A yeast tRNA mutant that causes pseudohyphal growth exhibits reduced rates of CAG codon translation

Summary

In Saccharomyces cerevisiae, the SUP70 gene encodes the CAG-decoding tRNA_{Gln}^{CUG}. A mutant allele, sup70-65, induces pseudohyphal growth on rich medium, an inappropriate nitrogen starvation response. This mutant tRNA is also a UAG nonsense suppressor via first base wobble. To investigate the basis of the pseudohyphal phenotype, 10 novel sup70 UAG suppressor alleles were identified, defining positions in the tRNA_{Gln}^{CUG} anticodon stem that restrict first base wobble. However, none conferred pseudohyphal growth, showing altered CUG anticodon presentation cannot itself induce pseudohyphal growth. Northern blot analysis revealed the sup70-65 tRNA_{Gln}^{CUG} is unstable, inefficiently charged, and 80% reduced in its effective concentration. A stochastic model simulation of translation predicted compromised expression of CAG-rich ORFs in the tRNA_{Gln}^{CUG}-depleted sup70-65 mutant. This prediction was validated by demonstrating that luciferase expression in the mutant was 60% reduced by introducing multiple tandem CAG (but not CAA) codons into this ORF. In addition, the sup70-65 pseudohyphal phenotype was partly complemented by overexpressing CAA-decoding tRNA_{Gln}^{UUG}, an inefficient wobble-decoder of CAG. We thus show that introducing codons decoded by a rare tRNA near the 5’ end of an ORF can reduce eukaryote translational expression, and that the mutant tRNA_{Gln}^{CUG} constitutive pseudohyphal differentiation phenotype correlates strongly with reduced CAG decoding efficiency.

Introduction

Organisms respond to changes in environment through controlling patterns of gene expression. Gene regulation is frequently exerted at the level of transcription, although it is well understood that translational control, as well as regulation of mRNA and protein stability, also play important roles in setting the steady-state level of protein expression for any given gene. Many examples of translational control operate by controlling the ability of an mRNA to recruit ribosomal subunits during the translation initiation process. In both prokaryotes and eukaryotes, secondary structure elements within the 5’ untranslated region (5’UTR), often subject to specific binding by RNA-binding proteins, regulate ribosome access to the AUG codon and subsequently, the open reading frame (Ikemura, 1982; Sharp and Li, 1987; Kuhn and Hentze, 1992; Klausner et al., 1993; Vega Laso et al., 1993; Dong et al., 1996; Grunberg-Manago, 1999).

There are however a growing number of examples where the translational efficiency of an mRNA is regulated at the level of translation elongation, particularly in organisms where there is biased codon composition within genes (Sharp and Li, 1987). Biased codon usage is matched by corresponding bias within the decoding tRNA population (Ikemura, 1982; Dong et al., 1996), thus rare codons are decoded slowly by cognate, low abundance tRNAs. Such slow decoding may cause ribosomal queuing, which in turn can extend back to the 5’ end of an mRNA and impact upon ribosome recruitment at the initiation level. Alternatively, slow decoding of a rare codon could destabilize the parent mRNA through ssrA-mediated turnover in bacteria (Keiler et al., 1996) or via no-go decay in eukaryotes (Doma and Parker, 2006) reviewed in Buchan and Stansfield (2007). Evidence for rare codon regulation of gene expression comes from studies in a range of systems; replacing multiple rare codons with synonymous but frequently used counterparts in the Escherichia coli chloramphenicol acetyltransferase gene increases its expression levels (Komar et al., 1999), while the sequential introduction of rare AGG codons into an open reading frame proportionately reduces expression of the encoded protein (Rosenberg et al., 1993). These observations are supported by modelling of translation that indicate that rare codon placement in open reading frames...
can be highly influential in regulating protein productivity from a given mRNA (Tuller et al., 2010).

Other, natural examples of regulation via translation elongation are also known. The **bldA** mutants of *Streptomyces coelicolor* cannot form aerial mycelia during late stages of growth, and do not produce the expected growth stage-specific antibiotics (Merrick, 1976). **bldA** encodes the developmentally regulated rare leucine-decoding tRNA, whose cognate TTA codon is largely absent from genes expressed during exponential growth, but is present in **adpA**, the master regulator of mycelial production (Leskiw et al., 1991; Li et al., 2007). Replacement of TTA by the more common TTG cognate leucine codon restores high level **adpA** expression (Takano et al., 2003). The *Streptomyces* switch to secondary metabolism and aerial mycelium production is thus controlled by the developmental regulation of **bldA** tRNA.

In *Saccharomyces cerevisiae*, several tRNA mutants are known that display **bldA**-analogous developmental defects. Mutations in the *S. cerevisiae* single-copy **SUP70** gene, encoding the glutamine-decoding tRNA**GlnCUG**, generate a constitutive pseudohyphal growth phenotype (Murray et al., 1998). Whereas diploid wild-type yeast bud in a bipolar manner to produce separate ellipsoid cells, nitrogen starvation triggers cell elongation, and unipolar budding to produce long filamentous chains known as pseudohyphae (Gimeno et al., 1992). However, diploid **sup70-65** mutants undergo pseudohyphal growth even when grown on media containing an abundant source of nitrogen. It was hypothesized that the tRNA**GlnCUG** mutations somehow impair the sensing of the cell nitrogen supply, although there is evidence this is not via the mitogen-activated protein kinase (MAPK) cascade or the cyclic AMP-dependent Protein Kinase A (PKA) pathway known to signal pseudohyphal growth (Pan and Heitman, 1999) (Murray et al., 1998). Thus the mechanistic basis of how **SUP70** tRNA mutations trigger pseudohyphal growth is unclear.

In order to address the mechanistic basis of the tRNA**GlnCUG** pseudohyphal growth phenotype it is necessary to identify the mechanism by which the **sup70** tRNA mutations signal to nitrogen sensing machinery. Previous studies of the **sup70** alleles, including measured expression levels of **CAG** codon-enriched β-galactosidase reporter genes, indicated that the mutants were probably not compromised in their ability to translate CAG codons (Murray et al., 1998). However, we have now re-investigated this in much more detail using a broader range of methods, and discovered that the **sup70-65** tRNA is in fact inefficiently charged with glutamine, and furthermore is unstable, leading to a large reduction in the global capacity to decode CAG during translation. We furthermore show that introduction of additional CAG codons at the 5′ end of an ORF significantly compromises reporter expression in a **sup70** mutant, revealing a clear signature of translational elongation defects during CAG decoding in this genetic background. The work thus establishes the clear principle that altering codon decoding rates during eukaryote translation elongation can significantly impact on gene expression, probably through the establishment of ribosomal queues that modulate ribosome recruitment. The study establishes the further principle that altering the translational decoding rate of the CAG codon generates a highly specific pseudohyphal growth phenotype in yeast.

**Results**

**Yeast SUP70 tRNA gene mutations cause pseudohyphal growth in N-replete liquid medium**

Provided with sufficient nitrogen in the growth medium, diploid *S. cerevisiae* grows as ellipsoid cells that bud in a bipolar pattern to form round, smooth colonies on solid agar medium. Under limiting nitrogen conditions on solid medium, diploid *S. cerevisiae* with a Σ127Bb genetic background will switch to pseudohyphal growth, budding in a unipolar manner to produce chains of elongated cells that radiate from the colony circumference to give the margins of their colonies a ruffled appearance (Gimeno et al., 1992). It has been previously reported that control over pseudohyphal growth is however lost in strains carrying specific mutations in the **SUP70** gene encoding tRNA**GlnCUG**. The **sup70-65** and **sup70-33** alleles trigger pseudohyphal growth on both nitrogen-limited and nitrogen-replete solid medium (Murray et al., 1998). In the first instance, we sought to further characterize this phenotype by analysing the behaviour of strains carrying these alleles on solid and in liquid medium.

