Yeast Eukaryotic Initiation Factor 4B (eIF4B) Enhances Complex Assembly between eIF4A and eIF4G in Vivo

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Background: Mammalian eIF4B stimulates eIF4A helicase activity, but its function in promoting translation initiation in yeast is unclear.

Results: Yeast eIF4B enhances eIF4G/eIF4A association in vivo and in vitro.

Conclusion: Yeast eIF4B stimulates eIF4F assembly by promoting an eIF4G HEAT domain conformation conducive for binding eIF4A.

Significance: A new function is established for eIF4B of supporting eIF4F assembly for mRNA activation.

Translation initiation factor eIF4F (eukaryotic initiation factor 4F), composed of eIF4E, eIF4G, and eIF4A, binds to the m7G cap structure of mRNA and stimulates recruitment of the 43S preinitiation complex and subsequent scanning to the initiation codon. The HEAT domain of eIF4G stabilizes the active conformation of eIF4A required for its RNA helicase activity. Mammalian eIF4B also stimulates eIF4A activity, but this function appears to be lacking in yeast, making it unclear how yeast eIF4B (yeIF4B/Tif3) stimulates translation. We identified Ts mutants that appear to be lacking in yeast, making it unclear how yeast eIF4B stimulates translation.

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In mammals, the ATPase and helicase activities of eIF4A are also stimulated by eIF4B (4), which contains multiple ssRNA-binding domains (15, 16); however, the activation mechanism is poorly understood. Direct interaction of eIF4B with eIF4A is not readily detected, although efficient complex formation occurs in the presence of ssRNA and ATP (17–19). Hence, eIF4B might stimulate helicase activity by enhancing domain closure in the eIF4A-ATP-mRNA complex, by stabilizing a conformation of this complex incompatible with mRNA duplex formation, or by helping to recruit eIF4A-ATP to single-stranded stretches in the mRNA. There is also evidence that eIF4B increases the efficiency of coupling ATP hydrolysis to duplex unwinding by eIF4A (20).

Beyond stimulating eIF4A helicase activity, it was suggested that mammalian eIF4B can promote ribosome attachment more directly by binding simultaneously to mRNA and 18S rRNA through its multiple RNA-binding domains (21) or by forming a protein bridge between the eIF4F-mRNP and eIF3 in the PIC (22). Other proposals envision a role for eIF4B in conferring 5′–3′ directionality to scanning. In one scenario, eIF4B interacts with eIF4A-eIF4G complexes bound to unwound mRNA at the exit channel of the 40S subunit to prevent backsliding of the PIC (23). In another model, eIF4A-eIF4G complexes disrupt helical structures in advance of the PIC, and eIF4B prevents reannealing of the unwound mRNA until it feeds into the 40S entry channel (18). Both models feature an eIF4G-eIF4A-eIF4F heterotrimer; however, there is evidence that eIF4A binding by eIF4G and eIF4B is mutually exclusive and that prior association with eIF4G enables eIF4A to form a stable mRNP with eIF4B following its dissociation from eIF4G (19).

Budding yeast harbors two eIF4G isoforms (1 and 2) that are ~50% identical in sequence and related to mammalian eIF4G (24), except that the yeast proteins contain only one HEAT domain. The two yeast isoforms are functionally redundant (25), and either is sufficient for cell viability (24). The interactions of eIF4E and eIF4A with their respective binding domains in eIF4G1 or eIF4G2 are essential for efficient translation in vivo (9–11, 26). Yeast eIF4A and the yeast homolog of eIF4B (yeIF4B/Tif3) enhance the translation of reporter mRNAs with structured leaders but are also required for efficient translation of mRNAs with short unstructured 5′-UTRs (27, 28). Although yeIF4B is nonessential, tif3Δ cells grow poorly, especially at low temperatures (28).

The available genetic evidence is consistent with the possibility that yeIF4B stimulates translation in vivo by promoting eIF4F function (28, 29), and we showed previously that eIF4F and yeIF4B are both required for rapid recruitment of 43S PICs on native capped mRNAs in vitro (30). Surprisingly, however, yeIF4B does not stimulate eIF4A helicase activity in vitro (31), although yeast eIF4A can be activated by mammalian eIF4B (27) and mammalian eIF4B can functionally replace yeIF4B in a cell-free translation system (28). Thus, either the conditions required for activation of yeast eIF4A by yeIF4B remain to be identified, or this is not the critical function of yeIF4B. Moreover, direct interaction of yeIF4B with eIF4A or eIF4G has not been described. Hence, it is currently unclear how yeIF4B stimulates 48S PIC assembly and whether this function involves eIF4A-dependent or eIF4A-independent activities.

Interestingly, despite a relatively high affinity of yeast eIF4A for eIF4G in vitro (10, 30), interaction between eIF4G and eIF4A at native levels has not been detected in cell extracts under conditions where the eIF4G-eIF4G interaction, of similar affinity, is readily observed (9, 33–35). This situation stands in contrast to the relative ease of isolating intact eIF4F from mammalian cells (36). Accordingly, it has been suggested that eIF4G-eIF4A interaction in yeast cells is transient, being modulated by post-translational modifications or regulatory proteins, and depends on prior interaction of eIF4G with another factor that can expose the eIF4A binding site in the HEAT domain (10).

In the present study, we uncovered evidence for interaction between yeast eIF4G and yeIF4B in vivo that can restore complex formation between eIF4A and mutant eIF4G proteins harboring particular HEAT domain substitutions, and we reconstituted the ability of yeIF4B to rescue eIF4A-eIF4G interaction for one such mutant using purified components. We also found that yeIF4B enhances eIF4A-eIF4G interaction in vivo even in the case of WT eIF4G. Our results suggest that one aspect of yeIF4B function is to promote binding of eIF4A to the eIF4GeIF4E subassembly of eIF4F with attendant recruitment of eIF4A to the cap structure of mRNA to promote 43S PIC attachment and subsequent scanning for the start codon.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—All plasmids employed in this study are listed in Tables 1 and 2, and yeast strains are described in Table 3. Mutations in tif631-HA-Bam or tif632-HA were introduced into pEP88 or pEP41, respectively, by the QuikChange II site-directed mutagenesis system (Agilent Technologies) and verified by sequencing the entire coding sequence. Yeast strains YAS2282 and YAS2069 were purchased from the American Type Culture Collection (Manassas, VA). All novel yeast strains in Table 3 were constructed by introducing a TRP1 CEN4 plasmid with the appropriate TIF4631-HA or TIF4632-HA allele into YAS2282. The resulting transformants were replica-plated on 5-fluoroorotic acid medium lacking tryptophan (FOA−Trp) and incubated at 30 °C to evict the resident URA3 plasmid containing WT TIF4632 (37–39). No visible growth on FOA−Trp plates after 5–7 days indicated a lethal phenotype.

