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Role of *Saccharomyces cerevisiae TAN1* (tRNA acetyltransferase) in eukaryotic initiation factor 2B (eIF2B)-mediated translation control and stress response

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Abstract Eukaryotic initiation factor 2B (eIF2B) controls the first step of translation by catalyzing guanine nucleotide exchange on eukaryotic initiation factor 2 (eIF2). Mutations in the genes encoding eIF2B subunits inhibit the nucleotide exchange and eventually slow down the process of translation, causing vanishing white matter disease. We constructed a *Saccharomyces cerevisiae* genomic DNA library in YEp24 vector and screened it for the identification of extragenic suppressors of eIF2B mutations, corresponding to human eIF2B mutations. We found a suppressor-II (Sup-II) genomic clone, as suppressor of eIF2Bβ (*gcd7-201*) mutation. Identification of Sup-II reveals the presence of truncated *SEC15*, full-length *TAN1* (tRNA acetyltransferase), full-length *EMC4*, full-length YGL230C (putative protein) and truncated *SAP4* genes. Full-length *TAN1* (tRNA acetyltransferase) gene, subcloned into pEG(KG) vector and overexpressed in *gcd7-201 gcn2Δ* strain, suppresses the slow-growth (Slg$^-$) and general control derepression (Gcd$^-$) phenotype of *gcd7-201 gcn2Δ* mutation, but YGL230C did not show any effect. A GST-Tan1p fusion protein of 60 kDa was detected by western blotting using α-GST antibodies. Interestingly, Tan1p overexpression also suppresses the temperature-sensitive (Ts$^-$), Slg$^-$ and Gcd$^-$ phenotype of eIF2Bγ (*gcd1-502*) mutant. Role of Tan1p protein in eIF2B-mediated translation regulation was also studied. Results revealed that Tan1p overexpression confers resistance to *GCD7 GCN2*, *gcd7-201 gcn2Δ*, *GCD7 gcn2Δ* growth defect under ethanol, H_2O_2 and caffeine stress. No resistance to DMSO-, NaCl- and DTT-mediated growth defect upon *GCD7 gcn2Δ*, *GCD7 GCN2*, *gcd7-201 gcn2Δ* was observed by overexpression of *TAN1*. Hence, we proposed that Tan1p is involved directly or indirectly in regulating eIF2B-mediated translation.

Introduction

Eukaryotic initiation factor 2B (eIF2B) is a decameric complex containing two copies each of α, β, γ, δ and ε subunits encoded by *GCN3*, *GCD7*, *GCD1*, *GCD2* and *GCD6*, respectively, in yeast (Wortham and Proud 2015; Dever et al. 2016). The α, β and δ subunits of eIF2B constitute a regulatory sub-complex, while the γ and ε subunits form catalytic sub-complex, the latter playing a role in catalyzing the GDP–GTP exchange on its substrate eukaryotic initiation factor 2 (eIF2) (Pavitt et al. 1998). Global protein synthesis is reduced upon phosphorylation of eIF2α at serine 51 (S51) position under stress condition. eIF2B binds tightly to the phosphorylated eIF2 and slows down the process of translation (Krishnamoorthy et al. 2001; Pavitt et al. 1998; Rowlands et al. 1988). *Saccharomyces cerevisiae* has developed a well-defined mechanism to overcome stress by translation of general control nonderepressible (*GCN4*) mRNA, stimulated under stress conditions, resulting in transcription of numerous stress-related genes (Natarajan et al. 2001). In *S. cerevisiae*, Gcn2p is the only enzyme that phosphorylates eIF2 in response to nutrient starvation and sodium or rapamycin exposure (Shenton et al. 2006). Volatile anesthetics inhibit protein synthesis via eIF2B in both *S.*

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cerevisiae and higher cells (Palmer et al. 2005). Fusel alcohols also inhibit translation initiation via eIF2B (Ashe et al. 2001). A neurodegenerative disease named differently CACH (childhood ataxia with central nervous system hypomyelination), VWM (leukoencephalopathy with vanishing white matter) or eRDs (eIF2B related disorders) vanishing white matter disease (Fogli and Boespflug-Tanguy 2006; Pronk et al. 2006; Schiffmann and Elroy-Stein 2006) is a rare leukoencephalopathy caused by mutations in the genes encoding different subunits of eIF2B (Hannig et al. 1990; Dever et al. 1993; De Aldana and Hinnebusch 1994; Pavitt et al. 1997). Mutations in all the genes encoding five eIF2B subunits are reported to derepress *GCN4* translation independent of eIF2 phosphorylation (Gcd⁻ phenotype) (Fogli and Boespflug-Tanguy 2006; Leegwater et al. 2001; Richardson et al. 2004; van der Knaap et al. 2002). eIF2B guanine nucleotide exchange activity in VWM patients is generally lower than normal cells (Horzinski et al. 2009). In some cases, cataracts, ovaries, kidneys and pancreas are also affected in vanishing white matter disease (van der Knaap et al. 2003; Fogli et al. 2003), but glial cells are severely affected. Function of different subunits of eIF2B in translation regulation in humans is not well understood; therefore, there is the unavailability of proper treatment for such a rare leukoencephalopathy. Identifying target suppressor proteins of eIF2B mutations can be a better approach to understand the complexity of the disease and might be useful in curing VWM disease.

Mutations in eIF2B genes corresponding to VWM disease make *S. cerevisiae* sensitive to amino acid starvation (Gcd⁻ phenotype) and show slow-growth phenotype (Slg⁻) and many time temperature-sensitive (Ts⁻) phenotype. eIF2B mutations impair the function of eukaryotic initiation factor 2B complex in many ways, such as by affecting the stability or integrity of the eIF2B complex, and altering eIF2–eIF2B interactions (Li et al. 2004; Richardson et al. 2004). However, some mutations do not affect eIF2–eIF2B interactions or GEF activity, but can still cause the VWM disease (Liu et al. 2011). In the present work, *TAN1* was identified as the extragenic suppressor of *gcd7-201 gcn2Δ* and *gcd1-502 gcn2-101* mutations of *S. cerevisiae* eIF2B subunits. Defect in protein folding is a major cause of many diseases, including cystic fibrosis and neurodegenerative disorders like Huntington's, Alzheimer's and prion diseases. eIF2B protein complex containing eIF2Bβ^{V341D} (human eIF2Bβ^{V316D}) mutation is unstable with reduced GEF activity, as a result of which eIF2Bβ^{V341D} exhibits a reduced growth rate and a defect in global translation (Gcd⁻) in *S. cerevisiae*. Moreover, eIF2Bδ is excluded from eIF2Bβ^{V341D} complexes (Richardson et al. 2004). The function of eIF2Bβ^{v341D} can be rescued by overexpression of eIF2Bδ alone (Richardson et al. 2004). In addition to GEF

activity, eIF2B is also characterized by possessing GDI displacement factor (GDF) activity. GDF function of eIF2B depends upon the eIF2Bε and eIF2Bγ subunits (Jennings et al. 2013). *gcd1-502* mutant has defective GCD1 subunit and has a GDF defect, conferring slow growth. Moreover, previous studies have reported that the slow-growth (Slg⁻) phenotype of eIF2Bγ mutants (G12V) and *gcd1-502* (L480Q) strains analogous to a human eIF2B mutation (EIF2B3-G11V) is suppressed by overexpression of the three subunits of eIF2 (Dever et al. 1995). It has been shown that depletion of *GCD7* in *gcd7-td* (degron) leads to codepletion of *GCD2* (Dev et al. 2010), thus suggesting that GCD7 provides a platform for GCD2 binding in a pentameric eIF2B complex. Met-tRNAi^{Met} binding stabilizes GTP binding to eIF2 (Kapp and Lorsch 2004), and thus, Met-tRNAi^{Met} overexpression could provide insights into translation defects due to eIF2B mutations. One such example is overexpressing Met-tRNAi^{Met} suppresses the lethality of Gcd2 depletion (gcd2-td mutant) (Dev et al. 2010). Tan1p is required for the formation of modified nucleoside N^4-acetylcytidine (ac^{4}C) in tRNA specific for leucine and serine (Johansson and Bystrom 2004). The role of *TAN1* overexpression in eIF2B-mediated translational regulation under H₂O₂-, alcohol-, caffeine-, DMSO-, DTT- and salt-mediated stress was also studied. Our results suggested that *TAN1* overexpression imparts resistance to the H₂O₂, ethanol and caffeine stress. Thus, *TAN1* regulates eIF2B-mediated translation regulation by an unrecognized mechanism.

