A Yeast Purification System for Human Translation Initiation Factors eIF2 and eIF2Bε and Their Use in the Diagnosis of CACH/VWM Disease

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Abstract

Recessive inherited mutations in any of five subunits of the general protein synthesis factor eIF2B are responsible for a white matter neurodegenerative disease with a large clinical spectrum. The classical form is called Childhood Ataxia with CNS hypomyelination (CACH) or Vanishing White Matter Leukoencephalopathy (VWM). eIF2B-related disorders affect glial cells, despite the fact that eIF2B is a ubiquitous protein that functions as a guanine-nucleotide exchange factor (GEF) for its partner protein eIF2 in the translation initiation process in all eukaryotic cells. Decreased eIF2B activity measured by a GEF assay in patients’ immortalised lymphocytic cells provides a biochemical diagnostic assay but is limited by the availability of eIF2 protein, which is classically purified from a mammalian cell source by column chromatography. Here we describe the generation of a recombinant expression system to produce purified human eIF2 from yeast cells. We demonstrate that human eIF2 can function in yeast cells in place of the equivalent yeast factor. We purify human eIF2 and the C-terminal domain of human eIF2Bε using affinity chromatography from engineered yeast cells and find that both function in a GEF assay; the first demonstration that this human eIF2Bε domain has GEF function. We show that CACH/VWM mutations within this domain reduce its activity. Finally we demonstrate that the recombinant eIF2 functions similarly to eIF2 purified from rat liver in GEF assays with CACH/VWM eIF2B-mutated patient derived lymphocytic cells.

Introduction

Childhood Ataxia with CNS hypomyelination (CACH) or Vanishing White Matter Leukoencephalopathy (VWM) (OMIM #603896) was described in the 1990s [1,2]. It is a fatal childhood onset white matter disease with a chronic progressive course exacerbated by acute episodes [3,4]. Inherited mutations in any of the five genes encoding subunits of the general protein synthesis initiation factor eIF2B (EIF2B1-5) cause CACH/VWM [3,5]. The subsequent description of a large clinical spectrum of the disease from neonatal to adult onset or even asymptomatic forms led to the concept of eIF2B-related disorders that are recognized by peculiar magnetic resonance imaging (MRI) abnormalities [6]. Well over 100 different, mainly missense, mutations have been presently reported [4]. Their consequences on the eIF2B complex have been demonstrated in yeast and in humans [7,8,9]. Abnormalities in glial cell development have been suggested by studies of patient samples [10,11,12,13,14,15,16] and studies of a mouse model [17]. One recent suggested explanation is that altered expression of splicing regulatory factors in eIF2B mutated glial cells may cause altered splicing regulation of the important myelin proteins PLP and DM20 [18]. However many aspects of the disease are still not understood and no current therapy is available.

eIF2B is well established as a key regulated general translation initiation factor. It functions as a guanine nucleotide exchange factor (GEF) to accelerate the dissociation of GDP from its substrate eIF2-GDP in the first step of translation initiation to form eIF2-GTP [19]. This complex then binds to initiator methionyl tRNA (Met-tRNAiMet) to deliver it to 40S ribosomes in a reaction that is stimulated by several other translation factors and is required for each translation initiation event on almost all mRNAs [20,21]. During the initiation cycle eIF2-bound GTP is hydrolysed to GDP to inactivate eIF2 and reset the system. eIF2 is regulated by cellular stresses facilitating translational control in a wide variety of settings. Most widely studied is the activation of eIF2α
kinases that phosphorylate eIF2 on its alpha subunit at Ser51 [22]. This phosphorylation reaction converts eIF2 from substrate to inhibitor of eIF2B. When phosphorylated at Ser51, eIF2 binds with higher affinity to eIF2B, but without undergoing nucleotide exchange [19]. Genetic and biochemical studies using eIF2B from yeast identified mutations in three subunits of eIF2B (eIF2Bα,β,δ) that disrupt this regulation and define a potential regulatory interface between eIF2 and eIF2B that is critical for regulation [23]. Subsequent experiments verified that equivalent mutations in mammalian eIF2B also disrupt eIF2B regulation confirming conservation of this regulatory mechanism [24,25].

eIF2B activity is measured by a GEF assay that was first established in the 1980s [26,27]. eIF2 forms a stable complex with GDP in the presence of physiological concentrations of Mg++ ions. It requires significant amounts of eIF2 protein purified from rat liver, rabbit reticulocytes or mammalian cell lysates by rounds of protein-binding filters. Two versions of the assay have been used; monitoring the progressive decline in labelled eIF2 captured on the C-terminal 200 amino acids contained all the elements necessary for minimal exchange function. This region was termed the catalytic domain scat [32,33].

The cell extract format of the assay has been used to assess eIF2B activity in immortalised lymphocytic cells isolated from blood samples of CACH/VWM patients [9,10,34]. This provides a biochemical diagnostic assay to complement MRI and genetic analyses. The adoption of this GEF assay for diagnostic purposes is limited by the availability of purified eIF2 protein. We, and others, have previously developed a yeast cell expression system to overexpress and purify epitope tagged yeast eIF2, which was subsequently used for in vitro studies [29,35]. Generating recombinant systems for mammalian eIF2 has proved challenging because eIF2 possesses three different subunits in a 1:1:1 complex, and because popular expression host cells, including E. coli, appear refractory to expressing significant amounts of the gamma subunit. We therefore decided to develop a recombinant yeast cell system to purify active human eIF2 protein.