To construct the tifΔ yeast strain used in Fig. 6, C–F, one TIF3 allele in diploid strain BY4743 was disrupted by using the tifΔ::hisG-URA3-hisG cassette obtained from plasmid...
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**TABLE 1**

| Yeast plasmid     | Relevant feature | Source                  |
|-------------------|------------------|-------------------------|
| pBAS2004          | TIF4632 URA3 CEN4 | Refs. 38 and 46         |
| pBAS2006          | TIF4632 TRP1 CEN4 | Ref. 38                 |
| YEpplac22         | sc TRP1 plasmid  | Ref. 47                 |
| pAS486            | p(HA)TIF4632 TRP1 CEN4 | Ref. 34                 |
| pEP41*            | TIF4632 TRP1 CEN4 | This study              |
| pEP88             | TIF4631-HA-Bam TRP1 CEN4 | Ref. 39                 |
| pEP91*            | tif4632-HA-Bam-L574F TRP1 CEN4 | This study              |
| pEP245*           | tif4631-HA-Bam-L614F TRP1 CEN4 | This study              |
| pEP256*           | tif4631-HA-Bam-W579A TRP1 CEN4 | This study              |
| pEP258*           | tif4632-HA-M570K TRP1 CEN4 | This study              |
| pEP259*           | tif4631-HA-Bam-B835A/F838A TRP1 CEN4 | This study              |
| pEP260*           | tif4632-HA-W539A TRP1 CEN4 | This study              |
| pEP261*           | tif4632-HA-T578I TRP1 CEN4 | This study              |
| pBAS3342          | TIF1 in hc URA3 plasmid | Ref. 9                  |
| p3349             | TIF2 in hc URA3 plasmid | Ref. 48                 |
| p3350*           | TIF3 in hc URA3 plasmid | Ref. 48                 |
| YEpplac195        | hc URA3 plasmid  | Ref. 47                 |
| M3925             | trpl::KanMX3     | Ref. 40                 |
| pFJZ043           | tif3::hisG::URA3::hisG | This study              |
| pRS315            | lc LEU2 plasmid  | Ref. 49                 |
| pEP329*           | tif4632-HA-M570K in lc LEU2 plasmid | This study              |

* This plasmid is identical to pAS486 except that a HpaI site within the ORF was eliminated by substituting one nt (TTA to TTG at codon 209) without changing the encoded amino acid sequence.

* Mutations producing the indicated amino acid substitutions in tif4631-HA-Bam or tif4632-HA were introduced into pEP88 or pEP41 by the QuickChange II site-directed mutagenesis system (Agilent Technologies).

* The hc plasmids derived from YEpplac195 containing TIF2 or TIF3 (48) were renamed p3349 and p3350, respectively.

**TABLE 2**

| Bacterial plasmid | Relevant feature | Source |
|-------------------|------------------|--------|
| pEPB26            | P<sub>Trp</sub>-GST-TIF4632(504–914)-His6 P<sub>Trp</sub>-Cdc33 | This study |
| pEPB27            | P<sub>Trp</sub>-GST-TIF4632(504–914)-L574F-His6 P<sub>Trp</sub>-Cdc33 | This study |
| pEPB29            | P<sub>Trp</sub>-GST-TIF4632(504–914)-W539A-His6 P<sub>Trp</sub>-Cdc33 | This study |

* The FJ plasmids used in this study included pFJZ043, selecting for Ura<sup>+</sup> transformants, to produce strain FJZ001. Haploid strains FJZ052 (tif3Δ) and FJZ046 (tif3Δ) were obtained by isolating Ura<sup>+</sup> and Trp<sup>−</sup> ascospore clones, respectively, by tetrad dissection after sporulation of FJZ001 and then eliminating the URA3 marker from the Ura<sup>+</sup> isolate by counterselection on medium containing 5-FOA to generate the unmarked tif3Δ::hisG allele in FJZ052. The presence of tif3Δ in FJZ052 was verified by PCR analysis of chromosomal DNA using primers PZ001 (5′-AGTACCGATTGAGCTGACG-3′), PZ002 (5′-CGAGCTTGCTTATAGTCAT-3′), and PZ004 (5′-CCCGAATTCCAAAAGAGGAGGGAGTTG-3′). To construct trp1Δ strains FJZ102 (tif3Δ) and FJZ107 (WT), FJZ001 was transformed with the trp1Δ::KanMX3 fragment obtained from plasmid M3925 (40), followed by tetrad dissection of a sporulated Kan<sup>+</sup> diploid transformant to obtain a Trp<sup>−</sup> Ura<sup>−</sup> ascospore and subsequent counterselection on 5-FOA medium to evict URA3 from the tif3Δ::hisG-URA3::hisG allele.

Coimmunoprecipitation and Immunoblot Analysis—Yeast strains were grown in 100 ml of SC–Trp also lacking uracil (SC–Ura,–Trp) to an A<sub>600</sub> of 0.5–0.8, and whole cell extracts (WCEs) were prepared as described previously (39). For coimmunoprecipitation, 1–3 mg of prepared WCEs were diluted with lysis buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM 4-(2-aminophenyl) benzenesulfonyl fluoride, 1 mM DTT, Complete protease inhibitor mixture tablets (EDTA-free, Roche Applied Science)) to 500 μl and incubated with 12.5 μg of agarose-conjugated anti-HA antibodies overnight at 4 °C with rocking. Immune complexes captured on agarose beads were collected by centrifugation and washed twice with 500 μl of lysis buffer (without protease inhibitors). The complexes were then incubated at 26 °C for 30 min in the presence or absence of RNases A and T1, washed twice with 500 μl of lysis buffer (without protease inhibitors), and resuspended in 40 μl of 2× Laemmli sample buffer. After boiling for 5 min, samples were resolved by 4–20% SDS-PAGE, transferred to PVDF membranes, and probed with appropriate antibodies. Immune complexes were visualized by ECL (GE Healthcare) according to the manufacturer’s instructions. For Western blot analysis of WCEs for Fig. 1, E and F, extracts were prepared by trichloroacetic acid extraction, as described previously (41).

Antibodies—Rat polyclonal anti-Tif3, rabbit polyclonal anti-eIF4A, mouse monoclonal anti-PABP, rabbit polyclonal anti-eIF4E, anti-Rps2, and rabbit polyclonal anti-Ded1 antibodies were kind gifts from M. Altmann, P. Linder, M. S. Swanson, J. E. G. McCarthy, J. Warner, and T. Chang, respectively. Mouse monoclonal anti-HA antibodies for immunoblotting and immunoprecipitation were purchased from Roche Applied Science and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Antibodies directed against Tif32 (42) and Rps2 (43) were described previously.

