- thaw electrocompetent cells (EP-MAX™ 10B Competent Cells) on ice
- mix 20 μl of the cells and 1 μl of the ligation reaction in a clean microcentrifuge tube
- transfer the mixture into precooled electoporation cuvette
- apply the pulse in the Gene Pulser Xcell Electroporation System using the „E. coli 1mm“ setting (conditions : 1,8 kV; 25 μF; 200 Ω)
- immediately add 180 μl of SOC medium and transfer to a clean microcentrifuge tube
- incubate for 30 minutes at 37°C with shaking
- spread on LB/Amp plate

### 16.8.2 Transformation of *E. coli* by heat-shock method

This method was used for introducing plasmid DNA isolated by QIAprep Spin Miniprep Kit.

**Protocol:**

- thaw competent cells (Subcloning Efficiency$^{TM}$ DH5$\alpha^{TM}$ Competent Cells) on ice
- mix 100 µl of the cells with 1 µl of plasmid DNA (500 ng) and icubate on ice for 30 minutes
- heat shock cells at 42°C for 30 seconds
- add 200 µl of SOC medium and incubate for 30 minutes at 37°C with shaking
- spread on LB/Amp plate

### 16.8.3 Transformation of *S. cerevisiae* by LiAC transformation method

**Protocol:**

- grow the *S. cerevisiae* strain you want to transform in 50 ml of liquid media (SD or YPD) to $OD_{600}$ of approximately 0,5
- centrifuge at 2500 rpm for 3 minutes and discard supernatant
- wash the cells in 1 ml 1xLiAc/TE
- resuspend the cells in 200 µl 1xLiAc/TE
- add 20 µl of Salmon sperm DNA (Invitrogen)
- mix 50 µl of the cells with 1 µl of plasmid DNA (500 ng) and 300 µl PEG/LiAC/TE solution
- incubate at 30°C for 30 minutes with shaking
- incubate at 42°C for 15 minutes without shaking
- add 1 ml $dH_2O$ and centrifuge at 2500 rpm for 3 minutes, discard supernatant
- add 200 µl $dH_2O$ and spread on appropriate plate

# 17. Protein manipulation

## 17.1 WCE preparation for Nickel affinity purification

**Protocol:**

- grow yeast cell culture in 100 ml of liquid media to $OD_{600}$ of 1
- centrifuge at 4000 rpm for 5 minutes at 4°C, discard supernatant
- wash with 10 ml of ice cold $dH_2O$
- from this point always work on ice
- resuspend the cells in 1 ml per gram (wet weight) of cells in BB buffer
- add 4 mm diameter acid-washed glass beads equal to one half of the total volume of resuspended cells
- vortex for 30 seconds followed by 1 minute on ice, repeat 5 times
- centrifuge at 3000 rpm for 5 minutes at 4°C
- transfer supernatant into precooled microcentrifuge tube
- centrifuge at 13000 rpm for 2 minutes at 4°C
- carefully transfer supernatant into new precooled microcentrifuge tube
- centrifuge at 13000 rpm for 10 minutes at 4°C
- save the supernatant

## 17.2 WCE preparation for formaldehyde cross-linking followed by sucrose density gradient centrifugation experiment

**Protocol:**

- grow yeast cell culture in 100 ml of liquid media to $OD_{600}$ of 1
- put ice in a centrifuge tube ( 50 g per 100 ml of culture)
- add culture and shake vigorously
- immediately add 5,4 ml per 100 ml of culture of formaldehyde, shake and incubate on ice for 1 hour
- add 10 ml per 100 ml of culture of 2,5M glycine and shake
- centrifuge at 3500 rpm for 5 minutes at 4°C
- resuspend the cells in 10 ml of GF buffer
- centrifuge at 2500 rpm for 3 minutes at 4°C

- resuspend the cells in 1 ml per gram (wet weight) of cells in GF buffer
- add 4 mm diameter acid-washed glass beads equal to one half of the total volume of resuspended cells
- vortex for 30 seconds followed by 1 minute on ice, repeat 8 times
- centrifuge at 3000 rpm for 5 minutes at 4°C
- transfer supernatant into precooled microcentrifuge tube
- centrifuge at 13000 rpm for 2 minutes at 4°C
- carefully transfer supernatant into new precooled microcentrifuge tube
- centrifuge at 13000 rpm for 10 minutes at 4°C
- save the supernatant

## 17.3. SDS-PAGE

**Protocol:**

- mix samples for SDS-PAGE with 4x sample loading buffer and boil for 5 minutes at 95°C
- put Criterion Precast Gel into electroforetic tank and pour in SDS-PAGE running buffer
- load samples on Criterion Precast Gel
- load Precision Plus Protein$^{TM}$ Standarts Dual Color (Bio-Rad)
- apply current, voltage 200 V for 1 hour

## 17.4 Western Blotting

**Protocol:**

- gel after SDS-PAGE wash with $dH_2O$
- assemble the blotting device (cathode, plastic net, sponge, filter paper, gel, nitrocellulose membrane, filter paper, 2 sponges, plastic net, anode)
- fill the blotting tank with cool blotting buffer
- apply current, voltage 25 V for 1,5 hour
- reassemble the blotting device
- incubate the membrane in blocking solution (5% milk in TBS-T) for 1 hour
- cut the membrane apart if probing for different antibodies

- incubate every part of membrane with appropriate primary antibody diluted in blocking solution over night at 4°C
- wash the membrane strips with TBS-T twice for 10 minutes
- incubate the membrane strips with secondary antibody diluted in TBS-T for 1 hour
- wash the membrane strips with TBS-T twice for 10 minutes

## 17.5 Chemiluminiscent detection

**Protocol:**

- assemble the membrane strips
- incubate with SuperSignal West Femto Maximum Sensitivity Luminol and SuperSignal West Femto Maximum Sensitivity Stable Peroxidase Buffer (1:1 solution) for 1 minute
- take pictures in G:BOX iChemi