Accordingly, diploid strain MLD14 (**sup70-65/sup70-65**) and the corresponding wild-type MLD17 (both generous gifts from Prof. R.A. Singer) were grown on either solid rich (YPD) or minimal media (SLAD), the latter containing limiting ammonium sulphate and known to trigger pseudohyphal differentiation in competent yeast strains (Gimeno et al., 1992). However, we could find no evidence of pseudohyphal differentiation on agar. All colonies were smooth-edged, lacking chains of cells at the circumference, in contrast to the phenotype reported for another **sup70-65** homozygous diploid, LMD651U (Murray et al., 1998). However, in this study, when the MLD diploids were tested in the corresponding liquid media, the **sup70-65** strain, but not the wild-type, underwent clear and marked pseudohyphal-type differentiation. The mutant grew as chains of ovoid cells, varying in length between four and more than 10 cells per chain in both N-replete and N-limiting medium (Fig. 1A and B). In order to quantify this phenotype, we calculated a cumu-
Mutations in the yeast tRNAGlnCUG using the chain formation index (CFI) was derived (Fig. 1C: Experimental procedures) which allowed us to quantitatively compare chain formation under different conditions. Using this index, it was apparent that the degree of pseudohyphal-type differentiation in the mutant was significantly greater in N-limiting SLAD medium than that measured in SD or YPD media (Fig. 1C).

To further characterize the chain formation phenotype, we used live microscope observation of MLD14 growth in liquid SLAD medium over a 6 h period (Fig. 1D). Unexpectedly, the budding pattern was bipolar, and not unipolar as described for pseudohyphal growth (Gimeno et al., 1992). The daughter cells generally remained attached to the mother, accounting for the clustering, and were significantly elongated, with an axial ratio of 1.7, compared with the wild-type value of 1.5. The sup70-65 homozygote MLD14 thus exhibited an atypical pseudohyphal growth phenotype, revealed only in liquid medium.

The MLD strains that were tested above were derived from the Σ1278b background, known to form pseudohyphae. As a further test of the sup70-65 phenotype, we asked whether this tRNA mutant could trigger pseudohyphal-type growth in an S288C-background diploid, which carries a flo8-1 mutation preventing archetypal pseudohyphal growth (Liu et al., 1996). We therefore created a homozygous SUP70 knockout in the sequenced diploid strain BY4743, complemented with a plasmid-based copy of either SUP70 or sup70-65 (Fig. 2). While the SUP70 transformant presented a wild-type phenotype, surprisingly, complementation of the sup70 homozygous deletion with a plasmid-borne sup70-65 allele successfully induced a chain formation phenotype, albeit less pronounced than that measured in the MLD strain background (Σ1278b-derived). The detection of chain formation in the S288C-derived strain was despite the absence of functional Flo8p, another indication that the sup70-65 pseudohyphal growth form was atypical. Both strains were then transformed with an additional plasmid-borne copy of constitutively active RAS2Val19, a dominant mutation that constitutively activates the yeast RAS-cAMP pathway (Toda et al., 1985) and enhances pseudohyphal growth (Gimeno et al., 1992; Lorenz and Heitman, 1997) (Fig. 2B). However the degree of chain formation was not significantly enhanced by the mutant RAS2Val19 allele in either wild-type or sup70-65 mutant. Although it is formally possible that the Ras2 pathway is maximally activated in these strains, we consider it more likely that the enhanced Ras2 pathway signalling achieved using the Val19 mutant was not capable of inducing an additional liquid medium chain formation response, suggesting that this liquid medium chain phenotype was not being signalled via the RAS-cAMP pathway.
Novel tRNA mutants capable of nonsense suppression do not cause pseudohyphal growth

Wild-type tRNA_CUG can only very inefficiently decode UAG codons via first base wobble, because SUP70 amber (UAG) suppressor activity is detectable only when over-expressed on a multi-copy plasmid (Pure et al., 1985). However, the sup70-65 mutation is a single-copy UAG suppressor (Murray et al., 1998) indicating the mutant tRNA has an enhanced ability to wobble-decode U in the first codon position. The sup70-65 mutation creates an A–C nucleotide mismatch at the base of the tRNA_CUG anticodon stem (base pair nucleotides 31–39; Fig. 3). This presumably distorts the anticodon stem and increases the propensity to decode UAG via first base wobble. In order to identify further sup70 mutants capable of inducing pseudohyphal growth, we wanted to identify additional sup70 nonsense suppressor alleles. In this way, we sought to test the hypothesis that structural modifications that alter SUP70 tRNA_CUG presentation of the anticodon might generally cause a tRNA defect that triggers deregulated pseudohyphal growth.

A library of plasmid-borne tRNA_CUG mutants was therefore generated via PCR. This library was transformed into wild-type strain MLD17 that carries the trp1-1 amber mutation (UAG) in homozygous form and is thus tryptophan (Trp) auxotrophic. Trp prototrophic transformants identified sup70 nonsense suppressor alleles. Sequencing of these alleles revealed that almost all the amber suppressor mutants defined nucleotide substitutions in the anticodon stem and loop (Fig. 3A). Stem mutations disrupt base pairing and would be predicted to disrupt structure and orientation of the anticodon loop. Reassuringly, the sup70-65 mutation was re-isolated from the screen, as was a mutation creating a UAG-cognate anticodon (H2; Fig. 3A).

The nonsense suppressor phenotype of these novel alleles was quantified using a dicistronic readthrough assay in strain BY4743 (SUP70/SUP70; Fig. 3B). A UAG stop codon, placed in a poor nucleotide context for termination to increase the sensitivity of readthrough detection, separated the lacZ and firefly luciferase open reading frames in the readthrough assay vector. A baseline readthrough efficiency of 2% was measured in untransformed BY4743 (data not shown), due to the leakiness of the UAG codon used, and the natural propensity of SUP70 tRNA to decode UAG via first base wobble (Weiss et al., 1987). An additional copy of wild-type SUP70 increased readthrough levels to more than 3% (Fig. 3B). Excluding H2, the other mutants exhibited varying UAG suppressor efficiencies of between 4% and 9% confirming their UAG suppressor phenotypes. As expected, readthrough levels of mutant H2 were high, as it has a mutated anticodon 5′-CUA-3′ able to directly recognize the UAG stop codon. The sup70-33 mutation, although not a suppressor screen isolate, was also tested for readthrough activity since it also exhibits a pseudohyphal growth phenotype (Murray et al., 1998). As expected this allele was no more effective a suppressor than the wild-type SUP70 allele (data not shown).