Materials and methods

All the chemicals and reagents used were of molecular biology grade and were procured from Thermo Scientific, Himedia Labs, India; MP Biomedicals, USA; Fermentas Inc. USA; and Bio-Rad. USA.

S. cerevisiae strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. Standard laboratory strain *E. coli* (DH5α) used for plasmid isolation was cultured in nutrient broth (NB) and grown at 37 °C. YEp24 vector was used for the construction of genomic DNA library, and pEG(KG) was used for cloning and expression of *S. cerevisiae* genes. *S. cerevisiae* strains were cultured in YPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) or liquid media (without agar). Nutrient broth (NB, Himedia Labs, Mumbai) with 100 μg/ml ampicillin was used to culture the bacterial strains of *E. coli* (DH5α) harboring YEp24 or pEG(KG) at 37 °C. Plasmid DNA of YEp24 and pEG(KG) was isolated and used in transformations of yeast strains (Sambrook et al. 2009; Elble 1992). Transformants were selected on

Table 1 Description of *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source or reference
H4	*mata leu2-3 leu2-112 ura3-52*	Alan G. Hinnebusch
H1794	*mat@ leu2-3 leu2-112 ura3-52 gcd7-201 gcn2::LEU2*[p500]	Alan G. Hinnebusch
H1795	*mat@ leu2-3 leu2-112 ura3-52 GCD7 gcn2::LEU2*[p500]	Alan G. Hinnebusch
H1792	*mat@ ura3-52* gcd6-1 *gcn2::LEU2*[p500]	Alan G. Hinnebusch
H1793	*mat@ ura3-52 GCD6 gcn2::LEU2*[p500]	Alan G. Hinnebusch
H750	*mata gcn2::LEU2 leu2-3 leu2-112 ura3-52*	Alan G. Hinnebusch
H591	*mat@ gcd12-503 gcn2-101 gcn3-101 his1-29 ura3-52 ino1* (HIS4-lacZ, ura3-52)	Alan G. Hinnebusch
H70	*mat@ his1-29* gcn2-101 gcn3-101 ura3-52 gcd1-502 (HIS4-lacZ, ura3-52)	Alan G. Hinnebusch

synthetic complete (SC) medium without uracil supplementation at 30 °C.

Construction and screening of *S. cerevisiae* genomic DNA library

High molecular weight genomic DNA was isolated as described in Sambrook et al. (2009). Genomic DNA was partially digested with three different concentrations of *Sau3AI* enzyme and was gel purified by using a gel purification kit (Thermo Scientific). A total of 50 ng of purified genomic DNA fragments resulting from restriction digestion was pooled and ligated with 20 μg of shrimp alkaline phosphatase (1U)-treated plasmid YEp24 at *BamHI* site using T4 DNA ligase (Rose et al. 1987). The ligation reaction was performed at 16 °C for 16 h. DH5α strain was transformed by the heat-shock method with the ligation mix (Sambrook et al. 1989) and plated on NA medium containing ampicillin (100 μg/ml). Transformations were pooled into three groups, each representing ~13,575 cfu. All the three pools were grown independently and plasmid DNA was isolated (Sambrook et al. 1989). Transformation of *S. cerevisiae* eIF2B mutant strains (Table 1) showing slow-growth (Slg⁻) phenotype was performed independently with all the three pools of plasmids. Wild-type strains *GCD7 GCN2* and *GCD7 gcn2Δ GCD6 gcn2Δ* strains (Table 2) were transformed with YEp24 vector using LiAc method (Elble 1992). Transformants were plated on SC medium with 2% glucose and lacking uracil. Synthetic complete supplement mixture (SC) without uracil is used as a dropout supplement to select transformants containing uracil-based plasmid. The transformants showing colony size to that of vector transformed *GCD7 gcn2Δ* were compared by streaking and spot assay (Gunde and Barberis 2005) to that of vector transformed *gcd7-201 gcn2Δ* mutant strain. The transformants showing Slg⁺ and Gcd⁺ phenotype were further screened by spot assay of tenfold serially

diluted culture and by streaking. The plasmid DNA was rescued from the potential *gcd7-201 gcn2Δ* transformant (Slg⁺, Gcd⁺) as given by (Hoffman and Winston 1987). Rescued plasmids were transformed back into *gcd7-201 gcn2Δ* mutant yeast strain in order to reconfirm the Slg⁺ and Gcd⁺ phenotype. The insert present in the plasmid was sequenced on both the strands at Eurofins, Bangalore (http://www.eurofins.in/) using vector-specific primers (Table 3).

Functional characterization of suppressor protein

Full-length *TAN1* gene and YGL230C gene present in the plasmids were amplified from rescued plasmids using gene-specific primers (Table 3) followed by sub-cloning into pEG(KG) vector with a *GAL1* promoter and protease cleavable N-terminal GST tag at the *SalI/HindIII* and *HindIII/BamHI* restriction sites, respectively. The resulting recombinant plasmid DNA (100 ng) was transformed into *E. coli* DH5α by heat-shock method (Sambrook et al. 1989). Plasmid DNA was isolated from transformants and sequences were ensured by DNA sequencing. In order to verify the potential gene responsible for Slg⁺ and Gcd⁺ phenotype, pEG(KG)/*TAN1* plasmid and pEG(KG)/YGL230C plasmid were transformed into *gcd7-201 gcn2Δ* strain by LiAc method (Elble 1992) and grown on SC medium supplemented with 2% galactose and lacking uracil. *gcd7-201 gcn2Δ* strain and *GCD7 gcn2Δ* were transformed with pEG(KG) vector alone, grown on SC-Ura/Gal medium and were used as controls. pEG(KG) has a galactose-regulated promoter. Gal promoter is repressed in the presence of raffinose, but is induced by addition of galactose. So the gene cloned under Gal1 promoter can be switched on or off depending on the presence of either raffinose or galactose. The transformants were analyzed by isolation of plasmid DNA by HiPurA™ yeast plasmid DNA purification kit. The *TAN1* transformants were screened for Slg⁺ and Gcd⁺ phenotype by spot assay. Gcd

phenotype was also tested on SC medium containing 30 mM 3-AT (3-amino-triazole). 3-AT is a histidine analog. Amino acid starvation is commonly mimicked in yeast using 3-amino-triazole (3-AT). Addition of 3-AT to wild-type strains causes histidine starvation, which activates Gcn2p kinase, ultimately increasing GCN4 expression. Mutations in eIF2B subunits in gcn2Δ strains inhibit eIF2B activity. Reduction in eIF2B activity in gcn2Δ strains allows growth on media containing 3-AT. pEG(KG), a uracil-based plasmid containing TAN1, was evicted on 5-fluoroorotic acid (FOA)-containing medium. Colonies from FOA plate were replica plated on SC medium without uracil and containing 2% galactose. URA3 gene product converts 5-fluoroorotic acid (5-FOA) to a toxic product, 5-fluorouracil. Thus, yeast cells containing URA3 marker cannot grow on medium containing 5-FOA, but can grow on medium lacking uracil. This property is used to select for the loss of vectors carrying the wild-type URA marker (Zhang et al. 1997). Plates were incubated at 30 °C for 2 days and were checked for growth.