Results and Discussion

Human eIF2 Subunits Can Complement the Function of the Equivalent Yeast Gene

The translation initiation factor eIF2 performs critical roles in the initiation and control of protein synthesis in eukaryotic cells. eIF2 is composed of three non-identical subunits and must interact with GDP, GTP, Met-tRNA, eIF3, eIF5, eIF2B and 4OS ribosomes to perform its functions [35,36,37] as well several eIF2α protein kinases for regulation. eIF2 has an archaean homologue, but is not found in eubacteria [38]. eIF2 subunits are highly conserved between yeast and mammals, including humans (Figure 1A). We decided to develop a yeast system as an expression vehicle for human eIF2 (hEIF2). As a first step we obtained cDNA clones and subcloned them into yeast expression vectors, under the control of conditional (galactose carbon source inducible) yeast promoters and bearing short terminal epitope tags (Figure 1B and Materials and Methods). Each yeast eIF2 gene is essential (Su2, Su3, and Gcd11 encoding eIF2α,γ respectively). Strains bearing individual gene deletions covered by a plasmid copy of the yeast gene were used to assess the function of the human expression clones. Each human vector was introduced into its corresponding yeast deletion strain by transformation and plasmid shuffling was used to evict the covering plasmid. We found that plasmids bearing EIF2S1 encoding hEIF2α complemented a su2Δ strain (Figure 1C, compare lanes 2 and 3 with lane 1) and grew as well as wild type yeast. Similarly plasmids bearing EIF2S2 encoding hEIF2B complemented su2Δ strain (Figure 1C, compare lanes 5 and 6 with lane 4). However our initial EIF2S3 plasmids (encoding hEIF2γ) could not complement gcd11Δ (data not shown). As EIF2S3 contains a significant number of codons rarely used in yeast we obtained a commercially synthesised yeast-codon-optimized clone and subcloned this into a similar compatible yeast expression plasmid. This could complement the gcd11Δ strain, but the resulting strain grew poorly (Figure 1C, lane 8). Western blotting confirmed both deletion of endogenous yeast genes and expression of the corresponding hEIF2 subunits (Figure 1D). Because eIF2 functions as a heterotrimer, in these complemented cells heterologous eIF2 complexes should form, each with one human and two yeast subunits.

We were concerned by the slow growth of the EIF2S3 complemented strain (Figure 1C, lane 8), as this may indicate that the codon-optimized cDNA is not fully functional. Several explanations are possible. Firstly, hEIF2γ may not be expressed at a high enough level to form sufficient eIF2 complexes for rapid growth. Expression of hEIF2γ did not alter the expression levels of the yeast α and β subunits (Figure 1D). We observed that different EIF2S3 transformants grew at different rates. When the expression level of hEIF2γ was examined in a selection of these cells, we consistently found that transformants expressing the highest hEIF2γ levels grew more slowly than those with lower expression (Figure 2A and data not shown). These results are therefore more consistent with the idea that the slow-growth phenotype is related to excess levels of hEIF2γ. Excess free hEIF2γ may bind to and sequester one or more interacting factors into non- or partially functional complexes. This second idea however is unlikely as the slow-growth phenotype is recessive. hEIF2γ is only slow growing in the absence of yeast GCD11 (Figure 2B and data not shown).

Because hEIF2γ is the ‘core’ subunit that binds to both eIF2α and β [38], excess hEIF2γ may form some γα or βγ complexes and thereby reduce the level of full γβγ complexes. If so, then reducing excess hEIF2γ may ameliorate complex disruption and improve growth as observed. Alternatively, because eIF2 must interact with many yeast factors including translation factors (eIF2B, eIF5, eIF3) Met-tRNA and 4OS ribosomes, hEIF2γ may not interact effectively with one or more of these. We assessed whether transforming the hEIF2γ strain with additional copies of yeast eIF2-interactive factors could complement the growth phenotype by mass action. A panel of high copy plasmids expressing tRNA, Met-tRNA, eIF3 or eIF5 was transformed into the gcd11Δ strain expressing hEIF2γ (see Methods for plasmids assessed). Only excess eIF5 reproducibly suppressed the slow-growth phenotype (Figure 2B and data not shown). Western blotting confirmed that slow-growth suppression in these cells was not caused by reduced hEIF2γ expression (Figure 2C). eIF5 interacts with both eIF2B and γ [36] as part of the multifactor complex [39], 43S pre-initiation complex and free eIF2 [40,41]. It has GTPase acceleration and GDP-dissociation inhibitor functions [42]. While not conclusive, perhaps impaired interaction between the hybrid eIF2 and yeast eIF5 affects eIF2 or
eIF5 function and that this could be suppressed by mass action. For example excess eIF5 may prevent its premature loss from initiating ribosomes. Alternatively it may stabilise eIF2

We employed a yeast strain deleted for the only yeast eIF2 kinase GAL10. This means that unlike the proteins purified from mammalian sources, our recombinant eIF2 is homogeneously unphosphorylated at the key regulatory site, Ser 51 of the alpha subunit. Purification by a single step using Flag M2 affinity resin to bind eIF2α, or a single nickel agarose step to bind eIF2β, was not sufficient to purify eIF2 (Figure 4A). The single step Flag affinity purification recovered a mixture of αβγ trimers, excess eIF2α and residual contaminating proteins (Figure 4A lanes 2–7). Similarly a single-step his6 purification captured a mixture of αβγ trimers and excess eIF2β (Figure 4A, lanes 8–12 and 4B, lanes 2–6). These results are consistent with the known structure of the archaeal homologue of eIF2, αIF2βγ where both αIF2α and β each separately bind to αIF2γ [38] and with the idea that expression of heIF2γ in our cells is limiting, so that excess free heIF2α and β subunits are formed. We therefore adopted a two-step purification strategy employing nickel agarose, followed by Flag resin (Figure 4B) to purify heterotrimeric eIF2 free from excess α and β subunits (Figure 4B, lane 11). Because eIF2γ expression levels were limiting in our system we transformed in a second plasmid to boost the amounts of the full eIF2 complex. With this strategy we obtained eIF2 that was approximately 90% pure (Figure 4C).