Recombinant Protein Production and Purification—Tetramethylrhodamine-labeled eIF4A (eIF4A-RH) (6, 30), yELF4B (30, 44), and GST–eIF4G2(504–914) (WT, L574F, or W539A) (30, 39) were purified as described previously.
Fluorescence Anisotropy Assays—Fluorescence anisotropy measurements of equilibrium binding constants ($K_i$) were performed as described previously with minor modifications (6, 30, 45). Briefly, to examine the effect of yeIF4B on GST-elf4G2(504–914)-elf4A-RH complex formation, elf4A-RH (30 nM) was mixed with yeIF4B (1 mM) in 300 μl of 1× Recon buffer (30 mM HEPES-KOH (pH 7.4), 100 mM KCl, pH 7.6, 3 mM Mg(OAc)$_2$, 2 mM DTT) in a quartz cuvette. Micrococal nuclease-treated/purified GST-eIF4G2(504–914) proteins were added, and the fluorescence anisotropy of elf4A-RH was

### TABLE 3

| Strain       | Genotype                                                                 | Source |
|--------------|---------------------------------------------------------------------------|--------|
| YAS2282      | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pBAS2004 [pTIF4632 URA3 CEN] | Ref. 46 |
| YAS2069      | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pBAS2078 [pTIF4631 TRPI CEN] | Ref. 38 |
| YAS1951      | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pBAS2068 [pTIF4632 TRPI CEN] | Ref. 38 |
| EPY41        | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4632 HA TRPI CEN]   | This study |
| EPY81        | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4632 HA-L574F TRPI CEN] | This study |
| EPY88        | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4631 HA-His TRPI CEN]  | Ref. 39 |
| EPY198       | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4631 HA-L574F TRPI CEN] | This study |
| EPY90        | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4632 HA-L574F TRPI CEN] | This study |
| EPY191       | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4632 HA-L574F TRPI CEN] | This study |
| EPY139       | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4632 HA TRPI CEN]   | This study |
| EPY232       | MATa ade2 his3 leu2 trp1 ura3 pep4 his3::HIS3 tfj631::ten2 rigf632::ura3 pEP21 [pTIF4632 HA-L574F TRPI CEN] | This study |
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measured on a Spex Fluorolog-3 spectrophotometer (Horiba Jobin Yvon) with an excitation wavelength of 550 nm and emission wavelength of 580 nm. As controls, $K_d$ values for GST-eIF4G2(504–914)-eIF4A-RH complexes were determined in the absence of eIF4B. Dissociation constants were calculated by fitting the anisotropy data to a hyperbolic function using Kaleidagraph software (Synergy).

RESULTS

Overexpression of eIF4B Suppresses Particular eIF4G Mutations Affecting the eIF4A/eIF4G Interface—To shed light on the role of eIF4B in promoting eIF4F function in vivo, we isolated point mutations in the gene encoding eIF4G2 (TIF4632) that confer temperature-sensitive (Ts<sup>-</sup>) growth and screened them for suppression of this phenotype by overexpressing WT eIF4B from a high copy (hc) TIF3 plasmid. Point mutations were generated at random in a single-copy plasmid-borne HA-TIF4632 allele, encoding eIF4G2 with an N-terminal HA tag and driven by the TIF4632 promoter, and mutant alleles were introduced by plasmid-shuffling into a strain harboring disrupted copies of eIF4A and eIF4G1. Hence, the plasmid-encoded gene products are the only eIF4G proteins present in the cells and are expressed at levels similar to that of native eIF4G2. Ten different Ts<sup>-</sup> alleles were thus identified, six of them harboring at least one mutation in the HEAT domain. Overexpressing eIF4A from an hc TIF3 plasmid suppressed the Ts<sup>-</sup> phenotype of the mutants containing substitutions in the HEAT domain (data not shown), as expected if the mutations weaken eIF4G2-eIF4A interaction in the manner described previously for other HEAT domain substitutions (9).

Interestingly, the Ts<sup>-</sup> phenotype of one of the mutants was also suppressed by overexpressing eIF4B (data not shown), raising the possibility that a high concentration of eIF4B can overcome a defective interaction between eIF4A and the eIF4G2 mutant. Because the TIF4632 allele of interest harbored multiple mutations, site-directed mutagenesis was employed to generate a panel of alleles with single mutations. The outcome of this analysis was that the tif4632-L574F mutation (henceforth referred to as L574F) was found to be necessary and sufficient for the Ts<sup>-</sup> phenotype displayed by the original compound mutation (data not shown), and, importantly, the Ts<sup>-</sup> phenotype conferred by L574F is mitigated by overexpressing either eIF4B or eIF4A in the mutant cells (Fig. 1A, hc TIF3 and hc TIF1 versus vector, 36 °C).

The HEAT domains of eIF4G2 and eIF4G1 are highly similar (24), and the Leu residue in eIF4G2 altered by L574F in eIF4G1 aligns with Leu-614 of yeast eIF4G1 (Fig. 1D). Because a crystal structure of the complex between yeast eIF4A and the eIF4G1 HEAT domain is available (5), we examined the phenotype of the corresponding Phe substitution at Leu-614 of eIF4G1. Remarkably, the tif4631-L614F mutation likewise confers a Ts<sup>-</sup> phenotype that is efficiently suppressed by overexpressing eIF4B or eIF4A (Fig. 1B, 36 °C). As illustrated in Fig. 2, Leu-614 is located near the end of helix α1 of the HEAT domain of eIF4G1, close to several residues in α1 or the α1-α2 turn that make side chain contacts with residues in the eIF4A CTD (5). Because the side chain of Leu-614 projects into the HEAT domain rather than

FIGURE 1. Overexpression of eIF4B reduces the Ts<sup>-</sup> phenotypes of particular substitutions in the HEAT domains of eIF4G1 and eIF4G2. A–C, an hc plasmid containing TIF1 (pBAS3432), TIF3 (p3350), or an empty vector (YEpLac195) was introduced into the following yeast strains harboring the indicated tif4632-HA or tif4631-HA mutant alleles as the only source of eIF4G: EPY81 (tif4632-L574F), EPY245 (tif4631-L614F), and EPY258 (tif4632-M570K) in A–C, respectively. These transformants, plus an empty vector transformant of YEplac195, were cultured at 30 or 36 °C for 2 days. D, amino acid sequence alignments of known eIF4G and eIF4G-related proteins. E and F, WB analyses of HA-tagged eIF4G proteins in WCEs. Yeast strains harboring the indicated mutant alleles (E) or TIF4631-HA alleles (F) were cultured at 30 °C in SC-Trp to A<sub>600</sub> of 0.2–0.4 and shifted to the indicated temperatures for 7 h. WCEs were prepared under denaturing conditions and subjected to WB analysis with antibodies against HA or PABP (to provide a loading control). The following HA-tagged strains were analyzed: EPY41, EPY81, EPY258, EPY260, EPY261, EPY88, EPY245, EPY259, and EPY256. Two to three independent transformants from each strain were examined for the WB analysis. Untagged WT strains YAS1951 and YAS2069 were examined in parallel as controls.
outward toward eIF4A (Fig. 2), its replacement with Phe would presumably weaken eIF4G HEAT domain interactions with the eIF4A-CTD indirectly. It might perturb the orientation of helix α1 in a way that weakens its contacts with eIF4A, or it might alter the disposition of nearby residues in helix α3 that make additional contacts with eIF4A (5) (Fig. 2). We envisioned that interaction of yeIF4B with the mutant eIF4G-HEAT domain, driven by the elevated cellular concentration of yeIF4B, would compensate for such perturbations of the eIF4A-eIF4G interface, either by restoring the WT conformation of HEAT domain helices or by interacting simultaneously with eIF4A and eIF4G to bridge their interaction within a hypothetical eIF4A-eIF4G-eIF4B trimeric complex.