## 17.6 Staining and drying of polyacrylamid gels

**Protocol:**

- after running SDS-PAGE wash the gel 5 minutes in water
- incubate 1 hour with Gel Code® Blue Stain Reagent
- destain 1 hour in water
- put on wet filter paper and dry on gel dryer

## 17.7 Purification of GST fused proteins

**Protocol:**

- inoculate in 5ml of LBAmp BL21 cells bearing desired pGEX plasmid and grow over night at 37°C
- inoculate 2 ml from the overnight culture into 200ml of LBAmp, grow to OD600 of 0,7
- induce by 1mM IPTG and grow 2 hours

- harvest cells by centrifugation at 4750 rpm for 14 minutes at 4°C and wash 10 ml of ice cold water
- resuspend in 10 ml of ice cold 1xPBS
- sonicate 4x30s
- add 0,5 ml of 20% TritonX-100 and rotate 30 min at 4°C
- get rid of cell debris by centrifugation at 13000 rpm for 10 minutes in 4°C
- add 400 µl of glutathion-Sepharose™ (50% slurry) to the supernatant and rotate for 30 minutes in room temperature
- spin down at 1000 rpm for 5 minutes at 4°C
- wash 3 times with 5 ml of ice cold 1xPBS
- resuspend in 100 µl 1xPBS
- make 20 ml aliquots and froze them at -80°C
- use one aliquot for estimation of protein amount by SDS-PAGE: 4 dilutions of BSA (0,1 µg, 1 µg, 2 µg, 5 µg) and 1µl, 2 µl and 5 µl of the GST fusion protein

## 17.8 TnT® Quick Coupled Transcription/Translation System (Promega)

**Protocol**:

- upon removal from storage at -80°C, rapidly thaw the TnT® Quick Master Mix by hand and place on ice
- assemble the reaction components in a 0,5 ml microcentrifuge tube, gently mix by pipetting

| **Components** | **Standard Reaction** |
|---|---|
| TnT® Quick Master Mix | 40 µl |
| [35S]methionine (1000Ci/mmol at 10mCi/ml) | 2 µl |
| DNA template (0,5 µg) | 2,4 – 5 µl |
| Nuclease-Free water to a final volume of | 50 µl |

- incubate reaction at 30°C for 60-90 minutes

## 17.9 GST "pull-down" experiment

**Protocol:**

- before use add 1mM DTT and 1% fat free powder milk to buffer B
- for one reaction: 250 µl buffer B + GST fusion protein (1 – 5 µg; it should be the same amount of every GST fusion protein used in one assay; always make control with GST only) + 10 µl of TnT® reaction (17.8)
- vortex at low rpm
- incubate rotating for 2 hours in 4°C
- spin down at 500 rpm for 2 minutes, remove supernatant
- wash 3 times with 1 ml of ice cold 1xPBS
- run SDS-PAGE with the entire reaction, always load 20% Input (2 µl of the TnT® reaction)
- subject the dried gel to autoradiography

## 17.10 Nickel affinity purification experiment

**Protocol:**

- estimate the total protein concentration (mg/ml) in WCE (17.1) using the Bio-Rad Protein Assay Kit based on the Bradford dye-binding procedure (Bradford 1976)
- use 1 mg of total protein for the assay; aliquot 5% of the total protein used in each reaction to provide the "Input" reference sample in the subsequent analysis (prepare for SDS-PAGE as in 17.3)
- add buffer BB up to 200 µl and 7,5 µl of $Ni^{2+}$-NTA-silica resin (50% slurry)
- rotate at 4°C over night
- centrifuge the sample at 2200 rpm for 2 minutes to collect the resin
- remove the "Flow-through" supernatant reserving a 5% aliquot
- wash the resin 3 times with 1 ml of buffer BB
- resuspend the resin in 50 µl of elution buffer E
- vortex briefly and elute rotating for 20 minutes in 4°C
- collect the resin by centrifugation and save the "Eluate" supernatant
- analyze the purified proteins by SDS-PAGE and western blotting

## 17.11 Formaldehyde cross-linking followed by sucrose density gradient centrifugation experiment

### 17.11.1 Sucrose density gradient preparation

**Protocol:**

- gradients should be prepared day before the main experiment
- prepare Grad 30% and Grad 7,5% solutions according to the number of gradients you will use

| | Grad 30% | | | Grad 7,5% | | |
|---|---|---|---|---|---|---|
| number of gradients | 2 | 4 | 6 | 2 | 4 | 6 |
| GA solution | 5 ml | 9 ml | 13 ml | 15 ml | 25 ml | 35 ml |
| GB solution | 10 ml | 18 ml | 26 ml | 3 ml | 5 ml | 7 ml |
| 1M DTT | 15 µl | 27 µl | 39 µl | 18 µl | 30 µl | 42 µl |

- label Beckman Polyallomer tubes using special labeling toll
- put the tubes into a rack on the Gradient Master
- load Grad 7.5 % solution with a syringe up to the mark
- load Grad 30 % solution with a syringe under the Grad 7.5 % solution until the tube is full, this two solutions must not mix
- put caps on the tubes
- Run the Gradient Master (setting: Grad; SW41; 7,5-30%)
- gently remove caps and put tubes into buckets
- weigh the buckets with gradients and balance the opposite ones
- store in 4°C over night

### 17.11.2 Sucrose density gradient centrifugation experiment

- measure the absorbance at 260 nm of WCE prepared in 17.2 (100x dilution)
- count number of UNITS (absorbance x 100 = number of UNITS/ml)
- take 1 UNIT as an 5% input, add 10 µl GF, prepare for SDS-PAGE as in 17.3 and freeze for later use
- take 20 UNITS to a clean microcentrifuge tube and add GF so that the opposite samples have a same volume