We wished to assess nucleotide binding and exchange with our heIF2 protein. The nucleotide exchange factor eIF2B is specific for eIF2. eIF2B is composed of five distinct subunits. However previous work has shown that the largest subunit alone retains catalytic function and studies in yeast showed that the carboxy terminal domain alone (yeast residues 518–712) is the catalytic domain, termed cat [32]. Subsequently, deletion of residues 549–596 within human eIF2Bα resulted in a protein that could form eIF2B complexes, but had no GEF activity in vitro showing this region is critical for human eIF2B GEF function [8]. In addition X-ray crystallographic structure determination has shown that the human equivalent domain adopts the same stacked, paired α-helical structure as the yeast cat [33,44]. We therefore predicted that a construct bearing the equivalent residues of human eIF2Bα, residues 533–721 would comprise the human eIF2B catalytic domain (heIF2γ-cat). A yeast codon-optimized heIF2γ-cat cDNA was synthesized and expressed with tandem Flag and polyhistidine tags from a galactose inducible promoter vector in a suitable yeast strain host and the same purification scheme devised for heIF2 was

Co-expression of All Three helF2 Subunits Complements a Triple Yeast elf2 Gene Deletion Strain

Dever and colleagues recently reported construction of a triple elf2 gene deletion yeast strain complemented by a single plasmid expressing the yeast genes [43]. To assess if co-expression of all three heIF2 subunits could completely replace the yeast factor, we modified this strain to create a Δppl selectable marker (see Materials and Methods) and transformed in two heIF2 plasmids. One plasmid co-expressed both heIF2αβ and a second plasmid expressed heIF2γ. Plasmid shuffling with FOA generated yeast strains entirely supported by heIF2 (Figure 3A, lower panel). As expected, growth was carbon source dependent. When expression of heIF2 genes from P_{GAL} promoters was repressed by glucose, heIF2-dependent strains failed to grow (Figure 3A, upper panel). Western blotting confirmed that the yeast genes had been deleted and that higher expression of heIF2 conferred a slower rate of growth (Figure 3B). In conclusion, our heIF2 is functional and can replace the yeast protein in vivo.

Purification of Human eIF2 and Catalytic Domains

Because we appended small epitope tags to each heIF2 subunit, we used affinity chromatography to purify the protein complex. We wished to assess nucleotide binding and exchange with our heIF2 protein. The nucleotide exchange factor eIF2B is specific for eIF2. eIF2B is composed of five distinct subunits. However previous work has shown that the largest subunit alone retains catalytic function and studies in yeast showed that the carboxy terminal domain alone (yeast residues 518–712) is the catalytic domain, termed cat [32]. Subsequently, deletion of residues 549–596 within human eIF2Bα resulted in a protein that could form eIF2B complexes, but had no GEF activity in vitro showing this region is critical for human eIF2B GEF function [8]. In addition X-ray crystallographic structure determination has shown that the human equivalent domain adopts the same stacked, paired α-helical structure as the yeast cat [33,44]. We therefore predicted that a construct bearing the equivalent residues of human eIF2Bα, residues 533–721 would comprise the human eIF2B catalytic domain (heIF2γ-cat). A yeast codon-optimized heIF2γ-cat cDNA was synthesized and expressed with tandem Flag and polyhistidine tags from a galactose inducible promoter vector in a suitable yeast strain host and the same purification scheme devised for heIF2 was

Figure 1. Individual helF2 cDNAs replace the function of the corresponding yeast gene. A. Table comparing yeast and human eIF2 subunit proteins. B. Cartoon depiction of plasmids pAV1907 (α), pAV1901 (β), pAV1905 (α,β) and pAV1970 (γ), respectively that express the indicated human eIF2 subunits from either GAL1 or GAL10 promoters. N-terminal his6 (h) and flag (f) epitope tags are also shown. C. Growth of yeast strains following plasmid shuffling on YPGal medium showing this

| Subunit | Yeast | Human | % Ident | % Sim |
|---------|-------|-------|---------|-------|
| eIF2α   | SU12  | EIF2S1| 54      | 74    |
| eIF2β   | SU13  | EIF2S2| 40      | 55    |
| eIF2γ   | GCD11 | EIF2S3| 74      | 86    |

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used (Figure 4C). To assess the functions of purified heIF2 and h2Bc, we set up a standard nucleotide exchange assay with radiolabelled GDP (see Materials and Methods). In the absence of eIF2B, but in the presence of excess unlabelled GDP, heIF2 bound [3H]GDP at 10–30 μM with MgCl2 from 0.2–2 mM. This indicates that the nucleotide binding function of heIF2 is intact. When h2Bc was added, this stimulated release of [3H]GDP with first order kinetics (Figure 4C). This confirms that nucleotide binding to eIF2 is reversible and that residues 533–721 of human eIF2B contains the catalytic domain.