Having isolated a family of novel sup70 amber suppressor alleles, their ability to trigger un-regulated pseudohyphal differentiation was then tested by shuffling the plasmid-borne alleles into the MLD17 homozygous ΔSUP70 knockout strain. However, with the exception of the control sup70-65 allele, which exhibited a high chain
formation index, none of the newly identified mutants induced pseudohyphal growth (Fig. 3C). We did note however that when a homozygous SUP70 deletant is supported by a single plasmid-borne copy of the SUP70 gene, the chain formation index was increased, indicating that reducing the gene copy number of the wild-type tRNA\textsubscript{CUG}tRNA can also establish low levels of chain formation. Since nonsense suppressor sup70 alleles must exhibit an altered presentation of the CUG anticodon in such a way as to enhance G–U wobble base pairing at the first codon position, it was concluded that altered anticodon presentation is in itself insufficient to trigger pseudohyphal growth. The sup70-65 tRNA must therefore exhibit other defects in addition to its altered anticodon presentation.

Site-directed sup70 mutants identify tRNA structural rigidity and translational efficiency as important determinants of the pseudohyphal phenotype

In order to characterize the impact of the sup70-65 and sup70-33 mutations on the function of tRNA\textsubscript{CUG}, we created site-directed mutants (sup70-65\textsuperscript{c}; sup70-33\textsuperscript{c}) in which the substituted mutant nucleotide was preserved, but stem base pairing was restored, for example creating a, A\textsubscript{31}–U\textsubscript{39} pair to create allele sup70-65\textsuperscript{c} (Fig. 4A). Other variants were created in which a G–U wobble base pair interaction was created, to weaken stem interaction (sup70-65\textsuperscript{i}, sup70-33\textsuperscript{i}). Using these novel alleles, the role of nucleotide pair identity, and pair strength on the pseudohyphal growth phenotype could be tested.

First, we assessed the ability of the new alleles to suppress the UAG stop codon, since the sup70-65 allele is an amber suppressor. Plasmids carrying the alleles were transformed into strain MLD17, which carries the trp1-1\textsuperscript{am} allele, causing tryptophan prototrophy in strain MLD17. Mutant E2 was not further analysed.

B. Quantification of nonsense codon readthrough using a dicistronic vector system. Dominant SUP70 alleles with amber suppressor activity were transformed into a wild-type strain (BY4743) along with a vector carrying a dicistronic stop codon readthrough assay system to quantitatively stop codon readthrough in three independent cultures. Error bars represent ± 1 standard deviation.

C. The ability of the novel nonsense suppressor SUP70 alleles (A: excluding mutant H2 which could not support viability), and that of SUP70 and sup70-65 alleles to trigger pseudohyphal chain formation was assessed by shuffling the plasmid-borne alleles into strain MLD17–SUP70 (ΔSUP70/ΔSUP70) and measuring their chain formation index (CFI) in triplicate independent transformants, grown in SD medium. Error bars represent ± 1 standard deviation.

Fig. 3. Novel SUP70 amber suppressor mutations identify anticodon stem and loop positions that regulate first codon-position wobble.

A. Location of novel amber suppressor SUP70 mutations, all of which are single mutants with the exception of H5. All suppressed the trp1-1 allele, causing tryptophan prototrophy in strain MLD17. Mutant E2 was not further analysed.

B. Quantification of nonsense codon readthrough using a dicistronic vector system. Dominant SUP70 alleles with amber suppressor activity were transformed into a wild-type strain (BY4743) along with a vector carrying a dicistronic stop codon readthrough assay system to quantitatively stop codon readthrough in three independent cultures. Error bars represent ± 1 standard deviation.

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sup70-33 allele exhibited no measurable suppressor activity, the sup70-65 allele was an efficient suppressor. Repair of the sup70-65 tRNA anticodon stem by nucleotide substitution to create an A₃₁–U₃₉ pair did not however completely eliminate suppressor ability (sup70-65c: Fig. 4B), while in contrast the sup70-65i (G₃₁–U₃₉) mutant exhibited no suppressor activity. Thus nucleotide identity, as well as the existence of an intact stem, appears to affect anticodon presentation and thus first base wobble.

The pseudohyphal phenotypes of the novel mutants were subsequently tested by shuffling them into a homozygous ΔSUP70 strain background (Fig. 4C). The results showed that recreating a position 31–39 base-pair in the sup70-65c and sup70-65i alleles significantly reduced the formation of pseudohyphae, but did not completely restore the wild-type phenotype, indicating that base pair strength and/or nucleotide identity may be important determinants of the pseudohyphal growth phenotype. Likewise in the sup70-33 family mutants, restoration of base pairing at the 5–68 base-pair did not restore a wild-type phenotype, again indicating a role for base identity or the structural rigidity of the acceptor stem in signalling pseudohyphal responses.

Finally, we created three SUP70 mutants at tRNA base 33, a base position that is uridine in almost all tRNAs and which when mutated markedly degrades translational efficiency (Fig. 4A) (Santos et al., 1996). U33 may also be a recognition determinant for the glutaminyl-tRNA synthetase (Hayase et al., 1992). We reasoned that if inefficient CAG codon translation was the cause of the sup70-65 and –33 phenotypes (perhaps in the case of sup70-65 caused by anticodon loop distortion), then reconstituting poor decoding of CAG codons, or inefficient tRNA^Gln^CUG^Gin^ charging, via mutation at position 33 should also generate a pseudohyphal growth phenotype. The results did indeed confirm this; although the U33G mutant did not differ from the wild-type, both U33C and U33A mutants generate significantly more pseudohyphal cell chains than the wild-type (Fig. 4C).

From these findings, we conclude that tRNA structure, as well as the identity of specific nucleotides, influences both stop codon readthrough and the regulation of pseudohyphal growth. The results also provide a preliminary indication that reductions in the translational efficiency of tRNA^Gln^CUG may cause unregulated pseudohyphal growth.

**Overexpression of either tRNA^Gln^CUG or tRNA^Gln^UUG attenuates pseudohyphal differentiation**

Since alterations in tRNA structural rigidity, anticodon loop presentation and overall translational efficiency all trigger the pseudohyphal growth phenotype, it was hypothesized that the sup70 pseudohyphal phenotype is caused by a...
defect in the ability of tRNACUG to efficiently decode its cognate CAG codon. That being the case, it should be possible to repair the pseudohyphal growth phenotype by increasing the gene dosage of sup70-65 alleles. This was found to be the case, since introducing one extra sup70-65 gene copy on a centromeric plasmid into a sup70-65 mutant partly repaired the phenotype, and introducing multiple extra copies using a 2μ-based vector completely repaired the phenotype (Fig. 5A).

Third base U-G wobble is thought to be restricted in eukaryotes, thus the CAA-decoding tRNAUUG is a poor decoder of CAG codons. However, it is known that if overexpressed, the CAA-decoding tRNAUUGGln can suppress a SUP70 (tRNACUGGln) gene knockout in yeast, because the mcm5-s2-modified UUG anticodon has nonetheless some weak ability to third-base wobble-decode the CAG codon (Johansson et al., 2008). We therefore tested whether driving improved CAG codon translation in a sup70-65 mutant via overexpression of tRNAUUGGln (gene tQ(UUG)C) on a multi-copy plasmid could suppress constitutive pseudohyphal growth.