- carefully load samples on the top of the gradients so that the opposite ones are balanced
- screw on the caps
- put in the ultracentrifuge and run at 41000 rpm for 5 hours at 4°C in vacuum
- after the centrifugation process the gradients using Brandel BR-188 Density Gradient Fractionation System, collect 12 fractions (600 µl each)
- add 100 µl of 6x SDS-Loading Buffer to have 700 µl fractions and boil for 5 minutes at 95°C
- load 35 µl of the fractions on gel as in 17.3, do not forget to load the 5% input prepared earlier

# Results

## 18. Characterization of the HCR1-like domain (HLD) of the TIF32 subunit by "Domain swapping"

As described above in chapter 6.3, HCR1 and the HLD of TIF32 share significant sequential and functional homology. This observation prompted an idea that these two domains might have a similar function and thus might be able to substitute for each other *in vivo*. To test this idea, two recombinant vectors were constructed: the first one bearing the HLD coding segment under control of the *HCR1* promoter; and the second, carrying the *TIF32* gene with its HLD replaced by full-length *HCR1*. Both constructs were introduced into *S. cerevisiae* H423 and H428 strains deleted for *TIF32* or *HCR1* genes, respectively, and tested for functional complementation by plasmid shuffling. As shown in Fig. 11., the HLD alone expressed from the *HCR1* promoter partially suppressed slow growth phenotype of the *hcr1Δ* strain. The chimeric TIF32-HCR1 protein was not able to support growth of the *tif32Δ* strain. These results that were not pursued further indicate that the HLD of TIF32 and HCR1 seem to share some functional similarity, the nature of which will to be explored in the future.

### 18.1 Preparing of recombinant vectors YEpHCR1-DS, pRSTIF32-His-DS, YEpHLD and pRSTIF32-HCR1-His

YEpHCR1-DS was constructed using the QuikChange® Multi Site-Directed Mutagenesis Kit (16.5) according to the vendor's instructions. In step 1, PCR was performed with the kit-provided enzyme blend using primers DS HCR1-BHI and DS HCR1-NcoI, elongation time 14 minutes, and YEpLVHCR1 (Valášek et al. 1999) as a template. This subcloning step was done to introduce the *BamHI* site immediately preceding the ATG start codon and the *NcoI* site immediately followed by the stop codon of *HCR1* to facilitate further cloning. Insertion of restriction sites was then verified by digestion with *BamHI* and *NcoI*. Since the strategy involves PCR

amplification of the entire plasmid, an 1289bp *HindIII-SacI* fragment containing the *HCR1* gene was isolated from the mutagenized plasmid and used to replace the corresponding fragment in YEpLVHCR1 to produce YEpHCR1-DS. The entire *HindIII* to *SacI* fragment of YEpHCR1-DS was sequenced to confirm the presence of only the desired mutations.

pRSTIF32-His-DS was constructed using the QuikChange® Multi Site-Directed Mutagenesis Kit according to the vendor's instructions. In step 1, PCR was performed with the kit-provided enzyme blend using primers DS TIF32-BHI and DS TIF32-NcoI, elongation time 22 minutes, and pRSTIF32-His (Valášek et al. 2002) as a template. This subcloning step was done to introduce the *BamHI* site immediately followed by ATG start codon before *Asp483* and *NcoI* site after *Leu786* of *TIF32* to facilitate further cloning. Insertion of restriction sites was then verified by digestion with *BamHI* and *NcoI*. Since the strategy involves PCR amplification of the entire plasmid, an 1655bp *AatII-XbaI* fragment containing the HLD was isolated from the mutagenized plasmid and used to replace the corresponding fragment in pRSTIF32-His to produce pRSTIF32-His-DS. The entire *AatII* to *XbaI* fragment of pRSTIF32-His-DS was sequenced to confirm the presence of only the desired mutations.

YEpHLD was constructed by inserting 921bp *BamHI, NcoI* fragment from pRSTIF32-His-DS into YEpHCR1-DS cleaved with *BamHI, NcoI*.

pRSTIF32-HCR1-His was constructed by inserting 801bp *BamHI, NcoI* fragment from YEpHCR1-DS into pRSTIF32-His-DS cleaved with *BamHI, NcoI*.

## 18.2 Testing phenotype of chimeric TIF32-HCR1 construct

Recombinant vector pRSTIF32-HCR1-His was introduced into H423 strain deleted for *TIF32* by LiAC transformation. Transformed clones were selected on SD media lacking leucine and subsequently streaked out onto media containing 5-FOA to select for those that lost YCpTIF32-His, URA3-based covering plasmid. H423 strain transformed with pRSTIF32-His and pRS315 were used as positive and negative control respectively. No clones were able to grow on 5-FOA, hence the TIF32-HCR1 chimeric protein does not support growth of *tif32Δ* strain.

## 18.3 Testing phenotype of HLD expressed alone from HCR1 promoter

Recombinant vector YEpHLD was introduced into H428 strain deleted for *HCR1* by LiAC transformation. Transformed clones were selected on SD media lacking leucine. H428 strain transformed with YEpLVHCR1 and YEplac181 were used as positive and negative control respectively. Resulting transformants were tested for various phenotypes and displayed an ability to suppress Slg- phenotype of *hcr1Δ* strain. As seen in Figure 11., YEpHLD did suppress the Slg- phenotype of H428 cells albeit only partially. To further document this finding, doubling times were measured for the tested strain and controls in 30°C and 37°C. Whereas the empty vector control increase doubling time by ~2-fold compared to wt, cells expressing the HLD in high copy showed reproducibly only ~1.5-fold increase at 37°C (Tab. 2).