It should be noted that neither L574F nor L614F reduces the steady-state level of the cognate eIF4G protein in cells grown at 30 or 36 °C, as judged by Western blot (WB) analysis of WCEs (except for M570K) (Fig. 1, E and F). Indeed, the mutants (except for M570K) are expressed at levels somewhat higher than the corresponding WT proteins. Thus, the mechanism of suppression by yeIF4B overexpression does not involve rescue of unstable mutant eIF4G proteins.

We reasoned that if yeIF4B overexpression suppresses the L574F and L614F substitutions by simply bridging the interaction of eIF4A with eIF4G in a trimeric complex, then it might be possible to suppress other HEAT domain substitutions that weaken the eIF4A/eIF4G interface. To test this idea, we first used site-directed mutagenesis to identify M570K as the substitution responsible for the Ts− phenotype of a second compound mutation we had identified in eIF4G2 that is suppressed by overexpressed yeast eIF4B, which includes Trp-579, interacts with a hydrophobic groove on the surface of the eIF4A-CTD, outside of the primary eIF4A-eIF4G1-HEAT interface (Fig. 2).
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FIGURE 3. Overexpressing yef4B reduces the Ts" phenotype and rescues the interaction defect of eIF4A with particular eIF4G substitution mutants. A–C, growth phenotypes of the indicated ifg432 or ifg431 mutant strains harboring pc plasmids containing TIF3 or TIF1 or an empty vector were assessed as in Fig. 1, A–C, using the following strains: EPY278, EPY279, EPY281, EPY282, EPY283, EPY285, EPY286, EPY287, and EPY251. D–F, the strains described in A–C plus EPY288, EPY294/EPY252 (untagged WT TIF4632/1 strain harboring hc TIF3 plasmid), or EPY297/EPY255 (empty vector) were cultured at 30 °C in SC–Ura, –Trp to an Aoom of 0.5–0.8. WCEs were immunoprecipitated with anti-HA antibodies, and immune complexes were treated with RNases A and T1 and subjected to WB analysis using antibodies listed on the left. Aliquots of 2.5% input extracts (WCE lanes) or 12.5–37.5% immune complexes (IP: αHA lanes) were examined. Lanes 1 and 2 and lanes 7 and 8 in D–E and lanes 1 and 2 and lanes 8 and 9 in F contain samples from the untagged WT control strains. Samples in D–F were run on the same gels, respectively, but rearranged for comparative purposes. The rearrangements are indicated by dividing lines.

- We also made an Ile substitution of Thr-578 in the eIF4G2 residue Thr-578. In addition, we made single and double Ala substitutions of two residues in the last (10th) helix of the eIF4G HEAT domain that make direct contacts with the eIF4A-NTD, Arg-835 and Phe-838 in eIF4G1 (Fig. 2) and Arg-795 and Phe-798 in eIF4G2. The eIF4G1-T618I and eIF4G2-R795A/F798A substitutions were lethal (data not shown), whereas the corresponding eIF4G2-T578I and eIF4G1-R835A/F838A substitutions conferred Ts" phenotypes that were mitigated by eIF4A overexpression. These latter mutations were also partially suppressed by yef4B overexpression (Fig. 3, B and C, 36 °C); however, in contrast to the situation above for eIF4G2-L574F and eIF4G1-L614F (Fig. 1, A and B), the Ts" phenotypes of eIF4G2-T578I and eIF4G1-R835A/F838A were suppressed more efficiently by eIF4A than by yef4B overexpression (Fig. 3, B and C, hc TIF3 versus hc TIF3, 36 °C). Again, the less efficient suppression of these last mutants cannot be attributed to lower protein abundance because the steady-state levels of eIF4G2-T578I and eIF4G1-R835A/F838A are equal to or greater than those of the highly suppressible eIF4G2-L574F and eIF4G1-L614F mutants (Fig. 1, E and F).

The suppression of eIF4G-HEAT Mutations Is Correlated with Rescue of Native eIF4G-eIF4A Complexes in Cell Extracts—We wished to test our hypothesis that yef4B overexpression can rescue complex formation between eIF4A and a subset of eIF4G-HEAT mutants in vivo. Accordingly, we immunoprecipitated WCEs with anti-HA antibodies and measured by WB analysis the amounts of eIF4A that coimmunoprecipitate with HA-eIF4G proteins in the presence or absence of yef4B overexpression. As noted above, association of yeast eIF4A with WT HA-eIF4G has not been observed in whole cell extracts at native levels of eIF4A and eIF4G expression (9, 35). However, we succeeded in coimmunoprecipitating a small fraction of eIF4A with WT HA-eIF4G1 from WCEs in a manner judged to be specific by the failure to coimmunoprecipitate eIF4A with untagged eIF4G (Fig. 4B, lane 18 versus lane 13, αeIF4A). This interaction was at least partially resistant to treatment of immune complexes with RNases A and T1, as was the coimmunoprecipitation (co-IP) of eIF4E with HA-eIF4G1 (Fig. 4B, lane 27 versus lane 18, αeIF4A and αeIF4E). In agreement with our previous results, co-IP of PABP with HA-eIF4G1 was greatly reduced by RNase treatment (Fig. 4B, lane 27 versus lane 18, αPABP) because the PABP-eIF4G interaction does not withstand co-IP analysis without the stabilizing influence of polyadenylated mRNA (39). In some experiments, a small amount of yef4B was also recovered specifically with WT HA-eIF4G in RNase-treated immune complexes (Fig. 4B, lane 18 versus lane 13, αeIF4A).
**eIF4B Promotes eIF4G-eIF4A Assembly**

**FIGURE 4. Correlation between the ability of overexpressed yeIF4B to suppress the Ts− phenotype and rescue defective interaction of eIF4A-eIF4G conferred by eIF4G substitutions.** A, summary of the ability of yeIF4B overexpression to restore cell growth at 36 °C and the coimmunoprecipitation of native eIF4A-complex for different eIF4G mutants. Results from Figs. 1 and 3 and are summarized qualitatively. Note that increasing the abundance of the M570K variant above the WT level allows yeIF4B overexpression to partially suppress the Ts− phenotype and to restore the M570K-eIF4A interaction defect (see supplemental Fig. S1). B, the hc TIF1, hc TIF3, and empty vector transformants of the tif4631-HA strain harboring the same plasmids as negative controls (EPY251) were cultured as in Fig. 3, D–F, along with transformants of untagged WT TIF4631 strain harboring the same plasmids as negative controls (EPY252, EPY253, EPY254, and EPY255; lanes 1–4, 10–13, and 19–22). WCEs were immunoprecipitated with anti-HA antibodies. Immune complexes were incubated at 26 °C for 30 min in the presence (lanes 19–27) or absence (lanes 10–18) of RNases A and T1 and subjected to WB analysis with antibodies listed on the right. Aliquots of 2.5% input extracts (WCE lanes) or 12.5–37.5% immune complexes (IP: αHA lanes) were examined. C and D, similar to B except using transformants of the tif4632-HA-L574F and tif4632-HA-M570K mutants described in Fig. 1, A and C, respectively, and hc TIF3 or vector transformants of untagged WT TIF4632 strain and examining only RNase-treated immune complexes. Samples in B–D were run on the same gels, respectively, but rearranged for comparative purposes. The rearrangements are indicated by dividing lines.