Catalytic Domain CACH/VWM Mutants have Reduced GEF Activity

As stated in the introduction, mutations in eIF2B cause a genetic disease. Mutations have been found in all five subunits, including several within the catalytic domain. Catalytic domain mutations include missense alleles: P604S [45], I649T [46], E650K [47], M608I [48] and W684S [49] and small deletions: D610–613 [9] and D666–672 [47]. In all patients reported thus far, these alleles occur as compound heterozygotes with other mutations contributing to the disease pathology and the measured eIF2B activity from patient cells. Using a mammalian cell expression system, the Proud laboratory has pioneered in vitro biochemical analyses of eIF2B CACH/VWM mutants. Typically HEK293 cells are transformed with vectors overexpressing all five subunits of eIF2B, which are affinity purified and the resulting complexes analysed for complex formation and GEF activity. For example when analysed as part of 5-subunit complex W628R reduced activity to ~20% [8], I649T to ~40% and E650K to ~30% of wild type [49]. The latter two also apparently reduced association with the alpha (and possibly delta) subunits of eIF2B [49] indicating possible reduced complex integrity that could contribute to reduced activity. The D610–613 mutant dramatically reduced expression or subunit stability and activity to 25% [9].

To further test our recombinant heIF2 and h2Bc, we introduced several mutations into our h2Bc expression vector and purified these to homogeneity (Figure 5). We included mutations that had been analysed as part of 5-subunit eIF2B complexes (W628R, I649T and E650K) as well as mutations not analysed previously (P604S and D666–672). All mutant forms significantly reduced GEF activity (Figure 5), validating that our heIF2 and h2Bc behave as expected. It was perhaps surprising that the D666–672 mutant had only a modest reduction in activity, when compared with missense mutations that might be predicted to have a smaller impact on the overall structure. Examining the locations of the mutated residues on the human catalytic domain structure [33,44] suggests that most of the missense mutations affect residues predominantly buried internally within the structure. Macromolecular modelling of the impact of the D666–672 mutant, suggests that the normal domain fold can be adopted, but
that there is local disruption of part of one α-helix only (data not shown). The affected helix does not contribute to the surface regions of eIF2 identified previously as critical for direct eIF2 binding and activity [50]. This observation appears to fit with the modest reduction in GEF activity observed for h2BeIF2, but does not rule out that it may have a more significant defect in vivo in the context of the full five subunit protein complex.

**helf2** Functions in GEF Assays with Extracts from Patient-derived Cells

Extracts from patient-derived cells have been used as a source of eIF2B to provide biochemical diagnosis for eIF2B related disorders [10,34]. To assess whether our helf2 would perform as a substrate in GEF assays with patient cells, we performed assays with lymphoblastoid cell line extracts from 18 CACH/VWM patients and compared the results to those obtained previously with the rat eIF2 substrate classically used for this diagnostic assay (Table 1). Our panel of cell lines included mutations in four of the five eIF2B subunits and those previously shown to have a range of disease severity from severe, early onset to classical/mild forms of disease [34].

Firstly, we observed a decreased GEF activity (~87.2%) using the helf2 substrate for the patient cell lines which exhibited a decreased GEF activity under the previously described diagnostic threshold (77.5%) using rat eIF2 as substrate [34]. The GEF activity values using the helf2 substrate are marginally higher and show more variability (with a mean SD = 9.1% using helf2 in comparison to 4.8% using rat eIF2 with a correlation factor between the two assays of r = 0.78. The disease-diagnostic threshold used with this substrate should therefore be revised from 77.5% to 89.9%. Using this new value, the diagnostic impact of the GEF assay is identical to the assay using rat eIF2: the patients with GEF activity <77.5% with rat eIF2 exacerbated also a GEF activity <89.9% with yeast helf2 (Table 1). There are several possible reasons that may explain the observed minor difference between the two sources of eIF2. Firstly, there are minor sequence variations between the two species. Secondly there may be differences in post-translational modifications between the two preparations. For example, we assume that eIF2 from rat liver will be partially phosphorylated at ser51 by endogenous eIF2 alpha kinases, while helf2 from yeast is unphosphorylated. Phosphorylated eIF2 inhibits eIF2B reducing the observed activity. Specific mutations in the α, β and γ subunits alter the sensitivity of eIF2B to inhibition by phosphorylated eIF2 [23,24,25]. However it is not yet known whether disease-causing mutations alter eIF2B sensitivity to eIF2 phosphorylation.

Cells from two patients tested here (432-1 and 1074-1) exhibited normal GEF activity using the rat eIF2 substrate. Here lower GEF activity was found with the helf2 substrate, taking patient 432-1 just below the proposed diagnostic threshold (Table 1). A recent report also identified two severe CACH/VWM patients with eIF2B mutations, but no apparent defect in eIF2B activity [49]. However the eIF2B GEF activity for those patients was measured only in primary fibroblasts. Further studies are needed to assess the diagnostic value of measuring eIF2B GEF activity in fibroblasts. Discrepancies have been previously reported between measuring...
eIF2B defects in immortalised lymphocytic cells compared to primary fibroblasts [51]. In summary, the recombinant heIF2 performs as well as the previously used substrate in this assay and measurement of reduced GEF activity with heIF2 and extracts from immortalised lymphocytic cells is diagnostic for eIF2B-mediated disease.