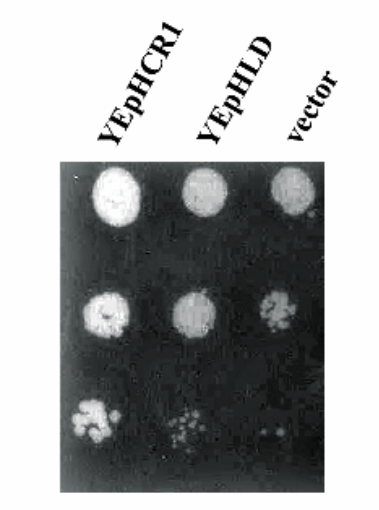


**FIGURE 11.** HLD can partially suppress Slg- phenotype of hcr1Δ cells. H428 transformants with YEpLVHCR1, YEpHLD and YEplac181 were spotted in three serial 10-fold dilutions on SD medium and incubated in 37°C for 2 days.

**TABLE 2.** Doubling times of H428 transformants in hours. WT – H428 transformed with YEpLVHCR1, HLD – H428 transformed with YEpHLD, EV – H428 transformed with YEplac181.

| | 30°C | 37°C |
|---|---|---|
| WT | 2,71 h | 2,15 h |
| **HLD** | **3,6 h** | **3,43 h** |
| EV | 4,2 h | 4,18 h |

# 19. Genetic and biochemical analysis of the critical determinants of the interaction between the PRT1-RRM and HCR1 as determined by NMR spectroscopy

As mentioned in chapter 6.2, NMR analysis of our collaborating group of P.J. Lukavsky revealed that the human eIF3b-RRM forms a hydrophobic pocket in which the critical Trp residue of eIF3j resides (L. ElAntak and P.J. Lukavsky, unpublished observations). Given a lack of feasible methodology to study protein-protein interactions and their physiological roles *in vivo* in higher eukaryotes, we turned our attention to the yeast model that is notoriously known to provide a large arsenal of well-established *in vivo* methods. First we had to test whether or not the nature of binding between human eIF3b and eIF3j subunits has remained conserved also in yeast. To do that, several *PRT1-RRM* mutants targeting critical pocket residues were constructed (Tab.3) and tested for binding to HCR1 *in vitro* and *in vivo*. Mutations in loop5 (L5) and in helix α1 of PRT1-RRM disrupted binding of HCR1 *in vitro* (Fig. 12.) and also eliminated HCR1 from MFC *in vivo* (Fig. 15.). Combined mutant *α1L5* showed Slg- phenotype at 37°C (Fig. 14) and also affected eIF3 association with 40S ribosome (Fig. 16).

**TABLE 3.** PRT1-RRM mutants created in this study

| **Mutation** | **Description** |
|---|---|
| PRT1-AALA | in loop 5, 114HRLF to AALA |
| PRT1-LFSK | in helix α1, 62LFSK to AAAE |
| PRT1-α1L5 | in helix α1 and loop 5, 114HRLF to AALA and 62LFSK to AAAE |

## 19.1 Construction of recombinant vectors pRSPRT1-His-XS, pRSPRT1-AALA-His, pRSPRT1-LFSK-His and pRSPRT1-α1L5-His

To construct pRSPRT1-His-XS, the following pair of primers was used with pKHN45 (Nielsen et al., 2006) as a template : AH-PRT1-BamHI and AH-PRT1-NotI-R. PCR product thus obtained was digested with *BamHI-NotI* and inserted into *BamHI-NotI* cleaved pKHN45. This subcloning step was done to remove the second *XbaI* and *SpeI* sites immediately following the stop codon of *PRT1* to facilitate subcloning of the RRM mutants.

pRSPRT1-AALA-His was generated by fusion PCR. The following two pairs of primers were used with pRSPRT1-His-XS as a template: AH-PRT1-ApaI and LV-RRM-AALA-R; and LV-RRM-AALA and AH-PRT1-XbaI-R, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification with primers AH-PRT1-ApaI and AH-PRT1-XbaI-R. The resulting PCR product was digested with *ApaI-XbaI* and inserted into *ApaI-XbaI* cleaved pRS-PRT1-His-XS producing pRSPRT1-AALA-His. The product was verified by restriction analysis and sequencing.

pRSPRT1-LFSK-His was generated by fusion PCR. The following two pairs of primers were used with pRSPRT1-His-XS as a template: AH-PRT1-ApaI and AH-PRT1-A1B-R; and AH-PRT1-A1B and AH-PRT1-XbaI-R respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification with primers AH-PRT1-ApaI and AH-PRT1-XbaI-R. The resulting PCR product was digested with *Apa*I-*Xba*I and inserted into *Apa*I-*Xba*I cleaved pRS-PRT1-His-XS producing pRSPRT1-LFSK-His. The product was verified by restriction analysis and sequencing.

pRSPRT1-α1L5-His was generated by fusion PCR. The following two pairs of primers were used with pRSPRT1-AALA-His as a template: AH-PRT1-ApaI and AH-PRT1-A1B-R; and AH-PRT1-A1B and AH-PRT1-XbaI-R respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification with primers AH-PRT1-ApaI and AH-PRT1-XbaI-R. The resulting PCR product was digested with *Apa*I-*Xba*I and inserted into *Apa*I-*Xba*I cleaved pRS-

PRT1-His-XS producing pRSPRT1-α1L5-His. The product was verified by restriction analysis and sequencing.

