Nucleic Acids Research, 2024, **52**, 3989–4001
https://doi.org/10.1093/nar/gkae053
Advance access publication date: 10 February 2024
RNA and RNA-protein complexes

An evolutionarily conserved phosphoserine-arginine salt bridge in the interface between ribosomal proteins uS4 and uS5 regulates translational accuracy in *Saccharomyces cerevisiae*

Kartikeya Joshi, Brooke Luisi, Grant Wunderlin, Sima Saleh, Anna Lilly, Temiloluwa Okusolubo and Philip J. Farabaugh *

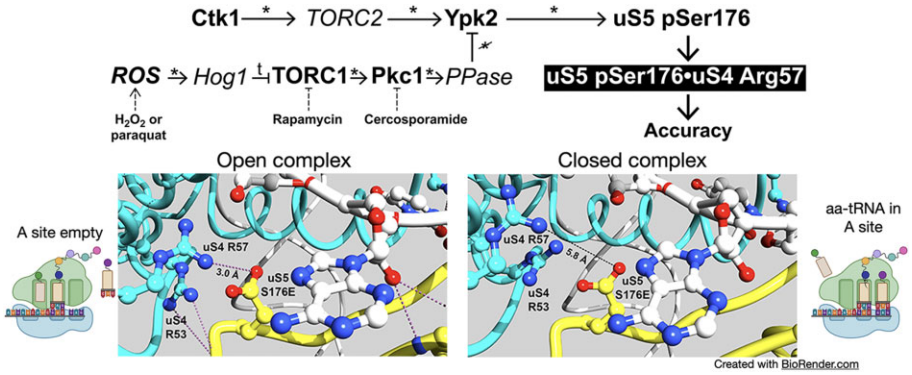
Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore 21250, USA

*To whom correspondence should be addressed. Tel: +1 410 455 3018; Fax: +1 410 455 3875; Email: farabaug@umbc.edu
Present addresses:
Kartikeya Joshi, AstraZeneca, Waltham, MA 02451, USA.
Brooke Luisi, The Johns Hopkins University Applied Physics Laboratory, Laurel, MD 20723, USA.
Anna Lilly, Fox Chase Cancer Center, Philadelphia, PA 19111, USA.
Temiloluwa Okusolubo, Laboratory of Sickle Cell Genetics and Pathophysiology, National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892, USA.

Abstract

Protein-protein and protein–rRNA interactions at the interface between ribosomal proteins uS4 and uS5 are thought to maintain the accuracy of protein synthesis by increasing selection of cognate aminoacyl-tRNAs. Selection involves a major conformational change—domain closure—that stabilizes aminoacyl-tRNA in the ribosomal acceptor (A) site. This has been thought a constitutive function of the ribosome ensuring consistent accuracy. Recently, the *Saccharomyces cerevisiae* Ctk1 cyclin-dependent kinase was demonstrated to ensure translational accuracy and Ser238 of uS5 proposed as its target. Surprisingly, Ser238 is outside the uS4-uS5 interface and no obvious mechanism has been proposed to explain its role. We show that the true target of Ctk1 regulation is another uS5 residue, Ser176, which lies in the interface opposite to Arg57 of uS4. Based on site specific mutagenesis, we propose that phospho-Ser176 forms a salt bridge with Arg57, which should increase selectivity by strengthening the interface. Genetic data show that Ctk1 regulates accuracy indirectly; the data suggest that the kinase Ypk2 directly phosphorylates Ser176. A second kinase pathway involving TORC1 and Pkc1 can inhibit this effect. The level of accuracy appears to depend on competitive action of these two pathways to regulate the level of Ser176 phosphorylation.