Western blot analysis

Whole-cell extracts (WCE) from induced and uninduced cultures of S. cerevisiae gcd7-201 gcn2Δ were prepared by a glass bead lysis method using Fast Prep (MP Biomedicals). For this, S. cerevisiae transformants were inoculated into SC medium (5 ml) supplemented with 2% raffinose (w/v) and incubated at 30 °C for 18 h. One percent of overnight grown primary culture was used to inoculate 10 ml SC medium supplemented with 2% raffinose (w/v) and incubated at 30 °C. At an A $_{600}$ of ~0.5, an aliquot was taken as the uninduced control, and the remaining culture was induced by the addition of 2% galactose (w/v) followed by continued incubation for an additional 3 h at 30 °C. The cells were harvested by centrifugation (6000 rpm for 10 min) and protein extracts were prepared using 20% trichloroacetic acid (TCA). Twenty micrograms of total protein was resolved by SDS–PAGE transferred to the nitrocellulose membrane (Millipore, Immobilon P 0.45 μm) by electroblotting and incubated at 4 °C for 1 h in blocking solution containing 5% nonfat dried milk. The membrane was incubated with anti-GST antibodies (1:5000, Abcam) for overnight at 4 °C. Immunoreactive proteins were detected by using primary antibody (α-GST for fusion protein and α-GCD6 for GCD6) and secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase (1:10000, Abcam) for 1 h and after three successive washes using PBST (phosphate-buffered saline containing Triton X-100) buffer, blots were developed using an enhanced chemiluminescence kit (ECL, Bio-Rad, Inc., USA).

Expression of TAN1 in eIF2Bε, eIF2Bγ, eIF2Bδ and GCN2 mutants

Transformation of eIF2ε (H1792), eIF2Bγ (H70), eIF2Bδ (H750) and GCN2 (H591) mutants (Table 1) with pEG(KG) or pEG(KG)/TAN1 was done, and they were grown in SC medium lacking uracil and containing 2% galactose for 2 days at 30 °C. Transformants were selected and analyzed for Slg$^+$ and Gcd$^+$ phenotype by streaking and spot assays on SC medium lacking uracil and containing 2% galactose or SC medium lacking uracil and containing 2% galactose and 30 mM 3AT, respectively. Plates were incubated for 2 days at 30 °C. Temperature-sensitive (Ts$^-$) phenotype of eIF2Bγ (H70) was also checked by incubation at 37 °C.

Effect of H$_2$O$_2$, ethanol and caffeine on the growth S. cerevisiae strains overexpressing TAN1

To determine the effect of H$_2$O$_2$, ethanol, caffeine, NaCl, DMSO and dithiothreitol (DTT), wild-type GCD7 GCN2, GCD7 gcn2Δ and gcd7-201 gcn2Δ strains of S. cerevisiae containing pEG(KG) or pEG(KG)/TAN1 were grown for 16 h at 30 °C with shaking in SC medium supplemented with either 2% galactose or raffinose (lacking uracil). SC medium was also supplemented with 4 mM H$_2$O$_2$ (Ring et al. 2008), 10% ethanol (Yamauchi and Izawa 2016), 20 mM caffeine (Wanke et al. 2008), 1.6% DMSO (Motlekar et al. 2009), 35 mM dithiothreitol (DTT) (Ring et al. 2008) and 1 M NaCl (Melamed et al. 2008) separately. Cell density at $A_{600 \text{ nm}}$ was measured by using UV visible spectrophotometer. S. cerevisiae transformants were also tested by spot and halo assays (Flower et al. 2005; Yang et al. 2006). Slg$^-$ and Gcd$^-$ phenotype was tested by spotting tenfold serially diluted cultures on SC medium supplemented with either 2% galactose or raffinose (lacking uracil) and 3-AT (30 mM) for Gcd$^-$ phenotype or containing H$_2$O$_2$ (4 mM), ethanol (10%), caffeine (30 mM), DMSO (1.6%), dithiothreitol (DTT) (35 mM) and NaCl (1 M). The plates were incubated at 30 °C for two days. In halo assay, filter disks of H$_2$O$_2$, ethanol, caffeine, DMSO, dithiothreitol (DTT) and NaCl were placed on SC agar medium containing either 2% galactose or raffinose (lacking uracil) with uniformly spread culture of S. cerevisiae mutant or wild-type strains containing pEG(KG) or pEG(KG)/TAN1. Petri plates were incubated at 30 °C for 2 days and observed for zone of inhibition of yeast cell growth. The S. cerevisiae GCD7 GCN2, GCD7 gcn2Δ and gcd7-201 gcn2Δ strains were streaked on YPD plates with or without H$_2$O$_2$, ethanol, caffeine, DMSO, dithiothreitol (DTT) and NaCl and were used as controls.

Results

Construction of *S. cerevisiae* genomic DNA library in YEp24 plasmid

Genomic DNA of the *S. cerevisiae* strain H4 (Table 1) and YEp24 plasmid DNA was isolated (Sambrook et al. 2009). DNA quality was checked by agarose gel electrophoresis (data not shown). For construction of genomic DNA library, restriction digestion of genomic DNA was done by using *Sau3AI* enzyme and YEp24 plasmid was digested with *BamHI* enzyme. Genomic DNA fragments of 0.5–6 kb were used for ligation reaction in 7.7 kb YEp24 plasmid.

Screening of genomic DNA library clones for rescuing slow-growth phenotype of eIF2B mutant strains

gcd7-201 gcn2Δ transformed with genomic DNA library having colony size equivalent to that of isogenic wild-type *GCD7 gcn2Δ* transformed with vector alone (Fig. 1a, c), but unlike vector transformed *gcd7-201 gcn2Δ* were observed (Fig. 1b). Comparing with the vector transformed mutant strains, we observed approximately 20 transformants showing growth comparable to the wild type. We only indicated eight of the transformants as representative colonies by pointing arrows. We screened all the 20 transformants for rescuing Slg$^+$ and Gcd$^+$ phenotype associated with mutations in different subunits of eIF2B. Further, we found one transformant which suppressed the Slg$^+$ and Gcd$^+$ phenotype of *gcd7-201 gcn2Δ*. A transformant named as Sup-II (suppressor clone II) restored growth (Fig. 1d) as well as Gcd$^-$ phenotype of *gcd7-201 gcn2Δ* (Fig. 1e). Sup-II showed very similar growth pattern to that of isogenic wild-type *GCD7 gcn2Δ* transformed with empty vector (Fig. 1d). In order to reconfirm the slow-growth suppression and Gcd$^+$ phenotype, Sup-II plasmid DNA was rescued from *gcd7-201 gcn2Δ* transformants (Fig. 1f) and transformed into *gcd7-201 gcn2Δ* strain. The transformants were restreaked to compare the growth. All the *gcd7-201 gcn2Δ* transformants were of uniform size and showed Slg$^+$ phenotype (Fig. 1g, h). The results suggested that a genomic clone (Sup-II) suppresses Slg$^-$ and Gcd$^-$ phenotype of *gcd7-201 gcn2Δ* mutant strain. The Sup-II clone was sequenced using YEp24-specific primers (Table 3). Further, only *EMC4* (∼573 bp), *TAN1* (∼928 bp) and YGL230C (∼444 bp) gene encoding for chaperone, tRNA acetyltransferase and putative protein, respectively, showed the complete ORF, whereas *SEC15* and *SAP4* were truncated. These results suggest that Sup-II harbors full-length *EMC4*, *TAN1* and YGL230C that rescue

the slow-growth and Gcd$^-$ phenotype of *gcd7-201 gcn2Δ* (Fig. 2a).