## 19.2 Constructing of recombinant vectors for GST pull-down experiment

*PRT1-RRM* mutants were cloned into pT7-7 (Tabor and Richardson 1987) vector under T7 promoter to be expressed in vitro by TnT® Quick Coupled Transcription/Translation System and tested for binding to GST fused HCR1.

pT7-prt1-rrm-AALA was made by inserting the *NdeI-HindIII* digested PCR product obtained with primers LVPNDEI-724 and LVPC136-724 using the template pRSPRT1-AALA-His into *NdeI-HindIII* digested pT7-7. The product was verified by restriction analysis and sequencing.

pT7-prt1-rrm-LFSK was made by inserting the *NdeI-HindIII* digested PCR product obtained with primers LVPNDEI-724 and LVPC136-724 using the template pRSPRT1-LFSK-His into *NdeI-HindIII* digested pT7-7. The product was verified by restriction analysis and sequencing.

pT7-prt1-rrm-α1L5 was made by inserting the *NdeI-HindIII* digested PCR product obtained with primers LVPNDEI-724 and LVPC136-724 using the template pRSPRT1-α1L5-His into *NdeI-HindIII* digested pT7-7. The product was verified by restriction analysis and sequencing.

## 19.3 Isolating GST-fused HCR1 and GST proteins for GST pull-down experiment

Plasmids pGEX-5x-3 and pGEX-HCR1 contain GST and GST fused HCR1 respectively under tac promoter, which is inducible by IPTG. After addition of IPTG to the growth media, proteins are expressed in high amounts.

Bacteria *E. coli* BL21 carrying this vectors were grown in LB media and then induced with IPTG. GST and GST-HCR1 proteins were isolated as described in chapter 17.7.

## 19.4 In vitro binding assay – "GST pull-down"

To investigate whether the critical determinants of PRT1–HCR1 interaction in yeast are similar in nature to those in humans, all three PRT1-RRM mutants and wild type PRT1-RRM were expressed *in vitro* by TnT® Quick Coupled Transcription/Translation System (17.8), labeled with 35S-methionine and tested for *in vitro* binding to GST fused HCR1. GST alone was used as a negative control. Neither of RRM mutants retained the ability to bind to HCR1 in vitro (Fig. 12) clearly illustrating that all the tested residues critically contribute to the PRT1-RRM – HCR1 protein-protein interaction.

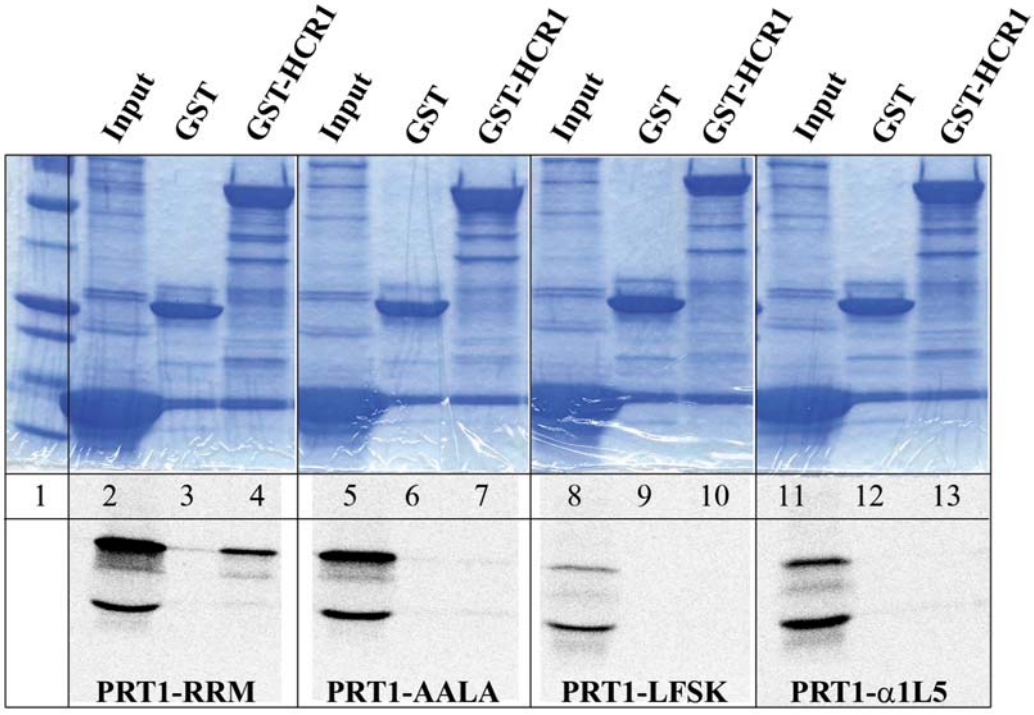


**FIGURE 12.** In vitro binding assay. Upper box displays amounts of GST and GST-HCR1 in each reaction. Lower box shows the same gel area after autoradiography. Only wt RRM was able to bind to GST-HCR1 (lane 4). (lane 1) Protein ladder, (lane 2,5,8,11) 20% Input of the TnT® reaction.

## 19.5 Testing phenotypes of *PRT1-RRM* mutants

Recombinant vectors pRSPRT1-AALA-His, pRSPRT1-LFSK-His and pRSPRT1-α1L5-His were introduced into H425 strain deleted for *PRT1* by LiAC transformation. Transformed clones were selected on SD media lacking leucine and

subsequently grown on media containing 5-FOA to select for those that lost the CR52 (Cigan et al. 1991) URA3-based covering plasmid. All mutant strains formed colonies on 5-FOA suggesting that neither mutation is lethal. H425 strain transformed with wt pRSPRT1-His and empty vector pRS315 were used as positive and negative controls, respectively.

Whereas the *PRT1-AALA* and *PRT1-LFSK* alleles showed no detectable growth defects (Fig.13), combination of both in *PRT1-α1L5* displayed Slg- phenotype at 37°C (Fig. 14) suggesting that the pocket residues are functionally important.