27 versus lane 22, eyeIF4B), which in all cases was increased dramatically on yeIF4B overexpression (lane 23 versus lane 19).

Having established an assay for native eIF4G-eIF4A complexes, we tested the prediction of our genetic analysis (summarized in Fig. 4A) that the L614F substitution weakens eIF4G1 association with eIF4A in a manner rescued by yeIF4B overexpression. Indeed, L614F clearly reduced the amount of coimmunoprecipitating eIF4A compared with that seen for WT eIF4G1, with or without RNase treatment (Fig. 4B, lane 17 versus lane 18 and lane 26 versus lane 27, eyeIF4A). Furthermore, overexpressing eIF4A1 (encoded by TIF1) or eIF4A2 (TIF2) rescued the co-IP of eIF4A with the L614F mutant (Fig. 4B,
elf4G Promotes elf4G elf4A Assembly

lanes 24 and 25 versus lane 26, elf4A). Note that this last conclusion should be restricted to RNase-treated complexes because overexpressed elf4A shows nonspecific association with immune complexes prepared with WCEs containing only untagged elf4G (Fig. 4B, lanes 11 and 12 versus lane 13, elf4A). Importantly, overexpressing elf4B also produced a marked increase in the amount of elf4A communoprecipitating with elf4G1-L614F (Fig. 4B, lane 23 versus lane 26, elf4A) without affecting the steady-state level of elf4A in the WCE (Fig. 4B, lanes 5 and 8, elf4A). Overexpressing elf4B had no effect on the amount of elf4A that communoprecipitated with elf4G1-L614F (Fig. 4B, lane 23 versus lane 26, elf4A), indicating that elf4B specifically stimulates elf4A association with the mutant elf4G1 protein. As mentioned above, analysis of the RNase-treated complexes revealed that overexpressing elf4B also increases its own association with elf4G1-L614F (Fig. 4B, lane 23 versus lane 26, elf4B). These findings support our conclusion that the L614F substitution in elf4G1 disrupts its interaction with elf4A in a manner rescued by elevated levels of elf4B in a manner associated with increased binding of elf4B to the mutant elf4G1 protein. The fact that the co-IP of elf4B with elf4G1 in cells overexpressing elf4B is resistant to RNase treatment (Fig. 4B, lane 14 versus lane 23, elf4B) suggests that the enhanced level of elf4B-elf4G complexes seen under these conditions does not depend on tethering of these two proteins to the same mRNA molecules.

We extended the co-IP analysis to include the corresponding elf4G2-L574F substitution, whose Ts− phenotype is also suppressed by elf4B overexpression (genetic data summarized in Fig. 4A), with similar results. In this and all subsequent experiments, we employed RNase treatment to eliminate nonspecific recovery of elf4A and elf4B in immune complexes when they are overexpressed. As shown in Fig. 4C, the L574F substitution reduces the amount of elf4A that communoprecipitates with the mutant HA-elf4G2 protein (lane 11 versus lane 12, elf4A), and overexpressing elf4A or elf4B increases the level of communoprecipitating elf4A above that seen for WT HA-elf4G2 (lanes 8–10 versus lanes 11 and 12, elf4A). Furthermore, overexpressing elf4B increases the amount of elf4B itself communoprecipitated with the elf4G2 mutant above that seen for WT elf4G2 (lane 8 versus lane 12, elf4B). Thus, suppression of the elf4G2-L574F substitution by elf4B overexpression is associated with restoration of its interaction with elf4A and also an elevated, RNase-resistant interaction with elf4B.

Quite different results were obtained for the elf4G2-M570K and elf4G2-W539A substitutions, whose Ts− phenotypes are not suppressed by elf4B overexpression (summarized in Fig. 4A). As shown in Fig. 4D, the M570K substitution evokes the expected reduction in the co-IP of elf4A with the mutant HA-elf4G2 protein (lane 13 versus lane 14, elf4A), and overexpressing elf4A increases the amount of communoprecipitating elf4A above that seen for WT HA-elf4G2 (lanes 11 and 12 versus lanes 13 and 14, elf4A). Moreover, overexpressing elf4B increases the amount of elf4B itself associated with the elf4G2-M570K mutant (lane 10 versus lane 13, elf4B); however, it does not increase the co-IP of elf4A with the mutant elf4G2 protein (lanes 10 and 13, elf4A). Similar results were obtained for the elf4G2-W539A substitution in Fig. 3D, where it can be seen that overexpressing elf4A, but not elf4B, rescues the impaired interaction of this elf4G2 variant with elf4A (lanes 9–12, elf4A), despite increased association of elf4B with the elf4G2 mutant (lane 9 versus lane 11, elf4B). Thus, in agreement with the genetic data (Fig. 4A), overexpressing elf4B does not restore elf4A association with the elf4G2-M570K or elf4G2-W539A variants in vivo, although these mutants interact effectively with elf4B itself.

We also conducted co-IP analysis for the elf4G2-T578I and elf4G1-R835A/F838A variants, whose genetic suppression by elf4B overexpression is appreciable but less extensive than that given by elf4A overexpression in the same mutants and also less extensive than that seen for the L614F/L574F substitutions of elf4G1/elf4G2 (as summarized in Fig. 4A). In both cases, overexpression of elf4B leads to detectable co-IP of elf4A with the mutant elf4G proteins; however, the amount of co-precipitating elf4A is below that achieved by overexpressing elf4A (Fig. 3, E (lanes 9–11) and F (lanes 10–13)). We found reproducibly in several independent experiments that the amounts of elf4A that communoprecipitated with these two mutant proteins on overexpressing elf4B versus overproducing elf4A differed more substantially than that seen for the elf4G1-L614F and elf4G2-L574F mutants discussed above. Thus, the relative efficiency of suppression of the Ts− phenotypes of different elf4G variants correlates well with the relative amounts of elf4A that communoprecipitated with the mutant proteins in cells overexpressing elf4B (summarized in Fig. 4A).

Finally, we examined the possibility that the failure of elf4B overexpression to rescue the elf4G2-M570K mutant might reflect the relatively lower abundance of this variant compared with all others we examined for suppression (Fig. 1, E and F). To this end, we introduced extra copies of the M570K allele on a second low copy plasmid, which predictably increased the steady-state level of this variant above that of WT elf4G2 (supplemental Fig. S1B, compare Sc versus Lc lanes). Introducing additional copies of M570K alone does not improve cell growth (supplemental Fig. S1A, row 2 versus row 4); nor does it increase the amount of elf4A that communoprecipitates with this elf4G2 mutant (supplemental Fig. S1C, lanes 12 and 13). However, overexpressing elf4B confers a detectable improvement of growth by the cells harboring extra copies of the M570K allele (supplemental Fig. S1A, row 1 versus row 3). Excess elf4B also increases the amount of elf4A that communoprecipitates with the M570K mutant (Fig. S1C, lane 11 versus lane 13). These findings extend the correlation summarized in Fig. 4A between suppression of the Ts− phenotypes of elf4G mutants and restoration of their association with elf4A on elf4B overexpression. Presumably, under the stimulatory influence of excess elf4B, the increased abundance of the elf4G2-M570K mutant achieved in these last experiments drives complex formation with elf4A by mass action.