Strain MLD14 (sup70-65/sup70-65) and the wild-type counterpart MLD17 (SUP70/SUP70) were therefore transformed with the multi-copy plasmid pTRNA-UUG carrying tQ(UUG)C and were assessed for pseudohyphal growth (Fig. 5B). The results show clearly that whereas the control vector left the chain formation index unaffected, transformation of the sup70-65 homozygous diploid with a multi-copy plasmid encoding the CAA-decoding tRNA significantly reduced pseudohyphal growth (Fig. 5B). These experiments suggested that pseudohyphal growth was being caused by the inefficient translation of CAG glutamine codons.

tRNACUG levels in sup70 pseudohyphal mutant strains are markedly reduced in comparison with wild-type

The evidence presented so far suggested that the sup70-65 tRNA might in some way be compromised in its ability to decode CAG codons, perhaps because its stability or charging level were affected by the mutation in the anticodon stem. We therefore used alkaline acrylamide denaturing gels, and Northern blot analysis to examine overall levels of tRNACUGGln as well as its levels of glutamine charging. The more abundant isoacceptor tRNAUUGGln as well as the unrelated tRNAAsw were analysed as control tRNAs, the latter also being used to normalize tRNACUGGln charging levels between samples (Fig. 6).

For reliable Northern blot analysis of both yeast glutaminyl tRNAs, it was important to first verify that the oligonucleotide probes used did not cross-react, since the nucleotide sequences of tRNACUGGln and tRNAUUGGln differ by only four nucleotides, including the anticodon difference. Accordingly, we overexpressed the genes encoding

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Fig. 5. Complementation of the pseudohyphal growth defect by additional copies of tRNAGlnCUG or tRNAGlnUUG.

A. Strains MLD17 and MLD14 were transformed with either a control multi-copy vector (2μ), a single-copy vector carrying the wild-type SUP70 gene (pSUP70), a single-copy vector carrying the sup70-65 allele (psup70-65) or a multi-copy vector psup70-65 [2μ] carrying the sup70-65 allele.

B. Strains MLD17 (SUP70/SUP70) and MLD14 (sup70-65/sup70-65) were transformed with either a control multi-copy vector or a multi-copy vector pTRNAUUGGln carrying the tRNACUGGln gene tQ(UUG). Pseudohyphal growth was quantified using the chain formation index, in triplicate independent transformant cultures. Error bars represent ± 1 standard deviation. Significance was calculated using a Student’s t-test (*P = 0.05, **P = 0.025, ***P = 0.005).
tRNAGlnCUG and tRNAGlnUUG in strain MLD17, and using probes specific for two of the four nucleotide differences, showed clearly that when tRNAGlnCUG was overexpressed, no additional tRNAGlnCUG was detected by the tRNAGlnUUG probe. The converse was also true, indicating the probes were specific under the conditions used (Fig. 6A, lanes 5–8).

Using these two probes and one for tRNAHis, Northern blot analysis was conducted. The results showed that tRNAHis and tRNAGlnCUG species existed predominantly in the charged form in both wild-type and mutant strains, and could be deacylated using alkaline conditions to produce a marked tRNA band-shift (Fig. 6A, lanes 1–4). However, detection of tRNAGlnCUG revealed that in the sup70-65 mutant, but not in the wild-type, probing for the mutant tRNA consistently detected a diffuse smear that was of much lower intensity than in the wild-type strain (Fig. 6A, lanes 3 and 4, top panel). Within this smear, 3–4 more faint bands could be weakly discerned, although this was blot dependent. Following deacylation, two bands were still detectable, indicating at least two separately migrating species of different mass. These results indicated that the sup70-65 mutation had rendered the mutant tRNAGlnCUG less abundant, and judged by the difficulty in resolving it as a single band on a denaturing gel, possibly degraded and existing in multiple forms.

Quantification of this Northern blot analysis, together with additional Northern blot slots of the mutant MLD14 (sup70-65/sup70-65) and MLD17 (SUP70/SUP70) strains (data not shown) revealed that in the mutant, charging levels are slightly reduced from 67% in the wild-type MLD17 strain, to 50% in the sup70-65 mutant (Fig. 6B). However, importantly, total tRNAGlnCUG levels in the mutant are reduced to 25% of that of wild-type MLD17 (Fig. 6C). Another sup70-65 strain, LMD651, and strain LMD6533LU, carrying an allele sup70-33 that is also known to produce uncontrolled pseudohyphal growth (Murray et al., 1998) were also analysed by Northern blot and again found to contain greatly reduced concentrations of tRNAGlnCUG relative to the control tRNAGlnUUG (Supplementary Fig. S2).

The reduced stability and charging of the mutant tRNA may be caused simply by resultant instability of the anticodon (sup70-65) or acceptor (sup70-33) stems, possibly enhanced by altered patterns of tRNA nucleoside modification. The combination of reduced overall tRNA level and reduced charging would reduce the overall level of charged tRNA available for CAG decoding in the sup70-65 mutant to 19% of wild-type (Fig. 6D), likely to have a significant effect on CAG codon translation.

The sup70-65 mutant exhibits reduced expression of reporters containing tandem CAG codons

The Northern blot analysis presented in this work suggests the sup70-65 mutant may be significantly compromised in its ability to efficiently translate CAG codons. We therefore wanted to test whether the introduction of additional tandem CAG codons at the 5’ end of the firefly
Fig. 7. Slow translation of CAG, but not CAA, codons in a sup70-65 tRNA mutant background.

A. Plasmid-borne luciferase genes were engineered to contain either 5 or 10 tandem copies of the CAA codon at the 5′ end of the ORF (5 × CAA, 10 × CAA). Counterpart plasmids express luciferase engineered with similar CAG codon arrays (5 × CAG, 10 × CAG).

B. Tandem CAA- or tandem CAG-luciferase constructs were transformed into IS700 derivative yeast strains (ΔSUP70/ΔSUP70). IS700 transformants were supported with plasmid pSUP70 (encoding tRNA_{CUG}, single-copy plasmid), pSUP70-2q (encoding tRNA_{CUG}, multi-copy plasmid) or pSUP70-2q (encoding tRNA_{CUG}, single-copy CEN plasmid). Luciferase enzyme levels were normalized using qRT-PCR measurement of luciferase mRNA, (ACT1 qRT-PCR used as loading control). Normalized luciferase levels were expressed relative to the 5 × CAA construct value for the wild-type strain. Error bars represent ± 1 standard error (n = 5). Significance was calculated using a Student’s t-test (*P = 0.05, **P = 0.025, ***P ≤ 0.005).

C. Ribosome flux along the tandem CAA or CAG constructs was simulated in the different tRNA backgrounds, using a dynamic TASEP model of translation elongation, responsive to individual tRNA concentrations. Luciferase production rates were normalized relative to the 5 × CAA construct value for the wild-type strain. Error bars represent ± 1 standard error (n = 1000 simulations). D–F. In further simulations, the luciferase production rate in the sup70-65 mutant was determined in response to different tRNA_{CUG}^{dsn} concentrations in the 5 × CAA (circles) and 5 × CAG variants (squares; D); the vertical dotted line represents the level of tRNA_{CUG}^{dsn} measured experimentally in the sup70-65 mutant background. The response of luciferase expression to different numbers of CAG codons in the tandem repeats in the sup70-65 background was also simulated (E). The ribosome density in the first 50 codons of luciferase was then recorded during simulations of translation in the sup70-65 mutant using either the 5 × CAA construct (dashed line) or the 5 × CAG construct (solid line).

Luciferase ORF would reduce luciferase expression. We reasoned that slow CAG codon translation would cause extensive ribosomal queuing in the 5′ untranslated region back to the 5′ end, negatively impacting upon recruitment of ribosomal subunits by the 5′ cap structure. Less firefly luciferase would therefore be expressed.