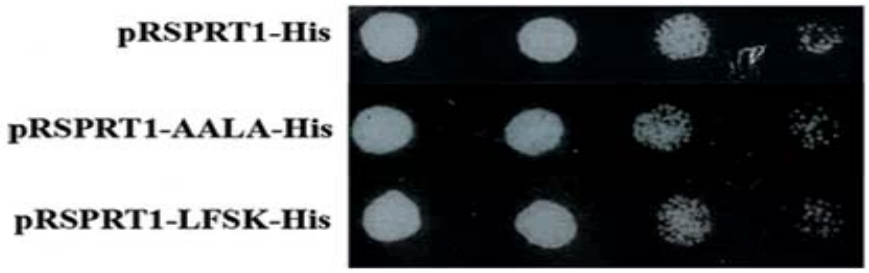


**FIGURE 13.** H425 transformants with pRSPRT1-His, pRSPRT1-AALA-His and pRSPRT1-LFSK-His were spotted in four serial 10-fold dilutions on SD medium and incubated in 37°C for 3 days. Neither of mutants showed any visible phenotype.

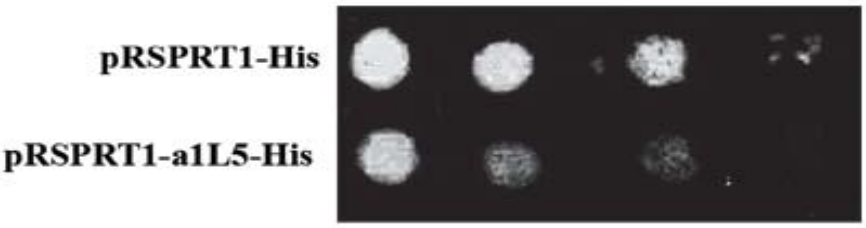


**FIGURE 14.** H425 transformants with pRSPRT1-His and pRSPRT1-α1L5-His were spotted in four serial 10-fold dilutions on SD medium and incubated in 37°C for 3 days. *PRT1-α1L5* mutant displayed slow growth phenotype.

## 19.6 “Nickel pull-down”

Effect of the PRT1-RRM mutations on the formation of entire eIF3-containing MFC was analyzed by $Ni^{2+}$ chelation chromatography using $His_8$-tagged PRT1 as a bait (see chapter 17.10). WCEs from transformants of the H425 strain expressing individual PRT1-RRM mutants were incubated with Ni-NTA silica resin and analyzed by SDS-PAGE followed by western blotting. H425 transformed with pRSPRT1-His and original H425 strain bearing untagged PRT1 were used as positive and negative controls, respectively. All tested MFC components copurified specifically with wt PRT1, as expected, but not with its untagged version. In contrast, all tested PRT1-RRM mutations specifically eliminated only HCR1 from MFC (Fig. 15), suggesting that mutated pocket residues are important for binding of HCR1, but not for the stability of MFC.

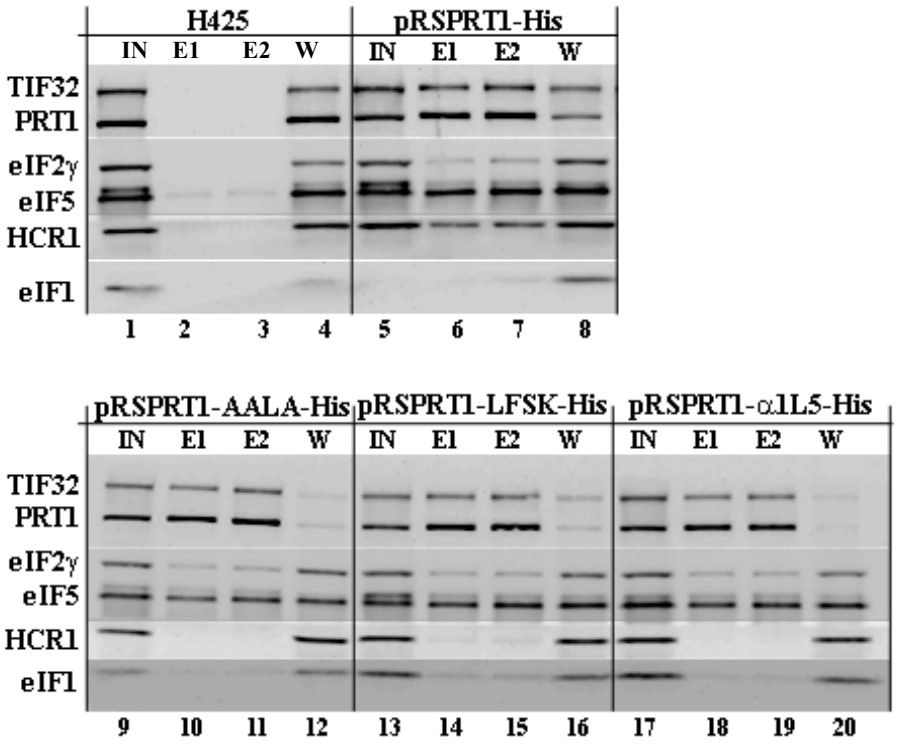


**FIGURE 15.** The helix α1 and loop 5 of PRT1-RRM are required for HCR1 association with MFC in vivo. WCEs were prepared from H425 bearing untagged PRT1 (lanes 1 to 4), and H425 transformants with pRSPRT1-His (lanes 5 to 8), pRSPRT1-AALA-His (lanes 9 to 12), pRSPRT1-LFSK-His (lanes 13 to 16) and pRSPRT1-α1L5-His (lanes 17 to 20). Lanes 1, 5, 9, 13 and 17 contained 5% of the input WCEs (IN); lanes 2, 6, 10, 14 and 18 contained 30% of fractions eluted from the resin (E1); lanes 3, 7, 11, 15 and 19 contained 60% of fractions eluted from the resin (E2); lanes 4, 8, 12, 16 and 20 contained 5% of the flow through (wash, W).