yeIF4B Can Partially Restore elf4A Association with the L574F Variant of the elf4G2-CTD in Vitro—We sought next to determine whether the ability of overexpressed elf4B to rescue elf4A-elf4G2-L574F complexes in vivo could be reconstituted in vitro with purified components. To this end, we con-
L574F variant of the eIF4G2 HEAT domain can partially rescue complex formation between eIF4A and the
when the same reaction was conducted in the presence of 1
W539A, variant of eIF4G2. In separate experiments, we found
selectively eIF4A interaction with the L574F, but not the
ability of increased concentrations of yeIF4B to rescue
displaying a
decreased by a factor of
/2o ft h e
yeIF4B, the
eIF4A-RH bound to WT GST-eIF4G2(504–914) with a
Kd
of
50 nM, within the range reported previously for yeast
eIF4A was overexpressed from a strong
GAL promoters (10). It
reduces the coimmunoprecipitation of eIF4A
with eIF4G2 by a factor of
/2349
C-terminal residues 504–914 (encompassing the HEAT domain) and eIF4A-RH, purified as recombinant proteins from
Escherichia coli. The binding affinity of eIF4A for the WT or
mutant eIF4G2 proteins was determined by measuring the increase in fluorescence anisotropy of eIF4A-RH upon titrating the unlabeled GST-eIF4G2(504–914) protein in the presence or absence of excess WT yeIF4B (Fig. 5A). In the absence of yeIF4B, eIF4A-RH bound to WT GST-eIF4G2(504–914) with a
Kd of ~50 nM, within the range reported previously for yeast
eIF4A interaction with eIF4G1 (10, 30). As expected, the affinity of eIF4A-RH for the L574F variant was significantly reduced, displaying a ~7-fold higher
Kd for the complex formed with GST-eIF4G2(504–914)–L574F (Fig. 5, B and C). Importantly, when the same reaction was conducted in the presence of 1
μM
yeIF4B, the
Kd for the complex formed with the L574F variant decreased by a factor of ~4, reaching a value within a factor of ~2 of the
Kd for the WT complex (Fig. 5, B and C). Thus, yeIF4B can partially rescue complex formation between eIF4A and the
L574F variant of the eIF4G2 HEAT domain in vitro.

We showed above that the growth defect conferred by the W539A substitution in eIF4G2 cannot be suppressed by yeIF4B
overexpression (Fig. 3A). Consistent with this, the W539A variant of GST-eIF4G2(504–914) showed no detectable binding to
eIF4A-RH either in the absence or presence of 1
μM
yeIF4B (Fig. 5, B and C). Hence, our in vitro binding assays recapitulate the ability of increased concentrations of yeIF4B to rescue selectively eIF4A interaction with the L574F, but not the
W539A, variant of eIF4G2. In separate experiments, we found that 1
μM
yeIF4B does not significantly reduce the
Kd for the complex formed between eIF4A-RH and wild-type GST-
eIF4G2(504–914) (Fig. 5C), underscoring the specificity of the effect of yeIF4B on eIF4A binding to the L574F variant. These results are consistent with our conclusion that yeIF4B does not simply bridge the interaction between eIF4A and eIF4G, because in this scenario it might be expected that yeIF4B would reduce the
Kd for the complex between eIF4A and WT GST-
eIF4G2(504–914) and also confer at least partial rescue of eIF4A interaction with the W539A variant.

**Evidence that yeIF4B Promotes Association between eIF4A and WT eIF4G in Vivo**—As noted above, interaction between eIF4G and eIF4A at native expression levels in yeast cell extracts had not been detected previously (9, 35), and only a small amount of eIF4GI-eIF4A complex was observed when eIF4A was overexpressed from a strong (GAL) promoter (10). It was speculated that the eIF4G-eIF4A interaction in yeast cells is transient and might depend on interaction of eIF4G with another factor that exposes the eIF4A binding site in the HEAT domain (10). In view of our finding that overexpressing yeIF4B rescues eIF4A interaction with certain eIF4G HEAT domain mutants, we explored whether yeIF4B has a role in promoting eIF4A interaction with WT eIF4G in vivo. Supporting this hypothesis, we found that overexpressing yeIF4B produces a significant ~2.5-fold increase in the amount of eIF4A coimmunoprecipitating with WT eIF4G2 (Fig. 6, A and B). Moreover, deletion of TIF3 reduces the coimmunoprecipitation of eIF4A
with eIF4G2 by a factor of ~4 (Fig. 6, C and D). Overexpressing or eliminating yeIF4B had little or no effect on the amounts of eIF4A in the WCEs; nor did it affect the amounts of eIF4E that coimmunoprecipitated with eIF4G2 (Fig. 6, A and C). These results suggest that yeIF4B promotes complex formation between eIF4A and wild-type eIF4G2 in vivo. Below we propose an explanation for the ability of yeIF4B to increase the amount of eIF4A associated with WT eIF4G2 in WCEs (Fig. 6, A and B) but not in reactions with purified proteins (Fig. 5C).
elf4B Promotes elf4G-elf4A Assembly

![Image of a diagram](image_url)

**FIGURE 6.** yef4B promotes association between elf4A and wild-type elf4G *in vivo*. A, overexpression of yef4B enhances association of elf4A with WT elf4G2-HA *in vivo*. Shown is co-IP analysis of WT elf4G2-HA using transformants of strain harboring hcl TIF3 plasmid (EPY139) or empty vector (EPY234) (lanes 3 and 4 and lanes 7 and 8) and cognate transformants of control untagged strains (EPY294 and EPY297) (lanes 1 and 2 and lanes 5 and 6), conducted as described in Fig. 3, D–F, except that antibodies against elf4a/elf4b and 40S protein Rps2 were included. B, seven independent co-IP experiments were conducted as in A, and WB signals for elf4A and elf4G2 were quantified by videodensitometry using National Institutes of Health ImageJ software. elf4a/elf4g2 ratios were calculated for the hcl TIF3 transformants and normalized to the corresponding ratios for empty vector transformants, and the mean ± S.E. (error bars) ratios are plotted. C, elimination of yef4B weakens association of elf4A with WT elf4G2-HA *in vivo*. Shown is co-IP analysis of transformants of isogenic WT (FJZ107) and tif3Δ (FJZ102) strains harboring TIF4632-HA plasmid (lanes 2 and 3 and lanes 5 and 6) and the WT strain harboring empty vector (Yclac22) (lanes 1 and 4) carried out as in A, D, quantification of results from eight independent co-IP experiments conducted as in C, E, growth phenotypes of transformants of tif3Δ (FJZ052) and WT (FJZ2046) strains harboring the indicated hcl plasmids were assessed as in Fig. 1, A–C, except that cells were incubated at 18 °C for 9 days (tif3Δ) or 4 days (WT). F, WB analysis of WCEs from the relevant tif3Δ strains described in E using antibodies against elf4a and elf3b/Ded1 (as loading controls). Samples in A and C were run on the same gels, respectively, but rearranged for comparative purposes. The rearrangements are indicated by dividing lines.