We accordingly constructed variants of firefly luciferase, with either 5 or 10 tandem copies of CAG codons at the 5′ end of the reporter ORF, and as a control, additional luciferase variants with 5 or 10 tandem CAA codons at their 5′ end (Fig. 7A). Luciferase expression was measured in five independent cultures, along with expression levels of luciferase mRNA using qRT-PCR, the latter used to normalize luciferase activity levels. Three different ΔSUP70 knockdown strains were employed, one supported by a wild-type SUP70 gene on a centromeric vector, one carrying a multi-copy SUP70 plasmid, and one carrying the sup70-65 gene on a CEN vector.

To inform this experimental investigation and aid the analysis of the results, we also employed a recently developed mathematical model of the translation elongation process, responsive to codon-specific translation rates caused by differing tRNA abundances (Ciandrini et al., 2010). Using this we simulated the translation of firefly luciferase with either 5 or 10 tandem CAA or CAG codons introduced at the 5′ end of the ORF, and using a median translation initiation rate typical for yeast (0.1 s⁻¹), derived from a genome-wide application of the model (L. Ciandrini, I. Stansfield, M.C. Romano, unpublished work). The modelling of translation in the strain carrying the sup70-65 gene was achieved by reducing the simulated levels of tRNA_{CUG} abundance fivefold according to the results of the Northern analysis (Fig. 6), and that of the multi-copy SUP70 strain by increasing simulated tRNA_{CUG}^{dsn} abundance 15-fold, in the typical range for a 2μ vector. The model predictions could then be directly compared with experimentally measured luciferase expression levels.

The results show clearly that tandem CAG codons at the 5′ end of luciferase reduce its expression in a wild-type cell significantly, by approximately 20%, as expected given the ninefold lower abundance of the CAG-decoding tRNA relative to the CAA-decoder tRNA_{UUG} (Fig. 7B). This was true whether 5 or 10 tandem CAG codons were incorporated. This result was however also predicted by the model simulation of translation (Fig. 7C), indicating that even 5 CAG codons at the 5′ end of an ORF are sufficient to cause significant queuing, which impacts upon ribosomal recruitment. The modelling also supported the experimental observation that queuing could not be further enhanced by the introduction of additional CAG codons.

This reduced expression should, we predicted, be repurposable by overexpressing the SUP70 gene, boosting the copy number of tRNA_{CUG}^{dsn} and reducing or eliminating ribosomal queuing spanning the 5′ end of ORF and 5′UTR regions. Indeed this was the case; although luciferase expression overall was slightly reduced in this strain relative to the wild-type, nevertheless the 10 × CAG-luciferase expression levels were almost restored to those of 10 × CAA-luciferase when the yeast was transformed with a multi-copy SUP70 plasmid. Again, this experimental result was confirmed in the model simulation (Fig. 7B and C). Importantly, although expression of the 5 × CAA and 10 × CAA controls in a sup70-65 background was little different from that in the wild-type strain, multiple tandem CAG codons in the mutant caused an even more marked inhibitory effect than was apparent in a wild-type cell, reducing luciferase expression to 40% of the control, corresponding CAA constructs (Fig. 7B). Simulating the reduced abundance of sup70-65 tRNA using the mathematical model also predicted a similar reduction in luciferase expression, to 40% of control (Fig. 7C).

Several observations warranted further explanation. First, we noted that the correspondence between the amount of active tRNA_{CUG}^{dsn} available for CAG decoding in the sup70-65 mutant (20% of wild-type; Fig. 6), and the consequential effect on expression of the (CAG)n-luc reporters (approximately 40% of the corresponding CAA construct; Fig. 7B) was non-proportionate. We argued the
Fold-change in luciferase expression

Model prediction: fold-change in luciferase expression

Fold concentration of tRNA_{CUG}^{Gln} relative to wild type

Number of CAG codons in tandem repeats

Luciferase expression rate
reason for this must lie in the dynamics of the ribosomal queues forming at CAG codons. What causes inhibition of translation is not the number of inhibitory codons per se, but rather whether a queue of ribosomes forms, and stretches back to the 5′ mRNA cap to inhibit the joining of new ribosomes. The extent of the queue that forms is a product of a delicate balance between the rate of translation initiation, defining how fast ribosomes are joining the queue, and the rate at which ribosomes bypass the run of slow codons and leave the queue. Added to this, how fast ribosomes translate through a CAG array is difficult to predict simply on the basis of tRNA_{CUG} concentration; while a ribosome x at a CAG codon is stalled by another downstream ribosome y also at a CAG codon and waiting to encounter a tRNA_{CUG}^Gln the more 5′ ribosome x may in fact encounter the correct tRNA first and thus become ‘unblocked’. Inhibition of translation by tandem codon arrays is a complex function of array size, tRNA concentration and distance from 5′ cap to array.

Using the computer model, we simulated the effect of a range of tRNA_{CUG} concentrations on luciferase production from the 5 × CAA and 5 × CAG constructs, and first confirmed that as measured experimentally, 5 × CAA luciferase expression was essentially unresponsive to changes in tRNA_{CUG} abundance. 5 × CAA luciferase expression was in fact only affected when tRNA_{CUG} was reduced to extremely low levels, significantly below the 0.2-fold wild-type level of tRNA_{CUG}^Gln (indicated by the dotted line) experimentally measured in the sup70-65.homozygous diploid used in this work formed a body of evidence that strongly suggested the contrary; that sup70-65 mutation represents an important example of how mutations in single-copy tRNAs can regulate specific phenotypes in eukaryote cells.

Discussion

Transfer RNA molecules play a key role in delivering amino acids from the cytoplasmic pool to the polysomes engaged in mRNA translation. Such a central process has obvious potential to control the flux of ribosomes on the mRNA, and thus, protein expression. However, with some notable exceptions such as the Streptomyces bldA tRNA mutants, the study of how gene expression can be regulated via tRNA effects on translation elongation has been somewhat neglected. To address this, in this work we have undertaken a detailed characterization of the mechanism behind the observation that certain mutants of the SUP70 gene, encoding yeast tRNA_{CUG}^Gln, can promote growth of the organism in pseudohyphal-like chains of cells (Murray et al., 1998). The original report identifying this novel link between the translational apparatus and a developmental process regulating cell shape successfully excluded some possible molecular mechanisms – for instance, showing tRNA_{CUG} effects are independent of the STE signalling pathway – but did not identify the molecular mechanism, speculating that the tRNA_{CUG} may have a non-translational signalling role (Murray et al., 1998).

Here we assemble a body of evidence that strongly supports the contrary; that sup70-65 mutant tRNA_{CUG} is defective in its ability to efficiently translate the CAG codon, and that the sup70-65 mutation represents an important example of how mutations in single-copy tRNAs can regulate specific phenotypes in eukaryote cells.

Early on in this investigation we determined that the pseudohyphal growth phenotype triggered by the sup70-65 tRNA mutation is atypical. The MLD14 homozygous sup70-65 diploid used in this work formed pseudohyphae constitutively in nitrogen-replete, rich liquid medium, but not on solid medium. This is quite distinct from the phenotype exhibited by the Σ1278b strain background, which forms foraging projections of cell chains that radiate outwards from colonies on nitrogen-limited solid SLAD medium (Gimeno et al., 1992).
live cell microscopy monitoring, our work showed that sup70-65 mutants form chains of cells via bipolar budding, and not in a unipolar manner as described for Σ1278b pseudohyphae. Although further characterization of the sup70-65 phenotype was beyond the scope of this study, it appears as if the chains of cells formed by the tRNA mutant may be more typical of the fusel alcohol-triggered pseudohyphae signalled via the Swe1 morphogenesis checkpoint, since fusel alcohols also produce pseudohyphal growth on complex liquid media (Dickinson, 1996; 2008; Martinez-Anaya et al., 2003). This would explain why there was no evidence for STE pathway involvement in sup70-65 chain formation (Murray et al., 1998).