Sub-cloning of potential suppressors into pEG(KG) (yeast expression vector)

TAN1 and YGL230C genes were amplified using gene-specific primers (Table 3) containing *SalI* restriction site in the forward primer and *HindIII* in the reverse primer for *TAN1* and *HindIII* and *BamHI* for YGL230C followed by cloning under GST expression vector at respective restriction sites. A PCR product of ∼ 950 bp was observed on agarose gel as expected for TAN1 gene (Fig. 2b). A PCR product of ∼ 450 bp was also observed on agarose gel as expected for YGL230C (data not shown). *TAN1* gene is present on chromosome VII of *S. cerevisiae* genome (http://www.yeastgenome.org/) (Fig. 2a). pEG(KG) vector of 9.3 kb containing GST tag under *GAL1* promoter was used for sub-cloning of *TAN1* and YGL230C (Fig. 2c). *TAN1* and YGL230C gene sequences were verified by sequencing, and complete and error-free sequences of 928bp and 444 bp were observed. Data are not shown for YGL230C.

TAN1 overexpression rescued slow-growth and Gcd$^-$ phenotype of *gcd7-201 gcn2Δ*

The *gcd7-201 gcn2Δ* strain was transformed with vector alone or with pEG(KG)/*TAN1* or pEG(KG)/YGL230C expression constructs and transformants were streaked on SC medium without uracil, but containing either raffinose (Fig. 3a) or galactose (Fig. 3b). As expected, *gcd7-201 gcn2Δ* transformed with vector alone or *TAN1* construct showed slow-growth phenotype on SC medium containing raffinose. Interestingly, *TAN1* overexpression rescued the slow growth of *gcd7-201 gcn2Δ*, when streaked on SC medium containing galactose (Fig. 3b). In contrast, *gcd7-201 gcn2Δ* transferred with pEG(KG)/YGL230C did not rescue the slow-growth phenotype (data not shown). In order to verify the results, *pEG(KG)/TAN1* a uracil-based plasmid was evicted on FOA containing medium that showed the original slow-growth phenotype of *gcd7-201 gcn2Δ* (Fig. 3c) and did not grow on SC medium without uracil supplementation (Fig. 3d). These data clearly suggest that overexpression of *TAN1* rescued the Slg$^-$ of *gcd7-201 gcn2Δ* mutant. Further *TAN1* overexpression was analyzed for rescuing Gcd$^-$ phenotype of *gcd7-201 gcn2Δ* mutant strain (Fig. 3e). As expected, *gcd7-201 gcn2Δ* transformed with vector alone showed slow growth in SC medium without uracil supplementation as well as Gcd$^-$ phenotype on medium containing 3-AT. Interestingly, Gcd$^-$ phenotype of *gcd7-201 gcn2Δ* was also rescued
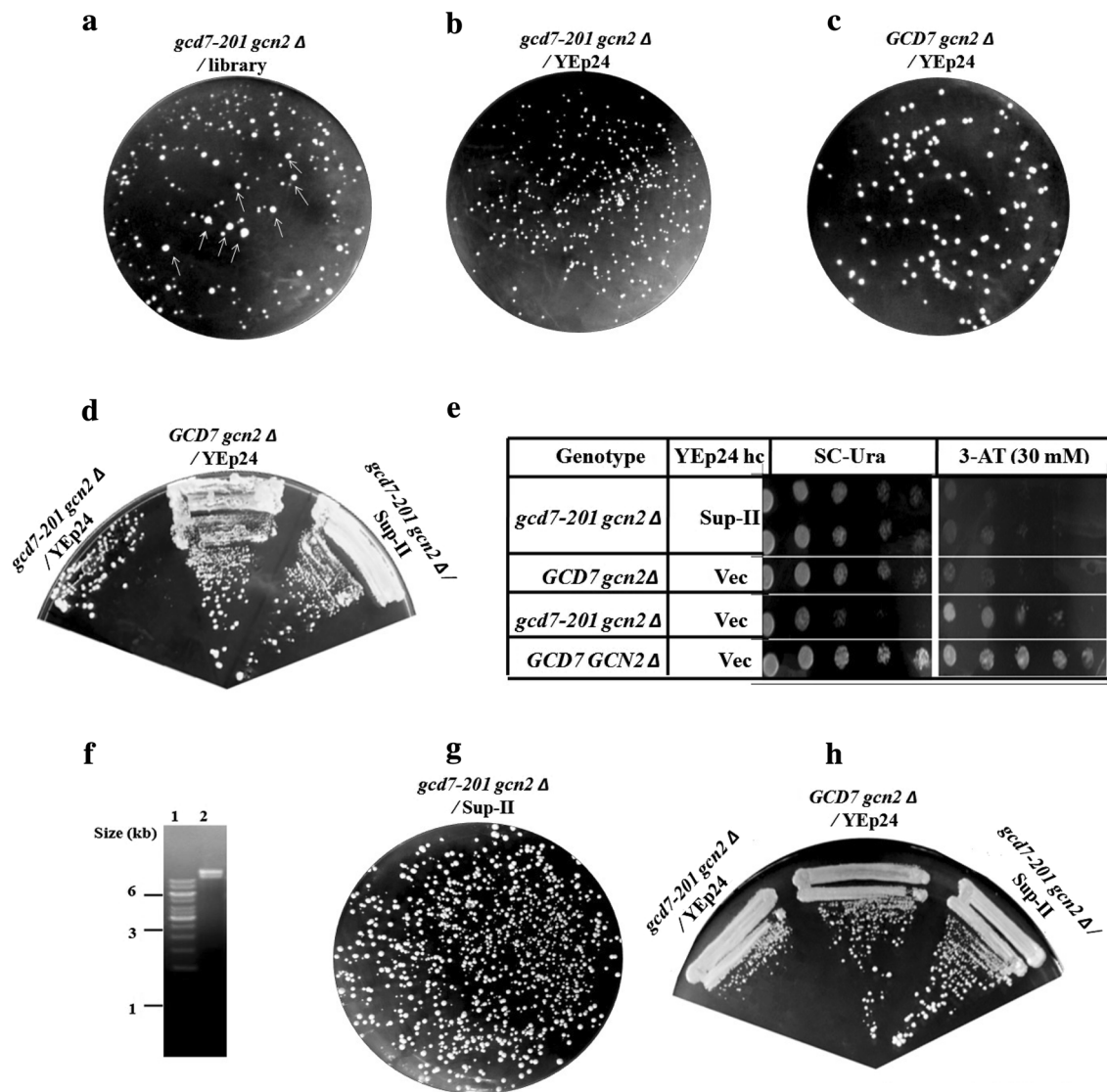
Fig. 1 Screening for extragenic suppressors of *gcd7-201 gcn2Δ* mutant strain transformed with **a** genomic DNA library. **b** (YEp24) vector and **c** *GCD7 gcn2Δ* transformed with (YEp24) vector. *S. cerevisiae* strains were plated on SC medium lacking uracil and incubated at 30 °C for 2 days. **d** *gcd7-201 gcn2 Δ* transformant showing Slg⁺ phenotype (Sup-II) was streaked along with *gcd7-201 gcn2Δ* and *GCD7 gcn2Δ* transformed with YEp24. **e** Serial dilutions of the transformant (Sup-II) showing Slg⁺ phenotype were spotted on SC-Ura and SC medium containing 30 mM-3AT. *GCD7 gcn2Δ* and *GCD7 GCN2Δ* transformed with vector were spotted as control. **f** The transformants (Sup-II) showing Slg⁺ and Gcd⁺ phenotype were used for rescuing plasmid as indicated in lane 2. **g** The rescued plasmid was retransformed into *gcd7-201 gcn2Δ* mutant strain and **h** further verified by streaking on SC-Ura medium as indicated