Graphical abstract



Introduction

Protein synthesis is under strong selection to maximize protein yield under all physiological conditions so that ribosome abundance or activity is regulated to match the translation capacity of the cell to the demand for protein synthesis. This regulation reflects the energy intensive nature of translation (1,2). This demand to maximize protein yield competes with a second demand to maintain translational accuracy (3). Misreading errors can replace correct amino acids during protein synthesis with alternatives that disrupt protein function, especially for those in active sites (4), or that cause misfolding of completed protein chains (5,6). Because of the potentially disruptive effect of inaccurate translation, cells employ a proteostasis network that maintains optimal accuracy of synthe-

Received: August 22, 2023. **Revised:** January 8, 2024. **Editorial Decision:** January 9, 2024. **Accepted:** February 8, 2024

sis, folding and degradation of misfolded proteins (7). Failure of this process in humans at any of these stages could lead to protein aggregation, which can result in neurodegenerative and neuromuscular disorders, or other diseases (8).

The question of how the accuracy of protein synthesis can be maintained arose very early in the study of ribosomes. The earliest mutants with altered translational accuracy expressed mutated 40S ribosomal proteins in bacteria (reviewed in 9). Hyperaccurate ribosomal '*res*' mutants in *E. coli*, identified by resistance to the error-inducing aminoglycoside antibiotic streptomycin, carry alterations in ribosomal protein uS12 (10). Error prone ribosomal ambiguity, '*ram*', mutants in *E. coli*, identified by suppressing the streptomycin dependence of some *rpsL* mutants, were originally identified in ribosomal protein uS4 (11) and later in uS5 (12). Similar accuracy mutants have been identified in uS4 and uS5 in *Saccharomyces cerevisiae* (13). The identification of mutations affecting ribosomal components demonstrated the ribosome has an intrinsic role in ensuring translational accuracy. A fundamental aspect of the function of the uS4, uS5 and uS12 proteins is the formation or disruption of protein-protein and protein-RNA interfaces during the structural transition of 'domain closure' when a cognate aminoacyl-tRNA (aa-tRNA) is accepted in the ribosomal A site (14). Domain closure involves rotation of the head domain of the small subunit toward the subunit interface and this movement both disrupts contacts at the uS4•uS5 interface and creates contacts between uS12 and two rRNA helices (h27 and h44) (14). Canonical error prone uS4 and uS5 mutants alter residues in the uS4•uS5 interface and the hyperaccurate uS12 mutants alter its contacts with the rRNA emphasizing the importance of the two interfaces in modulating accuracy (14). Zaher and Green (15) showed that error prone uS4 and hyperaccurate uS12 proteins alter accuracy in mechanistically distinct ways. Ribosomes with error-prone uS4 proteins decrease the rate of dissociation of near-cognate aa-tRNAs during the selection step whereas hyperaccurate uS12 proteins increase the rate of near-cognate aa-tRNA dissociation during the later proofreading step. This mechanistic difference implies that breakage of the two interfaces occur at distinct steps in the pathway to tRNA acceptance before and after irreversible GTP hydrolysis on elongation factor Tu (16).

Röther and Strässer (16) demonstrated a translational accuracy regulating role for the transcriptional cyclin dependent kinase Ctk1, identified for its role in transcriptional elongation (17), showing that Ctk1 associates with *S. cerevisiae* cytoplasmic ribosomes and its absence (*ctk1Δ*) increases sensitivity to translation inhibitors and increased translational errors measured *in vitro*. Ctk1 phosphorylated ribosomal protein uS5 *in vitro* at a putative target, uS5 Serine 238 (Ser238), which they identified as responsible for the Ctk1 accuracy effects. The idea that loss of phosphorylation of uS5 results in an error-prone phenotype is consistent with the role of uS5 as a canonical error modulating ribosomal protein, but the location of Ser238 far from the interface with uS4 implies that a putative mechanism of increased error could not involve destabilizing that uS4•uS5 interface. A Ser to Ala mutation blocking that Ser238 phosphorylation (*rps2-S238A*) increased *in vitro* misreading errors about 25%, seven-fold less than the *ctk1Δ* strain (16), which suggested to us that phosphorylation of other targets might be required to further increase accuracy.