One key finding of this work was the discovery that relative to a wild-type cell, tRNA<sub>CUG</sub> in the sup70-65 mutant is present at much reduced abundance (fivefold less), probably arising through instability of the mutant form of the tRNA (Fig. 6). Moreover, the pseudohyphal growth phenotype can be complemented by overexpression of tRNA<sub>UUG</sub> (Fig. 5), which is known to be able to inefficiently decode the CAG codon, albeit inefficiently (Johansson et al., 2008). This hints strongly that CAG codons are being decoded slowly in the sup70-65 mutant, simply because this already rare tRNA<sub>CUG</sub> is even further depleted. Further support for this hypothesis is provided by our observation that the pseudohyphal growth phenotype is even more marked (exhibiting larger chain formation indices) in a homozygous SUP70 delete diploid carrying a single plasmid borne gene copy of sup70-65, clear evidence of haplo-insufficiency (Fig. 3C).

In order to test the above conclusion more directly, we used reporter genes engineered to contain additional CAA or CAG codons; consistent with the hypothesis of slowed CAG translation, we show first that introduction of multiple, tandem CAG codons at the 5′ end of a firefly luciferase reporter ORF causes significant, 20%, decreases in expression level of the reporter, even in a wild-type cell. This is simply because the CAG- decoding tRNA is ninefold less abundant than CAA-decoding tRNA, inducing ribosomal queues at the 5′ end of the mRNA. Other researchers have also demonstrated that the introduction of multiple tandem CAG codons into the 5′ end of the luciferase ORF resulted in reduced translational expression in wild-type <i>S. cerevisiae</i> (Letzring et al., 2010). When the experiment was repeated in a sup70-65 mutant, expression of the CAG-engineered reporter was further reduced by 60% relative to the level achieved in a CAA-containing control construct (Fig. 7). A stochastic model of ribosomal dynamics with a single-codon resolution (Ciandrini et al., 2010) (L. Ciandrini, I. Stansfield and M.C. Romano, unpubl. work) has been used to simulate the translation of the engineered constructs. The results of the <i>in silico</i> translation are strongly consistent with the experimental results (Fig. 7C), and support the hypothesis that slow CAG decoding is the main cause of reduced expression of the codon-engineered luciferase genes in the sup70-65 mutant.

Curiously, in the original publication describing the properties of the sup70-65 tRNA, Murray et al. engineered a lacZ reporter gene to contain extra tandem CAG copies, and reported no difference in β-galactosidase expression between sup70-65 mutant and wild-type yeast (Murray et al., 1998). We are unable to explain this difference between their results and ours, but one possibility might lie in the other group’s choice of lacZ as a reporter. lacZ, a significantly longer ORF of over 1000 codons, already contains 43 CAG codons, representing a significant CAG queue threat, and this might have masked the compromised CAG codon translation we report here. In contrast, firefly luciferase only contains seven CAG codons and so is a more sensitive reporter with which to investigate CAG codon translation. In addition, these researchers showed that a hybrid tRNA comprising the backbone of the tRNA<sub>UUG</sub>, engineered to have a CUG anticodon, could suppress the pseudohyphal growth phenotype, and concluded that the CUG anticodon was a critical component for the (translation-independent) signal to the N-starvation pathway (Murray et al., 1998). This result is however entirely explicable by our data, because creation of such a hybrid tRNA will of course restore efficient CAG translation and thus complement the pseudohyphal growth phenotype.

Murray et al. second argue that the sup70-65 tRNA is unlikely to signal to the N-starvation pathway via compromised CAG translation, since the mutant tRNA is an efficient suppressor of the trp1-1 allele and must therefore be translationally competent (Murray et al., 1998). We too confirm that the sup70-65 tRNA<sub>CUG</sub> is an amber suppressor, despite its reduced abundance (Fig. 3), and we also agree that CAG translation must function at some level in the sup70-65 mutant, simply because SUP70 is an essential gene. However we argue that because nonsense suppression is a dominant phenotype, commitment to colony growth by a trp1-1 mutant yeast could result from very efficient suppression by a very small population of tRNAs. Thus the CAG translational inefficiency we demonstrate in this study is not necessarily incompatible with nonsense suppressor ability. Indeed, a related study of <i>E. coli</i> tRNA<sup>30th</sup> mutants found some with <i>3 x 10<sup>2</sup></i>-fold reduced specificity constant for the <i>E. coli</i> glutaminy l tRNA synthetase. They were thus poorly charged, but were nevertheless efficient amber suppressors (Jahn et al., 1991).

Since sup70-65 tRNA is both a trigger for pseudohyphal growth and an amber codon suppressor, we generated several novel nonsense suppressor tRNA<sup>30th</sup><sub>CUG</sub> alleles in an attempt to isolate novel pseudohyphal growth mutants. All but one of the mutations were located directly in either
the anticodon loop or the anticodon stem, identifying those tRNA nucleotides that regulate first base wobble (Fig. 4A). These results confirm a much earlier study describing an E. coli tRNA<sup>TM</sup> mutated to recognize a Gln CAG codon. In that paper, amber codon suppression via first base wobble could be enhanced by a range of anticodon stem mutations, many of which destabilized the host tRNA (Schultz and Yarus, 1994). The Schultz and Yarus study, and the results presented in this article, suggest that tRNA anticodons have been selected to minimize first base wobble. In our work, the screen also resulted in the independent re-isolation of the sup70-65 mutant, confirming its previously documented nonsense suppression phenotype (Murray et al., 1998), as well as an anticodon mutant (H2) directly cognate for the UAG stop codon. However in a ΔSUP70 background, none of the novel mutants triggered pseudohyphal growth. It seems as if generally distorting the anticodon stem or loop, while necessary to induce nonsense suppression, is not in itself sufficient to trigger pseudohyphal growth. sup70-65 is therefore unusual in exhibiting a combined phenotype, its mutation altering anticodon presentation and thus first base wobble. In addition this mutation also more generally reduces tRNA<sub>UGG</sub> abundance, probably through loss of tRNA stability as indicated by the Northern blot analysis (Fig. 6).