when *TAN1* was overexpressed (Fig. 3e). Overexpression of *TAN1* suppressed the Slg⁻ and Gcd⁻ phenotype of *gcd7-201 gcn2Δ*. This clearly suggests that Tan1p play a direct or indirect role in eIF2B-mediated amino acid starvation. Expression of GST-Tan1 was verified by western blotting. Whole-cell extracts (WCE) of *gcd7-201 gcn2Δ* mutant expressing GST-Tan1 or GST alone were analyzed by western blotting using anti-GST antibodies. As expected, a 26-kDa GST protein was detected in extracts expressing GST alone. On the other hand, ~60-kDa protein was detected in WCE of *gcd7-201 gcn2Δ* mutant transformed

with pEG(KG)/*TAN1* construct. α-GCD6 antibodies were used as an internal loading control (Fig. 3f).

Tan1p overexpression suppresses specific eIF2B mutations

To test whether Tan1p overexpression is specific, we transferred *S. cerevisiae* harboring mutations in different subunits which include H70 (*gcd1-502*), H1792 (*gcd6-1*), H591 (*gcd12-503*) and H750 (*gcn2::LEU2*). Interestingly, the *TAN1* overexpression rescued temperature-sensitive

Table 2 Description of nomenclature of *Saccharomyces cerevisiae* strains used in this study

Strain	Nomenclature used	Source or reference
H4	*GCD7 GCN2*	Alan G. Hinnebusch
H1794	*gcd7-201 gcn2Δ*	Alan G. Hinnebusch
H1795	*GCD7 gcn2Δ*	Alan G. Hinnebusch
H1792	*gcd6-1 gcn2Δ*	Alan G. Hinnebusch
H1793	*GCD6 gcn2Δ*	Alan G. Hinnebusch
H 7 5 0	g c n 2::L E U 2	Alan G. Hinnebusch
H591	*gcd12-503 gcn2-101*	Alan G. Hinnebusch
H70	*gcd1-502 gcn2-101*	Alan G. Hinnebusch
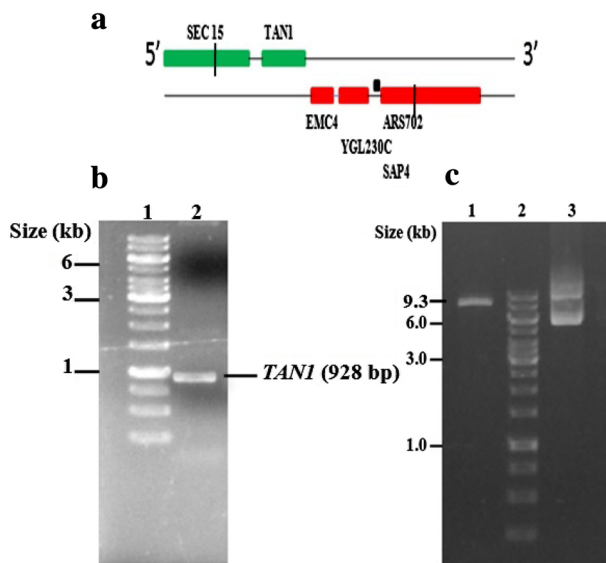
Fig. 2 PCR amplification of *TAN1* gene. **a** Schematic representation of Sup-II clone showing complete ORF of *EMC4*, *TAN1*, YGL230C and truncated *SEC15 and SAP4* genes on chromosome VII, **b** PCR amplification of the *TAN1* gene from genomic clone (Sup-II) and separated on 1% agarose gel, **c** pEG(KG) plasmid was isolated (lane 3) and digested with *XbaI* and *SalI* (lane 1). **b, c** Lane 1 represents molecular size marker (kb)

Table 3 Primers used in this study

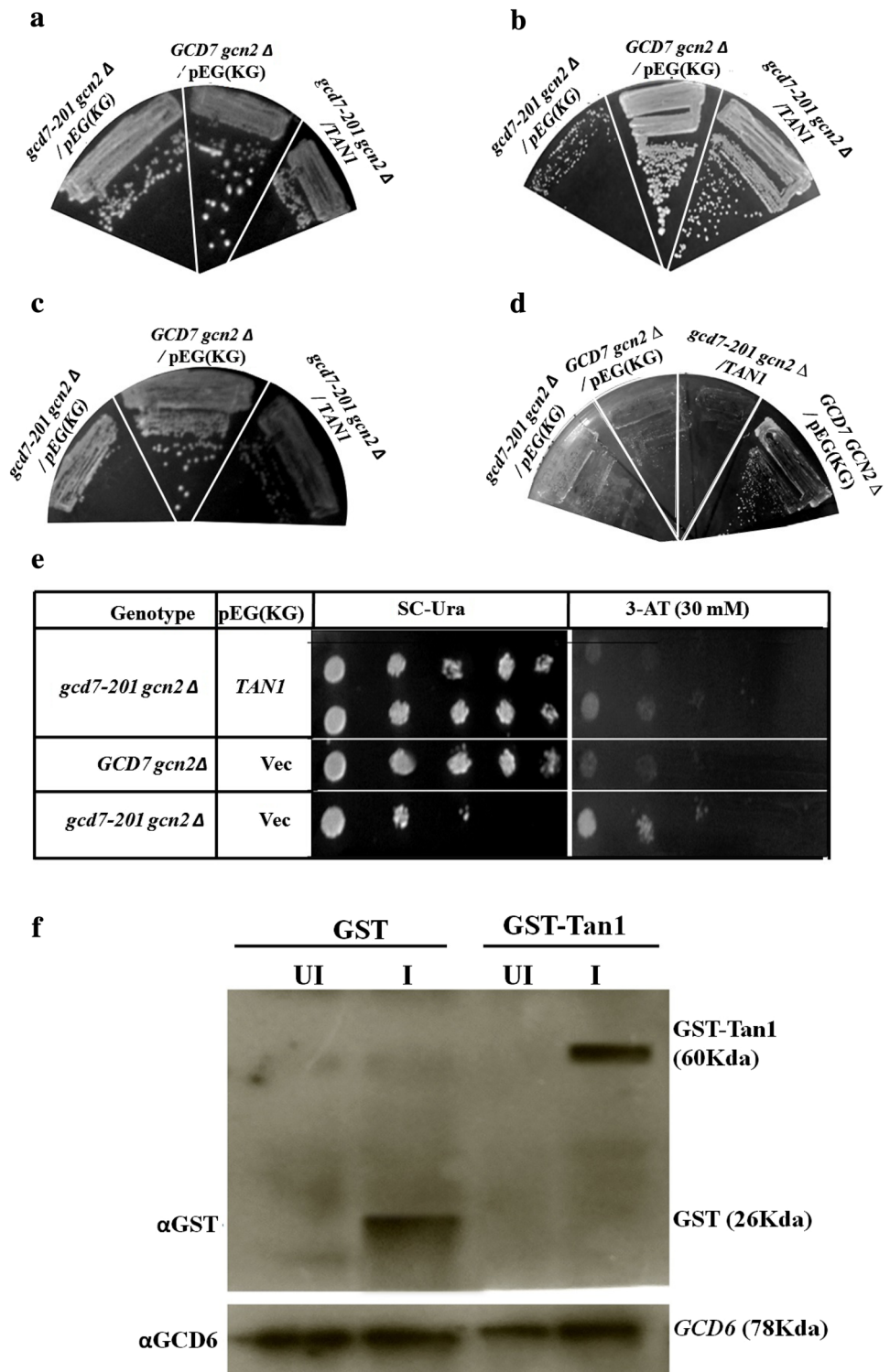| Name	Nucleotide sequence (5′–3′)	Source
YEp24-F	TGGAGCCACTATCGACTACG	This study
YEp24-R	GGCGATATAGGCGCCAGCAA	This study
TAN-F	TTGATGTCGACATGGGTGAAAAACGT	This study
TAN-R	CGCGCAAGCTTTTATTGTTTTACACTC	This study
UK-F	GCATGGATCCATGGGAATTATTACAC	This study
UK-R	GCAGAAGCTTCTAGAGAATACATTGG	This study