We envisioned that the stimulatory effect of yef4B on elf4G-elf4A complex formation would enhance the recruitment of elf4A to elf4F complexes bound to capped 5′-ends of mRNA and that this would represent one aspect of the stimulatory effect of yef4A on translation initiation *in vivo*. If so, then overexpressing elf4A, which is sufficient to enhance native elf4G-elf4A assembly (e.g. Fig. 4B, lanes 24 and 25 versus lane 27, elf4A), should mitigate the growth defect of tif3Δ cells. Consistent with this prediction, we found that overexpressing elf4A improved the growth at 18 °C of tif3Δ cells but did not affect the growth of WT TIF3 cells (Fig. 6E). We verified that elf4A was overexpressed in the tif3Δ transformants harboring he TIF1 (Fig. 6F). It is important to note, however, that tif3Δ cells overexpressing elf4A still grow much more slowly than isogenic WT TIF3 cells (Fig. 6E), indicating that yef4B has other critical functions in translation beyond stabilization of elf4A-elf4G complexes.

**DISCUSSION**

In this study, we demonstrated that various point mutations in the HEAT domains of elf4G1 and elf4G2 that weaken the binding to elf4A and hence are suppressed *in vivo* by overexpressing elf4A also are suppressed by overexpressing yef4B. Although suppression of Ts− phenotypes of elf4G HEAT mutations by elf4A overexpression was reported previously for other elf4G mutations (9), comparable suppression by yef4B overexpression is a novel finding of this study. In addition, we succeeded in establishing conditions for assaying the abundance of native elf4A-elf4G complexes in cell extracts and determined that these interactions are resistant to RNase treatment and, thus, persist without having these factors tethered to the same mRNA molecules. We are unsure why we have succeeded in specific coimmunoprecipitation of native elf4A and elf4G proteins where previous attempts have failed, but it might be attributable to using highly concentrated WCEs and omitting detergents from the wash buffers. In any event, using this assay, we demonstrated a robust correlation between suppression of the Ts− phenotypes of particular elf4G mutations and restoration of a WT level (or even greater) of elf4A association with elf4G in WCEs of cells overexpressing yef4B. This correlation strongly suggests that an excess of yef4B reduces the growth defects conferred by elf4G HEAT domain mutations by rescuing elf4A-elf4G assembly *in vivo*. We extended this correlation with the results of *in vitro* experiments on purified proteins, demonstrating that whereas both L574F and W539A substitutions on elf4G2 reduce its affinity for elf4A, detectable interaction with elf4A was enhanced by an elevated concentration of yef4B only for the L574F variant. These last findings are in accordance with the fact that the Ts− phenotype and defective interaction with elf4A conferred by L574F, but not that of W539A, are suppressed by yef4B overexpression *in vivo*. The elf4G1 residue corresponding to Trp-579 (Trp-579) lies in a segment outside of the HEAT domain that interacts with the elf4A-CTD (Fig. 2). Together, the results demonstrate that high concentrations of yef4B can mitigate the deleterious effects of HEAT domain mutations on the stability of native elf4G-elf4A complexes and the attendant defects in translation initiation produced by these mutations *in vivo*.

The ability of yef4B to restore elf4A association with the elf4G2-L574F mutant *in vitro* using purified proteins suggests that yef4B can interact directly with elf4G, elf4A, or both proteins in a manner that restores elf4G-elf4A association. The possibility of direct interaction of yef4B with elf4G is supported by our ability to detect coimmunoprecipitation of yef4B but not elf4A with elf4G2-M570K (expressed from a single-copy allele) and elf4G2-W539A upon yef4B overexpression (e.g. see Fig. 4D, lane 10). Because these elf4G-yeIF4B
complexes are resistant to an RNase treatment sufficient to significantly reduce association of PABP with eIF4G, the eIF4G-eIF4B interaction detected in these assays is not likely to be bridged by mRNA or PABP. In addition, we showed previously that immune complexes prepared under these conditions are devoid of detectable eIF3 or 40S subunits (39). Although we cannot rule out the possibility that another factor mediates or stabilizes the interaction between eIF4G and eIF4B observed in these immune complexes, our data are significant in providing the first evidence that yeIF4B is physically associated with eIF4G in native complexes that do not contain eIF4A, intact mRNA, or 43S PICs. As noted above, stimulation of eIF4A helicase activity by yeIF4B has not been demonstrated in vitro, and although genetic data are consistent with a role for eIF4B in promoting eIF4F function, it has been unclear whether yeIF4B acts in close cooperation with eIF4F or, rather, by a parallel mechanism. Our findings of mRNA-independent eIF4G-yeIF4B association in cell extracts suggest that yeIF4B stimulates eIF4F function in a manner involving direct contact between these two factors.

In addition to rescuing eIF4A association with certain eIF4G mutants, we observed that yeIF4B also promotes native complex formation between eIF4A and wild-type eIF4G2. Thus, overexpressing yeIF4B increased co-IP of eIF4A with WT eIF4G2, whereas the absence of yeIF4B in tfi3Δ cells markedly reduced detectable eIF4G2-eIF4A complexes. These findings suggest that at least one function of yeIF4B in vivo is to stimulate interaction of eIF4A with the HEAT domain of eIF4G, which is crucial for achieving the active conformation of eIF4A (5, 13, 14). Enhancing eIF4G-eIF4A association should also facilitate recruitment of eIF4A to eIF4F complexes at the 5′-ends of mRNAs, where eIF4A helicase function can produce a single-stranded RNA binding site for the 43S PIC. Our finding that eIF4A overexpression confers an appreciable, if modest, improvement of the growth of tfi3Δ cells is consistent with the idea that stimulating eIF4A-eIF4F assembly is one aspect of yeIF4B function in vivo, although the substantial growth defect remaining in these cells clearly indicates that yeIF4B has other important functions in the initiation pathway. Our model also helps to explain previous findings of Linder and co-workers (29) that the Ts− phenotype conferred by the A79V substitution in eIF4A is very efficiently suppressed by overexpressing yeIF4B. We envision that because the defect in eIF4A helicase activity is rate-limiting for translation in the A79V mutant, the ability of overexpressed yeIF4B to enhance eIF4A-eIF4G assembly provides a strong improvement of mutant cell growth.

How does yeIF4B overexpression promote association of eIF4A with HEAT mutants of eIF4G? Our findings that yeIF4B can be communoprecipitated with all of the eIF4G mutants we tested but rescues eIF4A association only for the HEAT domain mutants seems inconsistent with the possibility that yeIF4B merely bridges the eIF4G-eIF4A complex by binding simultaneously to both eIF4G and eIF4A. We cannot rule out the possibility that the W579A and W539A substitutions mapping outside of the HEAT domains of eIF4G1/eIF4G2 cannot be rescued by excess yeIF4B simply because they confer a stronger eIF4A binding defect than do the HEAT domain substitutions. However, this possibility seems less likely if one considers that the growth defects of the eIF4G1-W579A and eIF4G2-W539A mutants are less severe than those of eIF4G2-T578I and eIF4G1-R835A/F838A, whose association with eIF4A is rescued by excess yeIF4B. It is noteworthy that Nielsen et al. (19) found that interactions of mammalian eIF4A with eIF4G and eIF4B (in the presence of ssRNA) are mutually exclusive, precluding formation of a trimeric complex. Consistent with this, we showed here that yeIF4B does not reduce the Kd for the eIF4G-eIF4A complex formed with WT GST-eIF4G2(504–914), which might have been expected upon formation of a trimeric complex. Furthermore, whereas we have been able to detect the GST-eIF4G2(504–914)-eIF4A complex by native gel electrophoresis in vitro, the presence of yeIF4B at high concentrations did not alter the mobility of this binary complex, providing no evidence for the trimeric complex (data not shown).