In this study, we have provided strong evidence to suggest that the pseudohyphal phenotype triggered by sup70-65 is caused directly by a translational defect. We show first that sup70-65 tRNA is unstable and poorly charged. Knowing that the sup70-65 pseudohyphal growth phenotype is genetically recessive and therefore caused by loss of function, there is a clear link drawn between that phenotype and at the molecular level, a loss of the known function of tRNA in translation that we show here. Using the reporter CAG-engineered luciferase, we show that the unstable, defective tRNA is unable to translate CAG codons as efficiently as wild-type. Finally, we show that the pseudohyphal growth phenotype can be partly complemented by overexpression of tRNA<sub>UGG</sub><sup>Δin</sup>, known to weakly decode CAG codons (Johansson et al., 2008). Taken together, the evidence invites a very obvious conclusion; that reduced tRNA<sub>UGG</sub><sup>Δin</sup> abundance causes slower than normal translation of CAG codons, which in some way, signals pseudohyphal growth. The exact mechanism is however unclear; in unpublished work we have showed, for example, that sup70-65 mutants do not exhibit an altered unfolded protein response, known to play a role in pseudohyphal growth signals (Schröder et al., 2000); Murray et al. exclude STE pathway signaling in their earlier work (Murray et al., 1998), and defects in the nitrogen catabolite response in sup70-65 mutants were also excluded (Beeser and Cooper, 1999). We speculate instead that the sup70-65 mutation alters the efficiency of translation of the mRNA encoding a negative regulator of pseudohyphal growth, which we predict would contain key CAG codons towards the 5′ end of its open reading frame. Slower than normal translation of these codons would generate ribosomal queues on this and other mRNAs in the mutant tRNA background, compromising the ability of that mRNA to sequester ribosomes and thus reducing the translational expression of the putative negative regulator. In addition to Streptomyces bldA mutants, there are other precedents for similar mechanisms of tRNA regulation of gene expression. In yeast, the Elp complex is a conserved protein assembly responsible for U34 wobble position tRNA modifications such as mc<sup>5</sup> (Huang et al., 2005). Elp mutants show defects in the DNA damage response and telomeric silencing, but these defects can be complemented by overexpressing the tRNA targets of the Elp modifications, indicating translational regulation of telomeric silencing via the degree of tRNA modification (Chen et al., 2011). In fission yeast, the Elp complex activity appears to control the cell cycle through translational control of the protein kinase cdr2 expression, via lysine codon usage within the cdr2 ORF (Bauer et al., 2012). Thus specific alterations in the tRNA milieu, although exerting global effects on the translation apparatus, can nevertheless have defined phenotypic consequences for the expression of particular groups of genes, dependent upon codon content. Further work is ongoing in our laboratory to identify <i>S. cerevisiae</i> CAG-rich genes whose expression is SUP70-regulated.

**Experimental procedures**

<i>S. cerevisiae</i> strains and growth conditions

<i>Saccharomyces cerevisiae</i> strains MLD17 (MAT<sup>a</sup>/MAT<sup>a</sup> trp1-1/trp1-1 ura3-52/ura3-52 his3-11/his3-11 ade1/ade1), MLD15 (MAT<sup>a</sup>/MAT<sup>a</sup> sup70-65/SUP70 trpl-1/trp1-1 ura3-52/ura3-52 leu2-3112/LEU2 his3-11/his3-11 ade1/1ADE1), MLD14 (MAT<sup>a</sup>/MAT<sup>a</sup> sup70-65/SUP70 trpl-1/trp1-1 ura3-52/ura3-52 leu2-3112/LEU2 his3-11/his3-11 ade1/1ADE1), LMDWU (MAT<sup>a</sup>/MAT<sup>a</sup> SUP70/SUP70 trp1-1/trp1-1 leu2-3112/LEU2 ade1/1ADE1), LMD651U (MAT<sup>a</sup>/MAT<sup>a</sup> SUP70/SUP70 trp1-1/trp1-1 leu2-3112/LEU2 ade1/1ADE1), LMD653LU (MAT<sup>a</sup>/MAT<sup>a</sup> sup70-65/SUP70 trpl-1/trp1-1 leu2-3112/LEU2 ade1/1ADE1), LMD653LU (MAT<sup>a</sup>/MAT<sup>a</sup> sup70-65/SUP70 trpl-1/trp1-1 leu2-3112/LEU2 ade1/1ADE1) and LMD653LU (MAT<sup>a</sup>/MAT<sup>a</sup> sup70-65/SUP70 trpl-1/trp1-1 leu2-3112/LEU2 ade1/1ADE1) were used to investigate the causes of the pseudohyphal growth phenotype. These strains were provided by Dr L. Murray and Prof. R.A. Singer (Dalhousie University, Halifax, Canada). Strain BY4743 (MAT<sup>a</sup>/MAT<sup>a</sup> his3<sup>Δ1</sup>/his3<sup>Δ1</sup> leu2<sup>Δ0</sup>/leu2<sup>Δ0</sup> lys2<sup>Δ0</sup>/lys2<sup>Δ0</sup> met15<sup>Δ0</sup>/MET15 ura3<sup>Δ0</sup>/ura3<sup>Δ0</sup>) was employed as a wild-type S288C-derivative strain.

A sup70-65 knockout diploid strain MLD1 ΔSUP70 (MAT<sup>a</sup>/MAT<sup>a</sup> sup70::kanMX::MAT<sup>a</sup>/MAT<sup>a</sup> ade1/ade1 his3<sup>Δ1</sup>/his3<sup>Δ1</sup> trpl-1/trp1-1 ura3-52/ura3-52 [PAK01]) supported by a plasmid-borne copy of SUP70 was generated as follows. Using primers sup70-S1 and sup70-S2 (Table S1), heterozygous knockouts of the SUP70 gene were created in
BY4743 with either the kanMX (Wach et al., 1994) or natMX (Goldstein and McCusker, 1999) cassettes using the short-flanking homology method (generating strains BY4743 sup70::kanMX and BY4743 sup70::natMX). Using primers preS1 (situated in the upstream URA10 gene) and postS2 (hybridizing within the downstream SCS7 gene; Table S1), a kanMX cassette with SUP70-homologous long flanking regions was amplified from strains BY4743 sup70::kanMX genomic DNA and transformed into strain MLD17. Plasmid pAK01 (URA3, CEN) carrying the SUP70 allele was then transformed into the MLD17 sup70 heterozygous disruptant. Finally, primers preS1 and postS2 were used to amplify a natMX disruption cassette with SUP70 long flanking regions using BY4743 sup70::natMX DNA as a template; this was transformed into the MLD17 heterozygous sup70 diploid strain to generate strain MLD17 ΔSUP70. Antibiotic resistance, resistance to 5-fluoroorotic acid and diagnostic PCR were all used to verify successful deletion of both SUP70 gene copies.

A second SUP70 homozygous knockout strain supported by a plasmid-borne copy of SUP70 was generated using strain BY4743. One allele of SUP70 was deleted using a SUP70-kanMX knockout cassette as described above and support plasmid pAK01 transformed into the strain. The diploid strain was then sporulated. Two spores were selected that had geneticin-resistance, and 5-fluoroorotic acid resistance to 5-fluoroorotic acid and diagnostic PCR were all used to verify successful deletion of both SUP70 gene copies.

Supplementary Table S1. Plasmids pSUP70 (HIS3, CEN) carrying SUP70, pSUP70-33 (HIS3, CEN) carrying sup70-33 and pSUP70-65 (HIS3, CEN) carrying sup70-65 were created by PCR-amplifying the relevant SUP70 allele using primers sup70-S3 and sup70-S4 (Table S1), situated approximately 190 nt 5' and 160 nt 3' respectively from the SUP70 IRNA sequence, and cloning into pRS413 (Christianson et al., 1992) cut with NotI. Plasmids pAK01 (URA3, CEN) carrying the SUP70 gene, pSUP70-2µ (HIS3, 2µ multi-copy) carrying SUP70 and pSUP70-65-2µ (HIS3, 2µ multi-copy) carrying sup70-65 were created by amplifying the relevant cloned SUP70 allele with primers pRS-forward and pRS-reverse (Table S1) and using in vivo homologous recombination in yeast to repair Nott-gapped pRS416 or pRS423.