In conclusion, we describe a strategy for production of heterotrimeric recombinant human eIF2. We show that it can functionally replace yeast eIF2 in vivo and demonstrate its utility as a diagnostic tool for measuring the impact of eIF2B mutations that cause human disease. Employing recombinant heIF2 from yeast will remove the need to use animal tissue sources to obtain purified eIF2 for these purposes.

**Methods**

**Plasmid Constructions**

**Human eIF2 cDNA expression.** Human cDNAs encoding EIF2S1 (eIF2α) and EIF2S2 (eIF2β) were cloned with N-terminal Flag or his6 epitope tags respectively into the pBEVY series of plasmids bearing divergent promoters from the GAL1 and GAL10 genes (PGAL1 and PGAL10) that we have recently used with success elsewhere [52,53]. The cDNAs were cloned singly as Kpn1-EcoRI fragments downstream of PGAL1 creating pAV1901 (EIF2S2-his6) and pAV1907 (EIF2S1-Flag). In addition EIF2S1-Flag was cloned downstream of PGAL10 as a BamHI-Pst1 fragment in pAV1901 to create pAV1905 (EIF2S2-his6 and EIF2S1-Flag). EIF2S3 (eIF2γ) cDNA was codon-optimized and synthesized (GeneScript USA Inc.) and subcloned as a Xba1-Sal1 fragment into pBEVY-GL creating pAV1970 and pBEVY-GU creating pAV1974. All constructs were confirmed by DNA sequence analysis. Further details are shown in Table 2.

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**Figure 4.** Purification of heIF2, h2Bcat and GEF activity. A. and B.: Coomassie blue stained SDS-PAGE gel summaries of (A) single step partial-purification of heIF2 from yeast strain GP6452 cell extracts (lane 2) using Flag M2 (lanes 3–7) or nickel agarose resins (lanes 8–12) or (B) sequential two-step purification of heIF2 using first nickel agarose (lanes 3–6) and then Flag M2 affinity gel (lanes 7–11) that generated heterotrimeric eIF2. C. Left, coomassie blue stained SDS-PAGE gel of purified human elf2 labelled αβγ and h2Bcat. Right, GEF assays with wild type h2Bcat or elf2 alone.

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**Figure 5.** GEF activity of h2Bcat CACH/VWM mutants. Top, purified elf2B catalytic domains with indicated mutations both coomassie blue stained SDS-PAGE and Flag western blot are shown. Bottom: change in activity normalised to wild type (n = 4). 2 tailed T-test * p < 0.05, ** p < 0.0001.

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Human elf2Bc catalytic domain. The elf2Bc (eIF2Bc) catalytic domain (amino acids 533–721, termed here eIF2Bcat) was identified by sequence alignment of translated ORF from Genbank [NG_015826] with the yeast Saccharomyces cerevisiae Gcd6p catalytic domain [32,33]. The cDNA was codon optimised and custom synthesized (GeneScript USA Inc) with the 3’ catalytic domain [32,33]. The cDNA was codon optimised and identified by sequence alignment of translated ORF from Genbank (NG_015826) with the yeast Gcd6p catalytic domain (amino acids 533–721, termed here Gcd6p). The cDNA was codon optimised and custom synthesized (GeneScript USA Inc) with the 3’ catalytic domain [32,33].

Yeast (S. cerevisiae) Genetic Methods

All constructions employed standard methods and yeast media [54]. Transformations used the lithium acetate method. For serial dilution growth assays cells were grown in YPGal medium prior to shuffling, and FOA medium used 2% galactose in place of glucose. All strains used are summarized in Table 3.

To assess complementation of the slow-growth phenotype of strains expressing elf2Bc as the sole source of eIF2c, strains GP5613 or GP5614 were transformed with the following high copy plasmids to alter the levels of the indicated factors: tRNA<sup>Met</sup> pAV1345 (DFT4 LEU2); elf2Bc pAV1428 (GCD1 GCD6 URA3) and pAV1494 (GCD2 GCD7 GCD3 LEU2); elf5 pAV2015 (TIF5 URA4); elf3 pAV2112 (TIF3 NPI LEU2) and pAV2113 (PRT1 TIF3 TIF4 HR1 URA4). Only excess elf5 reproducibly suppressed the slow-growth phenotype. In some experiments other slow-growth suppressing colonies were obtained, but not reproducibly. Except for elf5, we assume that suppression was caused by altered elf2Bc expression similar to that shown in Figure 2A, rather than true suppressive effects of the transformed plasmid. In addition, combinations of overexpressed elf2B subunits were assessed: pAV1346 (SUP2 SUP3 URA4) and pAV1348 (GCD11 SUP2 SUP3 URA4) or the low copy plasmid pAV1280 (GCD11 CEN). Only plasmids bearing GCD11 suppressed slow-growth.