Cells commonly use protein phosphorylation to rapidly and reversibly regulate translational efficiency in response to changes in cell physiology, such as the availability of nutrients (18) or stress conditions (19). Examples include well-characterized kinases Gcn2 and related proteins (20), target of rapamycin complex 1 (TORC1) (21) and ribosomal protein eS6 kinase (S6K) (22), which corresponds to Ypk3 in *S. cerevisiae* (23). Phosphorylation of translation factors can directly reduce or increase their intrinsic activity or indirectly regulate their association with inhibitory factors (24,25). The role of Ctk1 is different from these other kinases in that it controls translational accuracy rather than protein output, implying the possibility of physiological control of accuracy through Ctk1.

Here we present data confirming that Ctk1 controls translational accuracy in *S. cerevisiae* measured *in vivo*. It does so, however, through a different target in uS5, Ser176. Our data support a model whereby phosphorylation of uS5 Ser176 within the uS4•uS5 interface results in creation of a salt bridge to a nearby residue, uS4 Arg57. The increased stability of the interface due to the salt bridge can explain increased translational accuracy since it would more strongly constrain the acceptance of cognate compared with near-cognate aa-tRNAs. Widespread conservation of necessary sequence motifs suggest that this molecular mechanism could function widely among eukaryotic species including fungi and metazoans. We identify an opposite role for the kinases TORC1 (26) and Pkc1 (27), which in the absence of Ctk1 enforce error-prone translation. Both the Ctk1 and TORC1/Pkc1 pathways function through a downstream AGC kinase, Ypk2 (28), a distant relative of Ypk3 (29), which is the putative direct kinase of uS5 Ser176.

Materials and methods

Strains and growth conditions

The *E. coli* strain used in this study for cloning and plasmid propagation is in DH5α (F⁻ Φ80lac*ZΔM15* Δ(lac*ZYA*-arg*F*)*U169* rec*A1* end*A1* hsd*R17* pho*A* sup*E44* λ– thi-*1* gyr*A96* rel*A1*) (30). The *S. cerevisiae* strains used are derivatives of BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) (31) and are listed in Supplementary Table S1. Yeast strains were grown either on Yeast peptone dextrose (YPD) media (Difco) or, when nutritional selection was required, on Yeast minimal media (SD) (1.7 g yeast nitrogen base w/o amino acids, 5 g ammonium sulphate, 2% glucose) supplemented with any nutritional requirements, as described (32). Yeast transformation was carried out using a lithium acetate high efficiency transformation protocol (33). Where indicated, cells were treated with various inhibitors of the kinases TORC1 (rapamycin, 2.7 nM) or protein kinase C (cercosporamide, 1.5 μM) or the inducers of oxidative stress, paraquat (750 μM) or hydrogen peroxide (H_2O_2, 1.5 mM).

In vivo reporter assays of translational accuracy

To determine the frequency of misreading errors *in vivo* we exploited a reporter plasmid system in which *E. coli lacZ* reporter gene expresses β-galactosidase. Mutant forms of the codon specifying the essential active site amino acid Glu 537 of β-galactosidase is replaced by any of several single nucleotide missense mutations. Misreading error frequencies for these codon mutants is calculated as value of the enzyme activity expressed from the mutant gene divided by the activity expressed from wild type gene in the same genetic background. This ratio for most Glu 537 mutants is about 10^{-6}; this is the background level of the assay system. Proteins expressed from other mutant genes have activities 10–100-fold higher. These

higher activities result from misreading of the mutant codon to insert the wild type Glu amino acid and the activities are proportional to the frequency of misreading error frequency (34,35). β-galactosidase protein assays were performed on yeast strains transformed with reporter plasmids and grown in selective minimal medium to an OD_{600} of 0.8−1.0. Transformant cells expressing wild-type β-galactosidase were diluted for assay 1000-fold compared to mutants and assayed to quantify β-galactosidase activity using the Promega β-Glo system according to manufacturer's specification, using 96-well LUMITRAC plates (Greiner Bio One). Activities in Relative Light Units were measured using a Modulus II Microplate Multimode Reader (Turner BioSystems) according to manufacturer's directions. Assays were performed on an average of six biological repeats (minimum of three, maximum of 24) of each strain tested. Each biological repeat was assayed in triplicate technical repeats. The data reported is the mean of all the data for each strain with error bars representing the standard error of the mean. The significance of any differences in activity between matched congenic strains was assessed by Student's two-tailed *t*-test assuming equal variance. In cases where many samples were tested against a single control, which can result in overprediction of significance, we used the Bonferroni correction to compensate for the repeated tests. We did not find evidence of false positive results.