Plasmids pJR7 (LEU2, CEN) in-frame UAG stop codon (pAC98-PDE2; Williams et al., 2004); pJR7 (LEU2, CEN) is identical to pJR5 but with insertional stop codon of lacZ and luc genes replaced by a glutamine CAG codon. Plasmids p5 × CAA-luc, p10 × CAA-luc, p5 × CAG-luc and p10 × CAG-luc represent yeast shuttle vectors (TRP1 CEN) that encode constitutively expressed firefly luciferase with tandem 5, or 10, tandem CAA, or CAG codons immediately after the cumulative sum curve. An exemplar plot of the cumulative sum showing these areas is shown in Fig. S1.

The S_L value for a given mutant was then expressed as a proportion of the corresponding wild-type S_L to produce a chain formation index, or CFI, allowing quantitative comparison of the chain formation capability.

\[ CFI = \frac{S_L \text{ (mutant)}}{S_L \text{ (wild type)}} \]

Plasmids

Primers used in plasmid construction are itemized in Table S1. Plasmids pSUP70 (HIS3, CEN) carrying SUP70, pSUP70-33 (HIS3, CEN) carrying sup70-33 and pSUP70-65 (HIS3, CEN) carrying sup70-65 were created by PCR-amplifying the relevant SUP70 allele using primers sup70-S3 and sup70-S4 (Table S1), situated approximately 190 nt 5' and 160 nt 3' respectively from the SUP70 IRNA sequence, and cloning into pRS413 (Christianson et al., 1992) cut with NotI. Plasmids pAK01 (URA3, CEN) carrying the SUP70 gene, pSUP70-2µ (HIS3, 2µ multi-copy) carrying SUP70 and pSUP70-65-2µ (HIS3, 2µ multi-copy) carrying sup70-65 were created by amplifying the relevant cloned SUP70 allele with primers pRS-forward and pRS-reverse (Table S1) and using in vivo homologous recombination in yeast to repair Nott-gapped pRS416 or pRS423.
downstream of the AUG translation initiation codon. They were created using a homologous recombination gap repair strategy in which a 5’ segment of firefly luciferase was amplified using a forward primer carrying a 5’ overhang encoding the tandem CAA or CAG codons. Using these PCR fragments, YCplac22-FL1 (TRP1 CEN fluc: Oliveira et al., 1993; Oliveira and McCarthy, 1995) cut with NdeI was gap-repaired using homologous recombination in yeast. For each PCR, the same 3’ primer was employed (lucR), partnered with either 5’ primer 5CAA-luc-F, 10CAA-luc-F, 5CAG-luc-F or 10CAG-luc-F.

**Generation of a mutant sup70 library**

The sup70 mutant library was generated by PCR-amplifying the SUP70 wild-type gene and approximately 400 nt of flanking vector sequence using primers pRS-forward and pRS-reverse and plasmid pSUP70 as a template (Table S1). The PCR reaction was made error-prone using non-equivalent concentrations of the dNTPs in the PCR reaction (0.144 mM dGTP, 0.144 mM dATP, 0.7 mM dTTP, 0.7 mM dCTP), combined with 0.3 mM MnCl2. Using both treatments ensured that a reasonable frequency of mutagenesis would be achieved within a small (72 nt) mutagenic target. The amplified, mutagenized library of sup70 fragments was co-transformed into MLD17 (trp1-1) with NotI-gapped pRS413. The vector sequences flanking the tRNA gene directed homologous recombination, and thus gap repair in vivo of pRS413, creating a mutagenized tRNA library.

**Stop codon readthrough assays**

To quantify readthrough of the UAG stop codon, yeast strains were transformed with either pJR5 or the control vector pJR7, which express β-galactosidase-luciferase fusion proteins. Dicistronic assays for stop codon readthrough were performed essentially as described (Forbes et al., 2007) with further modifications as detailed (Rato et al., 2011).

**tRNA preparation and RNA blots**

tRNA preparation was performed according to standard protocols (Hill and Struhl, 1986), with some modifications (Varshney et al., 1991). All tRNA preparations were stored in sodium acetate buffer (pH 4.6) at –80°C until used, except those that were to be deacylated, which were ethanol precipitated, washed with 70% ethanol then resuspended in 0.2 mM Tris-acetate, pH 9.0 and incubated for 1 h at 37°C. Charged and uncharged tRNAs were resolved electrophoretically on 40 cm denaturing 10% acrylamide gel [1 M sodium acetate (pH 4.8), 10% of 40% acrylamide/bis solution (19:1), 8 M urea]. Using semi-dry blotting, tRNAs within a 20 cm lower part of the gel was then transferred onto Amersham Hybond-N membrane at 8 V, 400 mA for 1 h, and fixed to the membrane using ultra-violet irradiation (120 mJ cm–2). For quantification of total tRNA, 100 pmol of tRNA preparation was slot-blotted onto Amersham Hybond-N membrane using a hybridization manifold and then immediately UV-cross-linked as above.

The probes to the respective tRNAs were end-labelled using polyadenylate kinase and standard methods (Sambrook and Russell, 2001), probes purified using G-25 spin columns, and hybridized to the blot membrane using standard conditions at 42°C (Sambrook and Russell, 2001). tRNA_UAG was detected using a 1:1 mix of SUP70 probe (5’-ttg ttc gga tca gaa cc-3’) and sup70-65 probe (5’-ttg ttc gga tca gaa tc-3’). tRNA_CUA was detected using the probe 5’-ttg ttc gga tca aac cc-3’, and tRNA_CUA was detected using the probe 5’-ttt cat cgg cca cca cg-3’. Washing was carried out using sequential 15 min. treatments with wash solution I (5 × SSC, 0.1% SDS), II (1 × SSC, 0.5% SDS) and III (0.1 × SSC, 1% SDS). The blot was exposed for 24–72 h to a phosphoimager screen and hybridization quantified using a Fuji FLA-3000 phosphoimager and Aida/2D v 2.0 densitometry software.

**Luciferase assays and qRT-PCR quantification of luciferase mRNA**

Luciferase assays were performed on lysates of five independent yeast cell cultures grown on SD medium until an optical cell density (600 nm) of 0.8 had been reached. Assays were performed using the Bright-Glo luciferase assay kit (Promega). qRT-PCR was carried out on five independent mRNA samples (Rneasy, Qiagen) following cDNA synthesis using Quantitect reverse transcriptase (Qiagen). Then, using SYBR Green methodology in a PCR reaction (LightCycler 480 SYBR Green I Master, Roche) using a Roche LightCycler 480 RT-PCR machine, the following were performed; using primers FLuc-f and FLuc-r (Table S1), amplification of a fragment of luciferase; in a parallel reaction, using primers ACT1-f and ACT1-r (Table S1), amplification of a fragment of the ACT1 cDNA as an internal loading control.

**Mathematical modelling of translation**

An extended Totally Asymmetric Simple Exclusion Process (TASEP) model of translation that had been previously developed (Ciandrini et al., 2010) was used to simulate translation on any given transcript. The stochastic model describes the kinetics of ribosomes on mRNAs, mimicking their bio- and mechanochemical cycle with a two-state dynamics. Yeast codon translation rates were estimated from data on tRNA abundances, assumed to be proportional to their gene copy numbers (Percudani et al., 1997) and adjusted to consider further corrections such as the wobble base pairing (L. Ciandrini, I. Stansfield and M.C. Romano, unpubl. work).

For each simulation, 1000 iterations were run, and the mean and standard error values for ribosomal density and translational efficiency (ribosomal ‘current’) were recorded. The model is freely available from the authors.

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Supporting information

Additional supporting information may be found in the online version of this article.