| Patient Number | Mutated gene | Gene mutations (protein substitutions) | elf2Bc GEF activity using elf2Bc (%)<sup>a</sup> | elf2Bc GEF activity using rat elf2Bc (%)<sup>b</sup> | elf2Bc/hel2Bc rat elf2Bc |
|---------------|--------------|----------------------------------------|-----------------------------------------------|-----------------------------------------------|--------------------------|
| 949-1         | Elf2Bc      | c.728C>T/c.728C>T (Pro243Leu/Pro243Leu) | 35.8                                          | 3.5                                           | 45.6                     |
| 1036-1        | Elf2Bc      | c.967C>T/c.1280C>T (Pro322Ser/Pro427Leu) | 44.5                                          | 9.9                                           | 30                       |
| 1838-1        | Elf2Bc      | c.818T>C/c.1346G>T (Met273Thr/Thr449Ile) | 44.5                                          | 2.1                                           | 46.8                     |
| 1758-1        | Elf2Bc      | c.338G>A/c.947G>A (Arg113His/Arg136Gln)  | 46.2                                          | 1.9                                           | 44.8                     |
| 570-2         | Elf2Bc      | c.626G>A/c.626G>A (Arg209Gln/Arg209Gln) | 49.1                                          | 12.8                                          | 52                       |
| 375-2         | Elf2Bc      | c.338G>A/c.1948G>A (Arg113His/Glu650Leu) | 56.2                                          | 3.1                                           | 59.4                     |
| 1348-1        | Elf2Bc      | c.338G>A/c.338G>A (Arg113His/Arg113His) | 56.6                                          | 11.6                                          | 61.6                     |
| 972-1         | Elf2Bc      | c.943C>T/c.271A>G (Arg315Cys/Thr91Ala)  | 56.6                                          | 2.4                                           | 60.3                     |
| 38            | Elf2Bc      | c.638A>G/c.638A>G (Glu213Gly/Glu213Gly)  | 57.5                                          | 4.0                                           | 40.3                     |
| 291-1<sup>c</sup> | Elf2Bc   | c.338G>A/c.1160A>G (Arg113His/Asp387Gly) | 58.6                                          | 17.7                                          | 41.5                     |
| 1878-1        | Elf2Bc      | c.338G>A/c.338G>A (Arg113His/Arg113His) | 59.5                                          | 4.5                                           | 48.2                     |
| 357-1         | Elf2Bc      | c.406C>T/c.1015C>T (Arg136Cys/Arg339Trp) | 63.7                                          | 17                                            | 44.5                     |
| 571-1         | Elf2Bc      | c.166T>C/c.944G>A (Phe56Val/Arg315His) | 63.9                                          | 15.2                                          | 40                       |
| 1963-1        | Elf2Bc      | c.338G>A/c.338G>A (Arg113His/Arg113His) | 64.4                                          | 14                                            | 69.2                     |
| 1152-1        | Elf2Bc      | c.134G>C/c.134G>C (Ala45Gly/Ala45Gly)  | 68                                            | 1.3                                           | 60.8                     |
| 432-1         | Elf2Bc      | c.338G>A/c.1884G>A (Arg113His/Thr628X) | 86.4                                          | 12.4                                          | 90.4                     |
| 1240-1        | Elf2Bc      | c.604G>A/c.1312C>T (Ala202Thr/Arg438X) | 87.2                                          | 26                                            | 67.7                     |
| 1074-1        | Elf2Bc      | c.338G>A/c.338G>A (Arg113His/Arg113His) | 92                                            | 5.5                                           | 108                      |

*Mean ± SD* SD standard deviation.

<sup>a</sup>Patient previously reported as Arg113His/Arg113His in error [34].

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Table 1. elf2Bc GEF activities measured in extracts from lymphoblastoid cell lines from indicated CACH/VWM patents using elf2Bc and rat elf2B substrates.
Table 2. Plasmids used or constructed for this study.

| Designation | Genes | Source/Construction summary |
|-------------|-------|----------------------------|
| pAV1228     | SU13 LEU2 CEN4 | A. Hinnebusch p920 |
| pAV1255     | SU12 LEU2 CEN4 | A. Hinnebusch p1097 |
| pAV1280     | GCD11 URA3 CEN4 | E. Hannig Ep293 |
| pAV1345     | IMT4 LEU2 2μm | A. Hinnebusch p1775 [58] |
| pAV1346     | SU12 SU1 URA3 2μm | A. Hinnebusch p1778 [58] |
| pAV1348     | GCD11 SU1 SU1 URA3 2μm | A. Hinnebusch p1780 [58] |
| pAV1427     | P_{GAL1}-Flag-His6-GCD6 URA3 leu2d 2μm | [59] |
| pAV1428     | GCD1-Flag-His6-GCD6 URA3 2μm | [59] |
| pAV1494     | GCD2 GCD7 GCN3 LEU2 2μm | [59] |
| pAV1702     | P_{GAL1}-P_{GAL10} 2μm LEU2 | pBEVY-GL dual promoter galactose expression vector [52] |
| pAV1703     | P_{GAL1}-P_{GAL10} 2μm TRP1 | pBEVY-GT dual promoter galactose expression vector [52] |
| pAV1704     | P_{GAL1}-P_{GAL10} 2μm URA3 | pBEVY-GU dual promoter galactose expression vector [52] |
| pAV1874     | TRP1 P_{GAL1}-Flag P_{GAL10}-Flag2-His6 2μm | Flag tagged human CDNAs amplified and cloned in pBEVY-T Note-Promoter mutations prevent expression. |
| pAV1901     | TRP1 P_{GAL1}-Flag2-His6 2μm | human EIF2S2-His6 digested from pAV1874 (KpnI/EcoRI) and ligated into pAV1703 |
| pAV1905     | TRP1 P_{GAL1}-Flag2-Flag-P_{GAL10}-Flag-His6-EIF252-His6 2μm | human EIF2S1-Flag from pAV1874 (BamHI/PstI) ligated into pAV1901 |
| pAV1907     | TRP1 P_{GAL1}-Flag2-Flag2-Flag P_{GAL10}-Flag 2μm | human EIF2S1-Flag KpnI/EcoRI digested from pAV1874 and ligated into pAV1703 |
| pAV1970     | P_{GAL1}-Flag2-Flag2 2μm LEU2 | Codon optimised EIF2S3 cloned into pAV1704 by XbaI/Sall digestion and ligation. |
| pAV1974     | P_{GAL1}-Flag2-Flag2 2μm URA3 | Codon optimised EIF2S3 cloned into pAV1702 by XbaI/Sall digestion and ligation. |
| pAV2015     | TIF5-Flag URA3 2μm | K. Asano KAB446 |
| pAV2075     | URA3 P_{GAL1}-Flag-His6-EIF2B5cat leu2d 2μm | Codon optimised commercially synthesized human EIF2B5 cDNA (codons 533–721) cloned MluI/BamHI into pAV2075 |
| pAV2095     | URA3 P_{GAL1}-Flag-His6-EIF2B5cat-leu2d 2μm | Site directed mutagenesis introduced mutation into EIF2B5 (533–721) cDNA as described for pAV2075. |
| pAV2096     | P_{GAL1}-Flag-His6-EIF2B5cat-M664–671 URA3 leu2d 2μm | Site directed mutagenesis as above |
| pAV2097     | P_{GAL1}-Flag-His6-EIF2B5cat-W628R URA3 leu2d 2μm | Site directed mutagenesis as above |
| pAV2098     | P_{GAL1}-Flag-His6-EIF2B5cat-P604S URA3 leu2d 2μm | Site directed mutagenesis as above |
| pAV2099     | P_{GAL1}-Flag-His6-EIF2B5cat-E664–671 URA3 leu2d 2μm | Site directed mutagenesis as above |
| pAV2112     | TIF23 NIP1-His6 LEU2 2μm | L. Valasek, Prague |
| pAV2113     | TIF31 TIF35 TIF34 HCR1 URA3 2μm | L. Valasek, Prague |