## 19.7 eIF3 association with 40S ribosome

To examine if the α1L5 mutation, disrupting the direct contact with HCR1, also affects association of mutant eIF3 with the 40S ribosome, binding of selected eIF3 subunits and other MFC components to the 40S was measured in WCEs obtained by formaldehyde cross-linking (Fig. 16.). H425 transformants with pRSPRT1-α1L5-His and pRSPRT1-His were grown in SD medium at 37°C to an $OD_{600}$ of ~1,5 and cross-linked with 2% HCHO prior to harvesting. WCE were prepared and subsequently separated on a 7,5% - 30% sucrose gradient by centrifugation at 41,000 rpm for 5 hours. Proteins from the collected fractions were subjected to Western analysis. Based on quantification of the Western signals, we observed ~45% decrease in the amounts of selected eIF3 subunits associated with 40S ribosomes in WCEs obtained from the *α1L5* cells compared to the wild type control. Similar but less pronounced reductions were also observed for eIF2 (~30%) and eIF5 (~25%). Amounts of the 40S-associated HCR1 were reduced only marginally (~15%).

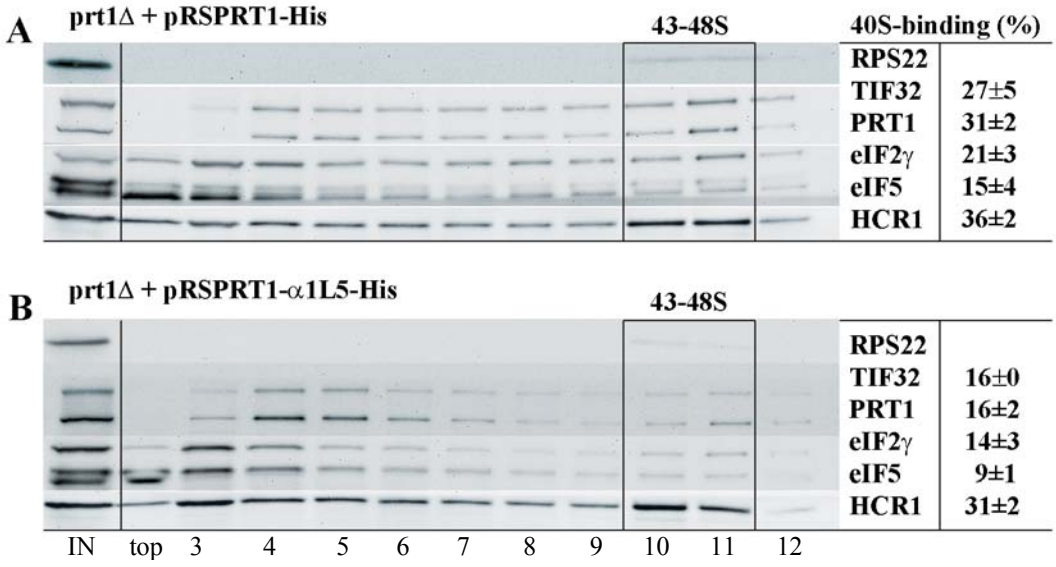


**FIGURE 16.** Destroying of the hydrophobic pocket of the PRT1-RRM reduces eIF3-binding to 40S ribosomes. (A) Strain H425 transformed with pRSPRT1-His, (B) Strain H425 transformed with pRSPRT1-α1L5-His. An aliquot of each WCE was analyzed in parallel (IN, 5% input); first two fractions were combined (top). Mean proportions of the total proteins found in fractions 10-11, calculated using NIH ImageJ from two independent experiments are shown in the right column. The calculations were done by Leoš Valášek, PhD.

# Discussion

## 20. What do the sequential homologues in HCR1 and the HLD of TIF32 have in common?

The HLD domain of TIF32, sharing a significant sequential homology with HCR1 (6.3), was found to be able to partially suppress the Slg- phenotype of *hcr1Δ* cells when expressed from the *HCR1* endogenous promoter on a high copy plasmid (Fig. 11). Susan Wagner from our laboratory has recently found that the C-terminal domain (CTD) of HCR1 specifically interacts with the TIF32-HLD and is entirely dispensable for wt growth unless combined with the *rpg1-1* mutation in TIF32 (this mutation occurs in the most homologous motif between HCR1 and the HLD called KERR – see chapter 6.3). In addition, both the HCR1-CTD and TIF32 are able to interact with the same ribosomal protein RPS2 occurring in the mRNA entry channel on the solvent side of the small ribosomal subunit (S. Wagner and L. Valášek, unpublished observations). These findings might suggest that the TIF32-HLD and the HCR1-CTD form a heterodimer, where they closely cooperate to perform an important function possibly involving mutual binding to RPS2. We speculate that this important function is probably somehow involved in mRNA recruitment to the 40S ribosome and/or subsequent scanning for the AUG start codon. Hence it is conceivable to suggest that the HLD domain expressed alone could form a pseudo-heterodimer with the internal HLD of TIF32 and thus partly substitute for HCR1 in the latter role.

Alternatively, besides TIF32, HCR1 binds also to the PRT1-RRM and might serve to stabilize their mutual interaction (Valášek et al. 2001b). Hence a partial suppression exerted by the HLD could also be explained by proposing that the HLD alone is capable of stabilizing the latter contact that is believed to be crucial for the MFC integrity (Valášek et al. 2001a).

Apart from translation initiation, HCR1 has been also shown to promote 40S ribosome biogenesis (Valášek et al., 2001b). Since the TIF32 roles seem to be restricted to translation initiation, it is highly unlikely that the HLD domain would

suppress a defect in ribosome biogenesis that is also caused by HCR1 deletion. Nevertheless, to clearly rule out this possibility, polysome profile analysis combined with pulse chase experiments detecting 40S biogenesis intermediates would need to be carried out.