(Ts⁻) phenotype of *gcd1-502* at 37 °C (Fig. 4a), but not that of H1792 (*gcd6-1*), H591 (*gcd12-503*) and H750 (*gcn2::LEU2*) (data not shown). *TAN1* overexpression also

rescued Slg⁻ and Gcd⁻ phenotype of *gcd1-502* (Fig. 4b). Thus, Tan1p causes strain-specific suppression of eIF2B mutants.

pEG(KG)/*TAN1* overexpression enhanced stress tolerance in *S. cerevisiae*

Unlike amino acid stress, *TAN1* overexpression protects *GCD7 GCN2*, *GCD7 gcn2Δ* and *gcd7-201 gcn2Δ* against H_2O_2-, ethanol- and caffeine-mediated stress. *GCD7 gcn2Δ*, *gcd7-201 gcn2Δ and GCD7 GCN2Δ* were streaked on YPD agar medium containing H_2O_2, ethanol and caffeine showed Slg⁻ phenotype (Fig. 5a). The *GCD7 GCN2*, *GCD7 gcn2Δ* and *gcd7-201 gcn2Δ S. cerevisiae* strains transformed with empty vector showed reduced growth (twofold) in the presence of 4 mM H_2O_2, 10% ethanol and 30 mM caffeine in the presence of either raffinose or galactose. *S. cerevisiae* strains *GCD7 GCN2*, *GCD7 gcn2Δ* and *gcd7-201 gcn2Δ* transformed with pEG(KG)/*TAN1* showed normal growth even in the presence of H_2O_2, ethanol and caffeine only in the presence of galactose (Fig. 5b, c, d), indicating that overexpression of Tan1p protects *S. cerevisiae* from H_2O_2-, ethanol- and caffeine-induced cell death. No significant protective effect was observed against DMSO, NaCl and DTT (data not shown). Halo assay shows zone of no growth surrounding the hydrogen peroxide-, caffeine- and alcohol-containing filter disks. Zone of inhibition was significantly reduced in *GCD7 GCN2*, *GCD7 gcn2Δ* and *gcd7-201 gcn2Δ* strains overexpressing *TAN1* in the presence of 2% galactose as compared to control cells (Fig. 6a, b, c). Using the cell spot assay, it was observed that different yeast strains (*GCD7 GCN2*, *GCD7 gcn2Δ* and *gcd7-201 gcn2Δ*) were protected from H_2O_2-, ethanol- and caffeine-mediated cell death only when Tan1p was expressed in galactose (Fig. 7a, b, c). These results strongly suggest that overexpression of *TAN1* is capable of preventing cell death due to H_2O_2, alcohol and caffeine. Thus, overexpression of *TAN1* repairs *GCD7 GCN2*-, *GCD7 gcn2Δ*- and *gcd7-201 gcn2Δ*-based defect in translation initiation in some or other way. Based on our results, we have proposed a new model (Fig. 8) explaining the amino acid starvation stress in eIF2B mutant of *S. cerevisiae* and H_2O_2, ethanol and caffeine stress-induced (wild-type and eIF2B mutant of *S. cerevisiae*) derepression of Gcn4p. The H_2O_2-, ethanol- and caffeine-mediated stress bypasses Gcn2p phosphorylation because similar results were observed in the presence or absence of Gcn2p. Overexpression of Tan1p modulates the eIF2B activity and in turn lowers *GCN4* expression. Our model is supported by previous reports such as butanol-mediated derepression of *GCN4* in a Gcn2p-independent manner (Ashe et al.

Rapamycin-induced stress has also shown to down regulate translation and *GCN4* induction (Barbet et al. 1996; Cherkasova and Hinnebusch 2003; Kubota et al. 2003). Moreover, oxidative stress (H$_2$O$_2$) inhibits general translation independent of Gcn2p or eIF2α phosphorylation (Knutsen et al. 2015).