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5 mM MgCl₂, 5 mM NaF, 10 mM Imidizole, 7 mM 2-mercaptoethanol, 10% Glycerol, 0.1% Triton X100, protease inhibitor tablet (Roche) and 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 μg/ml aprotinin and frozen under liquid nitrogen. Cells were lysed using a large cryogenic freezer mill (Spex Certiprep Ltd) and stored at –80°C prior to purification. All subsequent steps were performed at 4°C. Cells were thawed and cell debris removed by centrifugation at 5000 g (Sigma 4K15 centrifuge) and the resulting extract was clarified by successive rounds of centrifugation at 22,000 × g, 30 min, (Heraeus Biofuge Stratos) and 440,000 g, 1 hr, (Beckman ultracentrifuge T70.1 rotor). Nickel affinity chromatography (Qiaqen) was performed in batch mode with rotation for 2 hrs. Resin was collected by low speed centrifugation (2000 rpm), washed four times with Ni Wash buffer (as Lysis buffer, but with 100 mM KCl, 25 mM Imidizole) and eluted (2×1 hr) in Ni Elution buffer (as Ni Wash buffer, but with 500 mM Imidizole). Elutions were combined and dialysed against Flag Wash buffer (as Ni Wash buffer but lacking imidizole), then mixed with 1 ml prewashed Flag M2 agarose resin (Sigma) in batch binding mode for 2 hours. Following three washes in Flag Wash buffer, protein elutions were performed (2×30 min) in Flag Elution buffer [Flag Wash buffer with 0.4 mg/ml 3X Flag peptide (Sigma)]. Finally purified samples were dialysed into storage buffer [30 mM HEPES (pH7.5), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, 0.1% Igepal CA-630] then aliquoted and stored at –80°C. Typically a yield of 2.4 mg eIF2 was purified (Micro-BCA assay, Pierce).

eIF2B catalytic domain. Strain GP5448 contains plasmid pAV2075 for yeast expression codon-optimized galactose-induced expression of Flag and his6, tandem tagged eIF2Bcat. Strains GP5644-5648 similarly express specific mutant forms of the same protein (Table 3). Our purification scheme was performed as described for human eIF2, except on a smaller scale starting with 8–20 g wet weight cell pellet. Typically 300–500 μg was purified from 20 g starting cell pellet.

Western Blotting
Extracts from exponentially growing yeast cells were made using glass beads and a FastPrep-24 (MP Biomedicals). Typically
8 × 10⁴ cells of units were washed and resuspended in 200 μl Laemmli sample buffer, processed for 5 to 30 seconds at 6 ms⁻¹ setting in the FastPrep-24 at 4°C. 20 μl of each sample was resolved on 10% or 12% acrylamide SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies: Flag M2 (Abcam AB33207, 1:1000), Gcd11p (1:1000; E. Hannig, Texas), Tif5p (1:1000) [57], Tsa1p (Abcam AB33207, 1:1000; the human eIF2β antibody was raised to an epitope shared with yeast Tsa1p; epitope TIKPTVDDD; Tsa1p TIKPTVeDs), Sui2p (1:100; T. Dever, NIH), Sui3p (1:500) [50]. HRP conjugated secondary antibodies (Abcam) and enhanced chemiluminescence detection system (Pierce) were used.

**GFP Assays**

eIF2B GEF activity measured with purified h2Bcat. Activity was measured using a standard filter binding assay with eIF2 and radiolabelled GDP. eIF2-[³H]GDP binary complexes were formed in binary complex buffer [30 mM HEPES (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 1 mg/ml BSA, 1 mM DTT] with 30 pmol eIF2 and 0.5 μCi [³H]GDP (4.5 Ci mmol⁻¹) at 20°C for 10 min and stabilized by the addition of 1 mM MgCl₂. Nucleotide exchange was initiated by the addition of 2 μg h2Bcat and unlabelled GDP (2 nmol). Samples were removed at regular intervals and filtered through nitrocellulose filters, dried and counted by liquid scintillation.