Plasmid constructions and chromosomal gene editing

To analyze the role of protein–protein interactions between ribosomal proteins in translational accuracy, missense mutations were introduced by a variation on one-step gene disruption (36). A list of oligonucleotides used to introduce mutations into structural genes and target gene disruption are given in Supplementary Table S2. The method takes advantage of a set of plasmids carrying drug resistance cassettes: pFA6A-KanMX (resistance to 200 μg/ml G418), pFA6a-NatMX6 (resistance to 100 μg/ml nourseothricin), pFA6a-HphMX6 (resistance to 200 μg/ml hygromycin) and pFA6a-BleMX6 (resistance to 50 μg/ml phleomycin) (37). To introduce a missense mutation into a chromosomal gene, the mutation of choice was first introduced by QuickChange mutagenesis (Agilent). The mutated structural gene was amplified by PCR and inserted between the BamHI (5′ end) and PacI (3′ end) sites of the relevant pFA6a plasmid placing the drug resistance gene downstream of the structural gene in the intergenic region. A PCR fragment derived from this construct encompassing the structural gene, resistance gene and a sequence downstream directing integration into the intergenic region transformed into yeast inserts the drug resistance gene into the target gene, replacing varying amounts of the structural gene. The target gene region from candidate clones were PCR polymerized from total yeast DNA (38) and sequenced to demonstrate the presence of the resistance gene and to identify those carrying the site-directed mutation. The frequency of correct clones declined with its distance from the 3′ end of the gene. In all cases, a transformant carrying the resistance gene downstream of a wild type structural gene was used as a control to test the effect of the introduced mutation.

A deletion of the ribosomal protein gene *RPS9A* was created by inserting one of the *HphMX6* drug resistance gene in place of the chromosomal gene using one-step gene disruption (36). The desired resistance cassette was amplified from pFA6a-HphMX6 plasmid using oligonucleotides having a 20nt region complementarity adjacent to the resistance cassette at their 3′ end and 40 or 45nt of the region immediately flanking the target yeast gene at their 5′ end; because the complementarity between *RPS9A* and *RPS9B* ends in the coding region, the transformation targets only *RPS9A*. The PCR fragment was used to transform desired yeast strains, identifying transformants by the resulting drug resistance. The mutations were verified by direct sequencing of a PCR fragment derived from the gene region. The resulting mutations replace the coding region of the targeted gene with the drug resistance cassette flanked by 73 bp upstream and 50 bp of downstream of the MX6 region.

Structural analysis and modeling using UCSF chimera

Structural models of *S. cerevisiae* 40S ribosome subunits were derived using publicly available data at the Protein Data Bank at the Research Collaboratory for Structural Bioinformatics PDB (39). Structural models were created using UCSF Chimera (40). To model the effect of altering amino acids, we exploited the structure editing tool Rotamers, which predicts all likely conformations adopted by the replacing amino acid, testing for possible structural clashes and H-bonds among the conformers. The conformers were chosen to avoid clashes while forming H-bonds or salt bridges that conform to predictions based on *in vivo* accuracy effects. Where no H-bond or salt bridge is predicted among conformers that lack clashes, we show the conformer predicted to have the highest probability of formation.