Discussion

Eukaryotic initiation factor 2B (eIF2B) mutations lead to the translation dysregulation (Hannig et al. 1990; Dever et al. 1993; De Aldana and Hinnebusch 1994; Pavitt et al. 1997) causing a rare encephalopathy, vanishing white
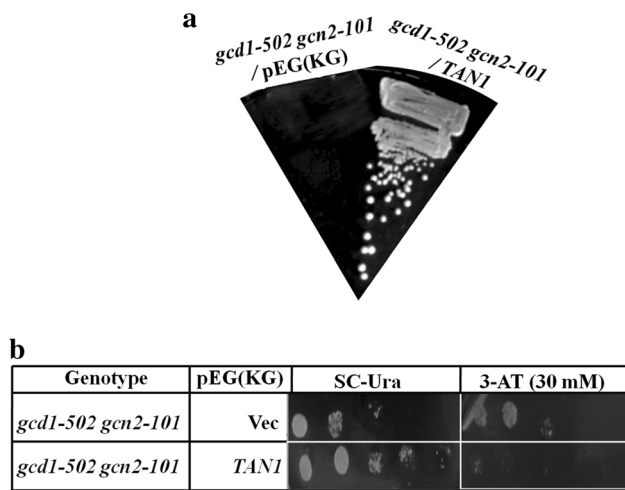
Fig. 4 Tan1p overexpression rescues Slg$^-$, Gcd$^-$ and Ts$^-$ phenotype of *gcd1-502 gcn2-101* strain. **a** *gcd1-502 gcn2-101* transformants showing Slg$^+$ phenotype were streaked along with *gcd1-502 gcn2-101* transformed with pEG(KG) vector alone. **b** Serial dilutions of the *gcd1-502 gcn2-101* transformants showing Slg$^+$ phenotype were spotted on SC-Ura or SC medium containing 3-AT (30 mM). *gcd1-502 gcn2-101* transformed with pEG(KG) was spotted as control. Plates were incubated for 2 days at 30 °C
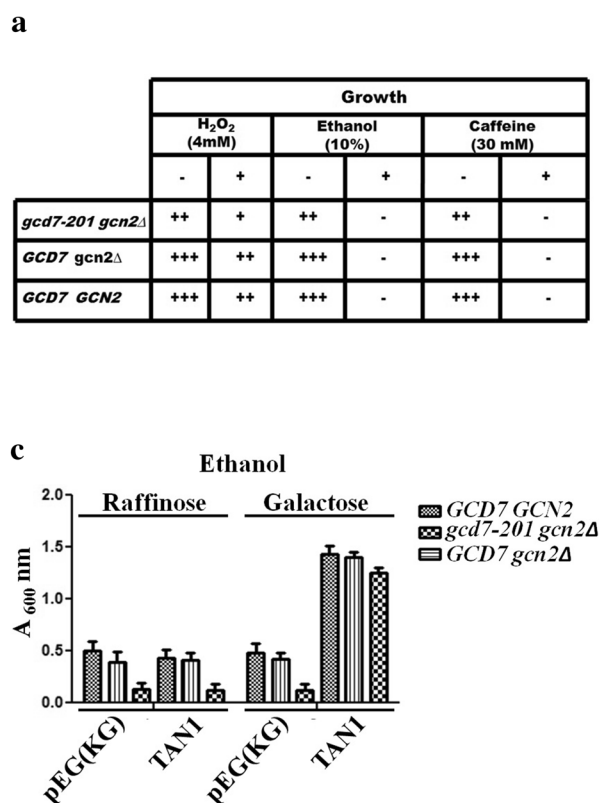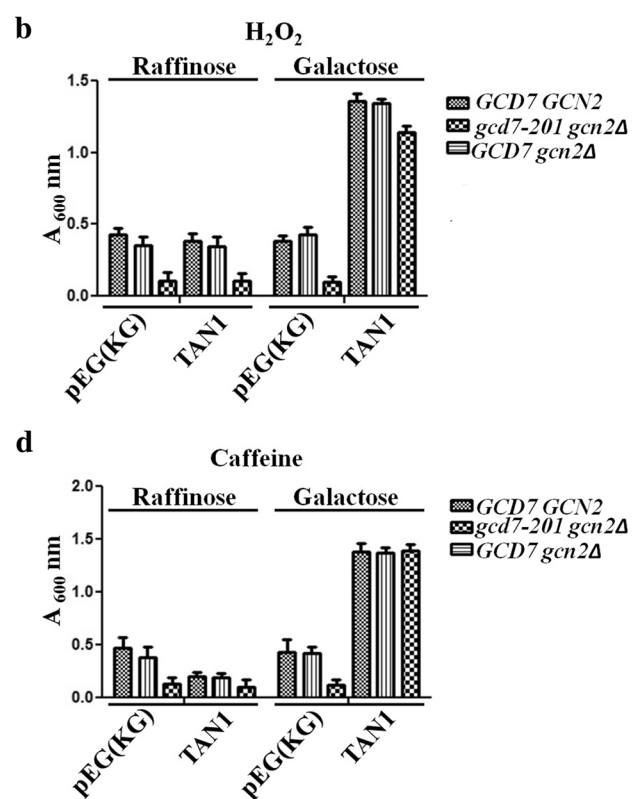
matter disease (VWM). In this study, we identified the extragenic suppressors of eIF2B mutations in *Saccharomyces cerevisiae*, corresponding to VWM. By screening the *S. cerevisiae* genomic DNA library, *TAN1* gene was observed to suppress the slow-growth (Slg$^-$) and general control derepression (Gcd$^-$) phenotype of *S. cerevisiae*, caused by *gcd7-201 gcn2Δ* and *gcd1-502 gcn2-101* mutations. It is also interesting to note that Tan1p overexpression rescued Slg$^-$, Gcd$^-$ and Ts$^-$ phenotypes of one of the regulatory eIF2B subunits and one of the catalytic eIF2B subunits. It has been already reported that overexpressing Met-tRNAi^{Met} suppresses the lethality of Gcd2 depletion (gcd2-td mutant) (Dev et al. 2010). Tan1p is a putative tRNA acetyltransferase required for the formation of modified nucleoside N^4-acetylcytidine (ac^{4}C) in tRNA and is important for tRNA stability (Johansson and Bystrom 2004). Tan1p protein abundance increases in response to DNA replication stress (Tkach et al. 2012). Thus, we also tested the effect of *TAN1* overexpression in *Saccharomyces cerevisiae* strains under different stress conditions (H$_2$O$_2$, ethanol, caffeine, DMSO, DTT and NaCl). *gcd7-201 gcn2Δ GCD7 GCN2* and *GCD7 gcn2Δ* strains were found to be more sensitive to H$_2$O$_2$, ethanol and caffeine and less

Fig. 5 *S. cerevisiae* strains overexpressing *TAN1* showed enhanced stress tolerance. **a** *gcd7-201 gcn2Δ*, *GCD7 gcn2Δ* and *GCN2 gcn2Δ S. cerevisiae* strains were streaked on YPD agar plates containing 4 mM H$_2$O$_2$, 10% ethanol and 30 mM caffeine. Number of plus signs (+) indicates comparative visible growth. Negative signs (−) indicates no visible growth. SC medium supplemented with **b** 4 mM H$_2$O$_2$, **c** 10% ethanol and **d** 30 mM caffeine in the presence of raffinose or galactose. Cultures were grown for 16 h at 30 °C. Cultures with raffinose supplementation were served as control. The cell density was obsereved by measuring absorbance at 600 nm (A_{600}). Data of three independent experiments were plotted with standard deviation
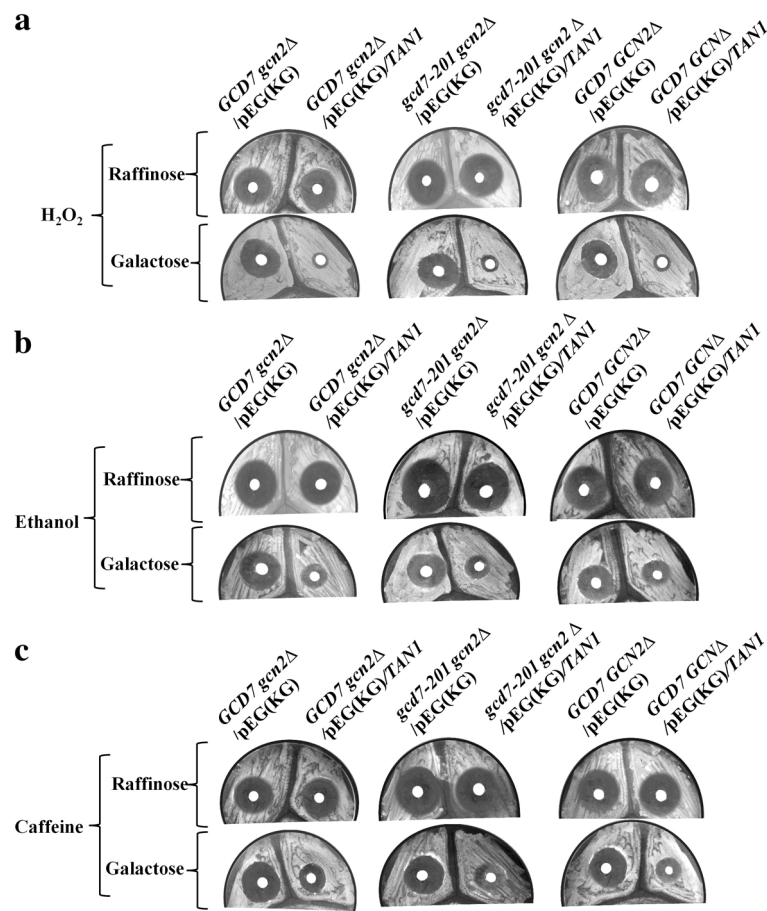
Fig. 6 Halo assay to test the effect of H₂O₂, ethanol and caffeine on the growth of eIF2B mutants. Hydrogen peroxide, ethanol and caffeine halo assays were performed with *gcd7-201 gcn2Δ*, *GCD7 gcn2Δ* and *GCD7 GCN2*. *S. cerevisiae* strains harboring either the empty vector or the *TAN1* gene-expressing plasmid were spread on SC medium, containing filter disks soaked in **a** H₂O₂ (4 mM), **b** ethanol (10%) and **c** caffeine (30 mM) in the presence of either raffinose or galactose. The plates were incubated at 30 °C for 2–3 days