**eIF2B GEF activity measured in patients cell extracts.** An Institutional Review Board of the participating centers (Comité de Protection des Personnes Sud-Est VI, 2009-A00188-49) approved the use of human subjects for this study. A written informed consent was obtained from all patients [34].

Activity measured with extracts from patient lymphoblastoid cell lines (lymphoblasts) as a source of eIF2B was performed with h2Bcat as described previously for eIF2 purified from rat liver [7,25] with the following modifications: the use of 1 μCi [³H]GDP (4.5 Ci mmol⁻¹) for eIF2-[³H]GDP binary complex formation, incubation of this mixture at 30°C for 30 min (instead of 10 min), and the [³H]GDP dissociation kinetics was monitored every 5 min (instead of every 2 min; from 0 to 15 min). Such comparative analyses were performed at least in triplicate for cells from 18 patients and matched controls.

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**Table 3. Yeast Strains Used in this study.**

| Designation | Genotype | Source/reference |
|-------------|----------|-----------------|
| GP3001      | MATα leu2-3 leu2-12 sui2Δ trp1Δ::M63 ura3-52 [SUI2 CEN LEU2] | Pavitt collection |
| GP3582      | MATα gcn11Δ::hisG leu2-3 leu2-112 ino1 ura3-52::His5-loa2 [URA3 CEN GCD11] | Pavitt collection, A. Hinnebusch NIH -F484 |
| GP3889      | MATα gcn2Δ leu2-3 leu2-112 pep4::LEU2 trp1Δ::M63 ura3-52 | Pavitt collection |
| GP4907      | MATα gcn2Δ leu2-3 leu2-112 ino1 sui3Δ His5-loa2:ura3-52 [SUI3 CEN LEU2] | K. Asano, KAY17 |
| GP5010      | trp1Δ::hisG in GP4907 | This Study |
| GP5012      | trp1Δ::hisG in GP3582 | This Study |
| GP5108      | [PGAL10::EIF251-Flag 2μm TRP1] plasmid shuffle in GP3001 | This Study |
| GP5109      | [PGAL10::EIF252-Flag PGAL1::EIF252-His6 2μm TRP1] plasmid shuffle in GP3001 | This Study |
| GP5110      | [PGAL10::EIF252-Flag His6 2μm TRP1] plasmid shuffle in GP5010 | This Study |
| GP5111      | [PGAL10::EIF251-Flag PGAL1::EIF252-His6 2μm TRP1] plasmid shuffle in GP5010 | This Study |
| GP5348      | GP3889 [PGAL-Flag-His6-EIF250cat] URA3 leu2Δ 2μm | This Study |
| GP5613      | [PGAL10::EIF253 2μm LEU2] FOA plasmid shuffle in GP5012 | This Study |
| GP5614      | [PGAL10::EIF253 2μm TRP1] FOA plasmid shuffle in GP5012 | This Study |
| GP5644      | GP3889 [PGAL10-Flag-His6-EIF250cat·His6] URA3 leu2Δ 2μm | This Study |
| GP5645      | GP3889 [PGAL10-Flag-His6-EIF250cat·His6] URA3 leu2Δ 2μm | This Study |
| GP5646      | GP3889 [PGAL10-Flag-His6-EIF250cat·His6·TRP1] FOA plasmid shuffle in GP5014 | This Study |
| GP5647      | GP3889 [PGAL10-Flag-His6-EIF250cat·His6·TRP1] FOA leu2Δ 2μm | This Study |
| GP5648      | GP3889 [PGAL10-Flag-His6-EIF250cat·His6·TRP1] FOA leu2Δ 2μm | This Study |
| GP5649      | GP3889 [PGAL10-Flag-His6-EIF250cat·His6·TRP1] FOA leu2Δ 2μm | This Study |
| GP5744      | GP5613 [URA3 2μm] | This Study |
| GP5755      | GP5613 [GCD11 CEN URA3] | This Study |
| GP5758      | GP5613 [TRP1 2μm] | This Study |
| GP5612      | MATα gcn11::Nat gcn2Δ::hisG his3Δ::met15Δ::pep4::HIS5 sui2Δ::hisG sui3Δ::KanMX ura3-10 [GCD11 SU13 URA3 2μm] | T. Dever J551 [43] |
| GP6124      | trp1Δ::hisG in GP6122 | This Study |
| GP6452      | GP3889 [PGAL10::EIF252 2μm LEU2] [PGAL10::EIF251-Flag PGAL1::EIF252-His6 2μm TRP1] [PGAL10::EIF253 2μm URA3] | This Study |
| GP6461      | [PGAL10::EIF251-Flag PGAL1::EIF252-His6 2μm TRP1] FOA plasmid shuffle in GP6124 (#1) | This Study |
| GP6462      | [PGAL10::EIF253 2μm LEU2] [PGAL10::EIF251-Flag PGAL1::EIF252-His6 2μm TRP1] FOA plasmid shuffle in GP6124 (#2) | This Study |

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Author Contributions

Conceived and designed the experiments: GDP GCS OBT.Performed the experiments: RAA AF MG. Analyzed the data: RAA AF GDP. Contributed reagents/materials/analysis tools: GCS OBT GDP. Wrote the paper: GDP AF OBT RAA GCS.

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