Results

Lack of Ctk1 kinase increases *in vivo* errors at error-prone codons

We have created systems to quantitatively measure the *in vivo* frequency of misreading in *Saccharomyces cerevisiae* by individual tRNAs (4,34,35,41,42). One such system (42) exploits an active site amino acid in the reporter protein β-galactosidase of *E. coli*, Glu537, the major nucleophile in the cleavage reaction digesting lactose to glucose and galactose (43). Altering an important active site amino acid like Glu537 can drastically reduce enzyme activity (34,42) but misreading of the mutant codons by a tRNA encoding the wild type amino acid generates a protein with full enzymatic activity. These misreading events are rare, and their frequency is proportional to the observed enzyme activity relative to that of wild type protein. Enzyme assays of proteins expressed from such mutant reporters provide highly reproducible and quantitative measures of misreading *in vivo*. Most single nucleotide mutants affecting Glu537 have activity no higher than a background of 2×10^{-6} times wild type. However, four mutants produce 10 to 100 times this background activity, indicating that they are misread much more frequently (34,42). Among these error-prone mutants, the mutant replacing GAA (Glu) with GGA (Gly) has the highest activity. We have demonstrated that this increased activity reflects translational misreading of the mutant codon by $tRNA^{Glu}$ (34,42) and have exploited this system to assess *in vivo* misreading frequency in this study.

To assess the utility of this *in vivo* error reporter system we tested several reported error modulating systems. One of

these was the regulation of translational errors in response to the Ctk1 kinase, originally identified as phosphorylating the C-terminal domain of the largest subunit of RNA polymerase II during transcription (17,44,45). Röther and Strässer (16) showed that it also associates with *S. cerevisiae* cytoplasmic ribosomes and its absence (*ctk1Δ*) increases sensitivity to translation inhibitors. They demonstrated that *in vitro*, Ctk1 phosphorylates ribosomal protein uS5 (encoded by *RPS2*) on Serine 238 (Ser238). A Ser to Ala mutation blocking that phosphorylation (*rps2-S238A*) increased *in vitro* misreading errors about 25% (16). This error frequency is seven-fold less than the *ctk1Δ* strain suggesting that there might be other targets of Ctk1 required to further increase accuracy.

We confirmed that the lack of the Ctk1 kinase (*ctk1::KanMX*) increased misreading in the four error-prone mutants of Glu537 relative to wild type (Figure 1). Errors increased the most (3.7-fold) for the GGA mutant but activity also increased from 2.0 to 2.9-fold for other error-prone β-galactosidase mutants (34). By contrast, the activities of the ten non-error prone β-galactosidase mutants were at the background level of the assay from extracts of wild type and *ctk1::KanMX* mutant background (Supplementary Figure S1). These data show that measurable translational errors are stimulated by the absence of Ctk1. The magnitude of the accuracy effect is equivalent to that of canonical error-prone mutations of ribosomal protein uS4, which we previously found to be 3.0-fold on average, or treatment with the error-inducing drug paromomycin, 2.7-fold on average (34).

Ctk1-dependent translational accuracy requires only the Ser176 of ribosomal protein uS5

To determine whether uS5 Ser238 was required for Ctk1-dependent accuracy, we tested the effect on accuracy of a Ser to Ala mutation in position 238 (*rps2-S238A*), which replaces the Serine with a non-phosphorylatable Alanine. As shown in Figure 2 and Supplementary Table S3, the Glu537 β-galactosidase reporter in presence of either the *rps2-S238A* mutant or *RPS2*$^+$ wild type allele produced normal levels of activity in *CTK1*$^+$ background, and in the absence of Ctk1 (*ctk1::KanMX*) we observed elevated activity indicative of increased error frequency. These data demonstrate that the high accuracy seen in the *CTK1*$^+$ background does not require Ser238. This result is inconsistent with Ser238 being the sole phosphorylation target involved in Ctk1-dependent accuracy.