sensitive to NaCl-mediated (Goossens et al. 2001), DMSO-mediated and DTT-mediated stress. But the growth was reduced to a greater extent in *gcd7-201 gcn2Δ* as compared to *GCD7 GCN2* cells, thus proposing the role of Tan1p in eIF2B-mediated translation regulation. This is in consistent with the previous reports of Gcn2p protein-dependent/independent Gcn4p protein induction and translation regulation. For example, butanol-mediated induction of *GCN4* by a Gcn2p-independent manner has already been reported (Ashe et al. 2001). Oxidative stress (H₂O₂) causes translation inhibition independent of eIF2α–p mediated by *GCN2* (Knutsen et al. 2015). Moreover, rapamycin causes translational inhibition (Barbet et al. 1996) by *GCN4*-mediated stress responses (Cherkasova and Hinnebusch 2003; Kubota et al. 2003). Thus, H₂O₂, ethanol and caffeine might activate Gcn4p in the presence of functional wild-type eIF2B. Interestingly, *TAN1* overexpression restored the cell growth of *GCD7 GCN2*, *gcd7-201 Gcn2Δ* strains under different stress conditions by directly or indirectly

modulating eIF2B GEF activity. Thus, H₂O₂, ethanol and caffeine might be used as chemical modulators to study eIF2B-mediated pathways leading to stress responses (Motlekar et al. 2009). Although the mechanism by which Tan1p protein functions in translation regulation is still unknown, the requirement of more stable tRNA specific for leucine and serine depends upon the expression of Tan1p protein. The stability of tRNA is maintained by various enzymes. For example, tRNA-modifying enzymes such as tRNA(m⁵U₅₄) methyltransferase Trm2p (Nordlund et al. 2000), pseudouridine (Ψ₅₅) synthase Pus4p (Becker et al. 1997), tRNA (m²G₂₆) dimethyltransferase Trm1p (Ellis et al. 1986) and the putative tRNA (Gm₁₈) 2′-O-ribose methyltransferase Trm3p (Cavaillé et al. 1999) are known for tRNA modification. Overexpression of Tan1p protein suppresses the Slg⁻ and Gcd⁻ phenotype of eIF2B mutants possibly by stabilizing the tRNA by recognizing general tRNA structure. This explanation is supported by the previous study explaining the in vitro binding of *E. coli* tRNA
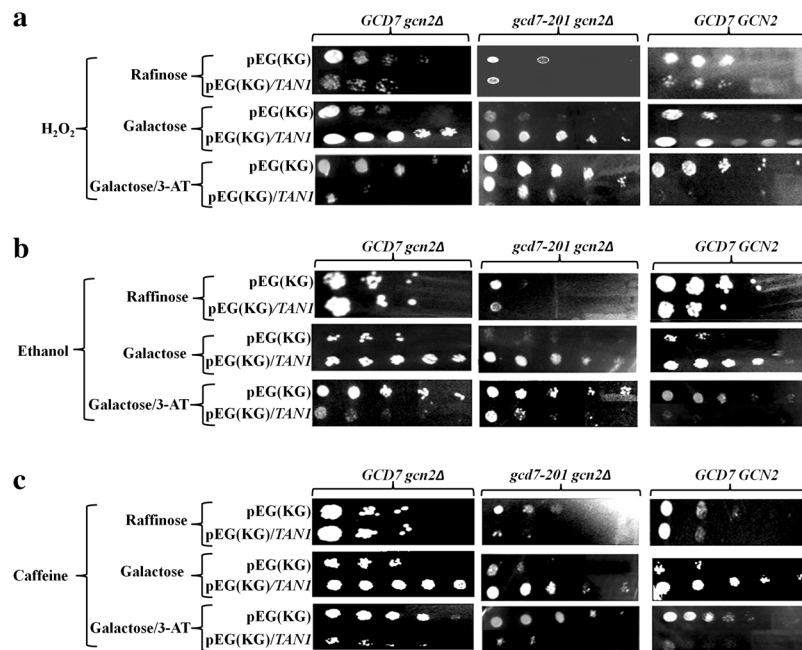
Fig. 7 Spot assay to test the effect of H₂O₂, ethanol and caffeine on the growth of eIF2B mutants. Hydrogen peroxide, ethanol and caffeine halo assays were performed with *gcd7-201 gcn2Δ*, *GCD7 gcn2Δ* and *GCD7 GCN2*. Tenfold serially diluted transformants harboring empty vector (pEG(KG)) or pEG(KG)-*TAN1* were spotted on SC-Ura or SC-Ura/3-AT agar plates supplemented with **a** H₂O₂ (4 mM), **b** ethanol (10%) and **c** caffeine (30 mM) in the presence of either raffinose or galactose. Cultures were grown for 2 days at 30 °C
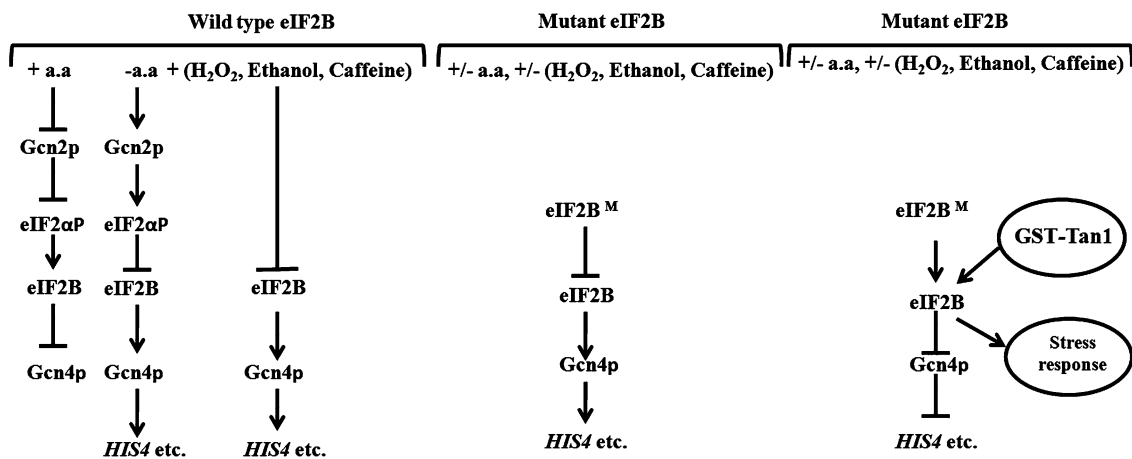

Fig. 8 Schematic model indicating the repression of *GCN4* by overexpression of GST-Tan1p via eIF2B. Panel I indicates that in the presence of amino acids (+a.a), Gcn2p is inhibited in wild-type cells that in turn inhibits Gcn4p expression. In contrast, amino acid starvation (−a.a) in wild-type cells leads to eIF2α phosphorylation and Gcn4p induction via activation of Gcn2p kinase. Similarly, the presence of H₂O₂, ethanol and caffeine in the growth medium inhibits eIF2B activity that in turn activates GCN4p. On the other hand, in panel II, eIF2B mutants grown in the presence or absence of a.a, H₂O₂, ethanol and caffeine inhibit eIF2B activity that can lead to Gcn4p activation. In panel III, the eIF2B mutants *gcd7-201 gcn2Δ*, *GCD7 gcn2Δ* and *GCD7 GCN2Δ* overexpressing Tan1p repress Gcn4p in the presence or absence of a.a, H₂O₂, ethanol and caffeine and rescue their Slg⁻, Gcd⁻ and Ts⁻ phenotype

(m¹G₃₇) methyltransferase to the tRNA species that are not methylated by the same enzyme (Redlak et al. 1997). On comparison with previous studies, it can be concluded that Tan1p protein might be involved in eIF2B-mediated translation regulation. More importantly, the resistance to different stress conditions brought upon the overexpression of *TAN1* in the wild-type and mutant yeast strains provides a facile model system to begin a detailed structural and functional analysis of its role in eIF2B-mediated translation regulation.

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