On the other hand, the fact that the chimeric TIF32-HCR1 protein did not support growth of *tif32Δ* strain does not necessarily mean that HCR1 can not substitute for the HLD's functions. The simplest interpretation of this negative result could be in improper folding of the chimeric TIF32 protein with HCR1 inserted in place of HLD. Taking into account that TIF32 is an essential protein, its larger missfolding would logically result in non viability.

Even though our data further extended an earlier believe that besides sequential homology, HCR1 and the HLD of TIF32 are also similar in function to some degree, more experimental data are certainly required to fully address the role of both of these eIF3 parts in translation initiation. Our laboratory in collaboration with the group of Alan G. Hinnebusch from NIH in Bethesda are currently striving to resolve this puzzle in a set of complementary approaches.

# 21. Defining the critical determinants of the PRT1-RRM-HCR1 interaction and their role in translation initiation

eIF3 plays critical roles in virtually all stages of translation initiation, during reinitiation, post-termination ribosomal recycling, and nonsense-mediated decay pathway (Hinnebusch, 2006; Pisarev et. al., 2007; Szamecz et. al., 2008; Isken et. al., 2008). In order to understand how the numerous functions of eIF3 are encoded in its conserved subunits and their interactions, high-resolution structural studies of protein-protein interactions of eIF3 subunits are imminent. Our collaborators from MCR in Cambridge revealed by NMR spectroscopy a first structure of an interaction among eIF3 subunits, between human eIF3b-RRM and eIF3j-NTD (L. ElAntak and P.J. Lukavsky, unpublished observations) (Fig. 8.). This interaction is driven by conserved charge complementarity between the subunits and an evolutionary conserved hydrophobic pocket on the backside of the eIF3b-RRM, which accommodates the strictly conserved Trp residue in the eIF3j-NTD.

My findings that specific mutations targeting the conserved residues of the hydrophobic pocket of the yeast eIF3b/PRT1-RRM disrupted its direct binding to eIF3j/HCR1 *in vitro* (Fig. 12.) and completely eliminated HCR1-association with the MFC *in vivo* (Fig. 15.) clearly point at the high evolutionary conservation of this interaction. It is noteworthy that the fact that the loss of HCR1 did not seem to significantly affect occurrence of TIF32 and PRT1 in the MFC (Fig. 15.) contradicts the theory mentioned in the previous chapter that HCR1 stabilizes their mutual contact required for the MFC stability and, hence, favors our pseudo-heterodimer idea. High degree of evolutionary conservation has gained further support by the recent data of Susan Wagner from our laboratory who showed that mutating the critical Trp and its neighboring acidic residues in HCR1 has the same effect as mutations in the pocket residues (S. Wagner and L. Valášek, unpublished observations).

Taken together, it is no surprise that the pocket residue mutations turned out to be important for viability of cells, as the combined α1L5 mutant displayed Slg- phenotype when grown at higher temperature (Fig. 14.). Since HCR1 is able to bind to 40S subunit on its own (Phan et al., 2001), it's elimination from the MFC does not necessarily need to cause any growth defect, providing that it will still be capable of carrying out its 40S-associated functions in close co-operation with other 40S-bound eIF3 subunits and MFC components. However, the α1L5 mutant significantly reduced 40S-association of the core subunits of eIF3 as well as eIFs 2 and 5 *in vivo* as shown by the formaldehyde cross-linking experiment (Fig. 16.). Even though HCR1 was able to bind to 40S subunit nearly to the same extend as in the wild type cells, as expected (Fig. 16.), it probably failed to fulfill its roles due to reduced ribosome-occupancy of eIF3.

Taken together, these findings indicate that the hydrophobic pocket of the PRT1-RRM is not important only for anchoring HCR1 to eIF3 but perhaps plays even more critical role in 40S-association of the whole eIF3 complex by creating yet another intermolecular bridge between eIF3 and 40S in addition to that already described in case of the TIF32-NTD (Valášek et al., 2003, Szamecz et al., 2008). What function(s) the evolutionary well conserved interaction between HCR1 and the PRT1-RRM performs on the 40S ribosome is again a subject of extensive studies in our laboratory.

# Conclusions

The initiation of translation in eukaryotes requires the coordinated action of at least 12 eukaryotic initiation factors (eIFs). Among them, eIF3 deserves a special attention owing to a broad range of functions that it is believed to promote. The research conducted here aimed at bringing a closer insight into functions of specific domains of several eIF3 subunits and the interactions among them. The middle HLD domain of TIF32 was proposed to share not only sequential but also functional homology with HCR1 as it was able to partially substitute for the loss of chromosomal copy of HCR1 when expressed alone under control of *HCR1* promoter.

Based on unpublished determination of the interaction interface between the RRM of human eIF3b/PRT1 and eIF3j/HCR1 by NMR spectroscopy in the laboratory of P.J. Lukavsky, we confirmed biochemically that also in yeast the conserved Trp residue in the N-terminal segment of HCR1 binds to the hydrophobic pocket of the PRT1-RRM formed by the helix α1 and the loop L5. This interaction was shown to be required for association of HCR1 with the rest of the MFC but not with the 40S ribosome. Besides evolutionary conservation of this contact and its importance for HCR1 binding to eIF3, the hydrophobic pocket residues turned out to be also required for anchoring the whole eIF3 to the small ribosomal subunit.

Detailed investigation of physiological roles of the eIF3 domains studied here, namely TIF32-HLD, PRT1-RRM and HCR1, as a part of the mutually interacting module of eIF3 is one of the main interests in our laboratory and, as a matter of fact, elucidation of molecular nature of functions of the RRM and the extreme C-terminal domains of PRT1 promoting its eIF3-scaffolding role will be the main goal of my doctoral thesis.

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