Finally, although overexpressing yeIF4B enhances complex formation between eIF4A and eIF4G in vivo, overexpressing eIF4A does not increase the abundance of eIF4B-eIF4G complexes (e.g. Fig. 4B, lanes 24–26). These findings do not support a model in which eIF4A binds more tightly to a yeIF4B-eIF4G subcomplex within a trimeric complex than it binds to eIF4G alone in eIF4A-eIF4G binary complexes.

Thus, in the absence of physical evidence for a yeIF4B-eIF4G-eIF4A trimeric complex, we propose instead that interaction of yeIF4B with the L574F or L614F mutant corrects the deleterious effects of these substitutions on the orientation of helix α1 or α3 in the HEAT domain, both of which harbor multiple points of contact with eIF4A, and thereby restores a conformation competent for binding eIF4A (Fig. 7). Dissociation of yeIF4B from the eIF4G HEAT domain would precede complex formation between eIF4G and eIF4A, such that a trimeric complex does not accumulate. In this view, interaction of yeIF4B with the eIF4G HEAT domain is transient and operates in a manner akin to a chaperone-substrate interaction. The lack of suppression of the single-copy M570K allele by yeIF4B overexpression probably reflects the combined effect of its relatively lower abundance and a greater disruption of HEAT domain structure by insertion of positively charged Lys deep into the space between helices α1 and α3 (Fig. 2), which also renders the protein more susceptible to proteolysis compared with the L574F variant.

The Ts− phenotypes of the eIF4G2-T578I and eIF4G1-R835A/F838A mutants were diminished by yeIF4B overexpression but less efficiently than by overexpressing eIF4A. Because these substitutions eliminate specific contacts at the eIF4A/eIF4G interface, their binding defects should be less responsive to yeIF4B overexpression if (as we postulate) yeIF4B acts primarily to promote the proper conformation of HEAT domain helices conducive to eIF4A binding. Indeed, it appeared from the co-IP analysis that yeIF4B overexpression was relatively less effective at enhancing eIF4A/eIF4G association for the T578I and R835A/F838A substitutions compared with the highly suppressible L574F and L614F substitutions. Finally, we propose that the Ts− phenotypes and eIF4A binding defects produced by eIF4G2-W539A and eIF4G1-W579A are insensitive to excess yeIF4B primarily because these residues lie outside of the HEAT domain.
It should be noted that excess yeIF4B did not fully suppress the Ts− phenotypes of the T578I and R835A/F838A mutants although the amounts of eIF4A associated with these variants upon yeIF4B overexpression were equal to or greater than that observed for WT eIF4G at native levels of factor expression (e.g. Fig. 3E, compare lanes 9 and 12, eIF4A). Accordingly, we conclude that the loss of direct contacts with eIF4A conferred by these eIF4G substitutions not only weakens the eIF4A–eIF4G complex but also impairs eIF4G function in modulating the conformation of eIF4A to promote the binding of substrates, ATP hydrolysis, or release of products and that the latter defect(s) persists even when eIF4A–eIF4G assembly is restored by overexpressing yeIF4B or eIF4A.

Our model in which yeIF4B promotes the productive conformation of the eIF4G HEAT domain is supported by our finding that overexpressing yeIF4B elevates native complexes formed between eIF4A and WT eIF4G, but can the model be reconciled with our ostensibly contradictory findings that yeIF4B has no effect on the stability (Kd) of the complex between eIF4A and the WT eIF4G2 HEAT domain in vitro? One explanation would be that the recombinant WT eIF4G2 HEAT domain expressed in bacteria spontaneously assumes a conformation conducive for eIF4A binding without prior interaction with yeIF4B, and because a trimeric complex cannot form, excess yeIF4B has no impact on the stability of the eIF4A-GST–eIF4G2(504–914) complex in vitro. In yeast cells, by contrast, a post-translational modification or interaction with an inhibitory factor would provoke an altered state or conformation of the HEAT domain incompatible with stable eIF4A binding, possibly mimicking the effects of the L574F and L614F substitutions. Transient interaction with yeIF4B would overcome these effects and stabilize the conformation conducive to stable complex formation with eIF4A.

![Image](image-url)

**FIGURE 7.** Hypothetical model for the ability of overexpressed yeIF4B to rescue eIF4A association with eIF4G2 HEAT domain mutants and promote assembly of WT eIF4G–eIF4A complexes in vivo. The HEAT domain of WT (A) or L574F mutant (B) eIF4G2 is depicted using yellow and orange shapes to represent helices 1–5 and 6–10, respectively, connected by a linker. The RecA-like domains of eIF4A are depicted using light and dark blue shapes that can be inserted into the pockets formed by the HEAT domain helices, as observed in the x-ray crystal structure of the eIF4G1–eIF4A complex described in the legend to Fig. 2. A, the L574F substitution in eIF4G2 distorts the packing of HEAT domain helices 1–3 in a manner that weakens their direct contacts with the eIF4A-CTD (i). Transient interaction of the mutant HEAT domain with yeIF4B in cells overexpressing the latter overcomes the moderate distortions conferred by L574F (ii) to achieve a conformation competent for stable complex formation with eIF4A (iii). B, in the case of WT eIF4G2, a hypothetical post-translational modification (green asterisk) or interacting protein (gray box) provokes a distortion of the WT HEAT domain in yeast cells, which might resemble that produced by the L574F substitution (i), and transient interaction with yeIF4B overcomes the putative modifications of the WT HEAT domain (ii) to stabilize the conformation conducive to stable complex formation with eIF4A (iii).
vivo by a post-translational modification or unknown regulatory factor and that interaction with another stimulatory factor would transiently expose the eIF4A binding site in eIF4G. Our results suggest that yeIF4B performs this predicted stimulatory function in vivo.

In conclusion, by uncovering a class of eIF4G HEAT domain mutations whose Ts - phenotypes are suppressed by yeIF4B overexpression and by elucidating the molecular mechanism of suppression, we detected a novel interaction between eIF4G and yeIF4B and revealed an unforeseen function for yeIF4B in promoting complex formation between eIF4G and eIF4A in vivo. This activity of yeIF4B should facilitate the known roles of eIF4G in stimulating eIF4A helicase activity and (in conjunction with eIF4E) of recruiting eIF4A to the capped 5'-ends of mRNA. Our results also suggest that yeIF4B is capable of reversing an inhibitory mechanism that appears to operate in WT yeast cells to limit association of eIF4A with the eIF4G-eIF4E subcomplex of eIF4F. It will be interesting to determine whether this novel function of yeIF4B is enlisted to regulate eIF4F assembly under specific physiological conditions.

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