Ribosomal protein uS5 is phosphorylated at other serines, including Ser176 and Ser181 (46,47), both of which, unlike Ser238, lie in the uS4●uS5 interface. Ser238 and Ser181 both match the minimal consensus for a cyclin dependent kinase site, S/T-P (48), and Ser176 matches a protein kinase A site, R-$X_{1\text{-}2}$-S (49). We performed the same accuracy test in strains carrying chromosomal Ser to Ala mutations of either site (*rps2-S176A* or *rps2-S181A*) in either a *CTK1*$^+$ or *ctk1::KanMX* background. The β-galactosidase activity in the *CTK1*$^+$ *rps2-S176A* background was significantly higher than *CTK1*$^+$ *RPS2*$^+$ ($P < 9 \times 10^{-5}$), and the activity was the same in the *ctk1::KanMX rps2-S176A* mutant strain whereas the activities of the *rps2-S181A* mutants were indistinguishable from the corresponding *RPS2*$^+$ mutants in either *CTK1*$^+$ or *ctk1::KanMX* backgrounds (Figure 2 and Supplementary Table S3). Because the normal high accuracy in the *CTK1*$^+$ background is eliminated when Ser176 is replaced by Ala but not Ser181 or Ser238, we conclude that Ser176 is necessary for the Ctk1 accuracy effect, consistent with Ser176 being a target of Ctk1. The *rps2-S176A* mutant phenocopies *ctk1::KanMX* showing that the lack of the putative Ser176 phosphorylation is sufficient to eliminate Ctk1-dependent accuracy. It is therefore unnecessary to posit other targets of Ctk1 to regulate missense accuracy.

To exclude the possibility of any regulatory crosstalk between the three serines we constructed Ser to Ala double mutants of Ser176 and either Ser181 or Ser238 and the triple mutant, either in the *CTK1*$^+$ or *ctk1::KanMX* backgrounds. Each of the strains had activities indistinguishable from either the *CTK1*$^+$ *rps2-S176A* or the *ctk1::KanMX RPS2*$^+$ mutant strains with one exception (Figure 2 and Supplementary Table S3). The activity of the triple mutant in the *ctk1::KanMX* background was statistically significantly higher ($P = 0.02$) suggesting that the presence of either Ser181 or Ser238 might slightly increase accuracy in the absence of Ser176. Because the statistical significance of this result is marginal, and the magnitude of the effect is rather small the effect may not be physiologically relevant. Overall, these data suggest that Ser176 is essential for the normal level of accuracy, but the Ser238 and Ser181 show no appreciable role in maintaining accuracy.

If Ser176 were the sole target of Ctk1 kinase, a phosphomimetic Ser to Glu mutant (S176E) should have an accurate phenotype even in the absence of Ctk1. We constructed three Ser to Glu mutations (S176E, S181E and S238E) in the *ctk1::KanMX* background and tested their effects singly or in combination. As expected, the *ctk1::KanMX rps2-S176E* mutation conferred a hyperaccurate phenotype, twice as accurate as the *CTK1*$^+$ *RPS2*$^+$ control (Figure 3 and Supplementary Table S3). The activities of *ctk1::KanMX rps2-S181E* and *ctk1::KanMX rps2-S238E* were not statistically different from the *ctk1::KanMX RPS2*$^+$, suggesting that phosphorylation at these sites does not contribute significantly to translational accuracy.

In summary, we find that uS5 Ser176, but not Ser238 or Ser181, is required for Ctk1-dependent accuracy suggesting that Ser176 is the actual target of Ctk1 on uS5. The accuracy of the *rps2-S176A* and *ctk1::KanMX* strains being identical implies that Ctk1-dependent accuracy requires no other target in uS5 or in any other of the over 200 direct or indirect targets of Ctk1 kinase (50).

Evidence for a salt bridge between phosphorylated uS5 Ser176 and Arg57 of uS4.

It is highly significant that the proposed target of Ctk1-regulated phosphorylation resides in the interface between ribosomal proteins uS4 and uS5. This interface was identified as contributing to translational accuracy; uS4 and uS5 mutants targeting residues in a close interface between these two proteins can alter the frequency of translational misreading (9,15). A model to explain this effect derived from comparison of the bacterial 'open' ribosomal conformation before recruitment of an aminoacyl-tRNAs to the ribosomal A site (51) to the 'closed' conformation adopted when an aminoacyl-tRNA has been successfully recruited to the A site (52). This comparison showed the uS4–uS5 interface dissociates during this transition, which was interpreted as suggesting that loss of stabilizing energy of this interface favors normal accurate selection of cognate tRNAs (14). One model for the accu-

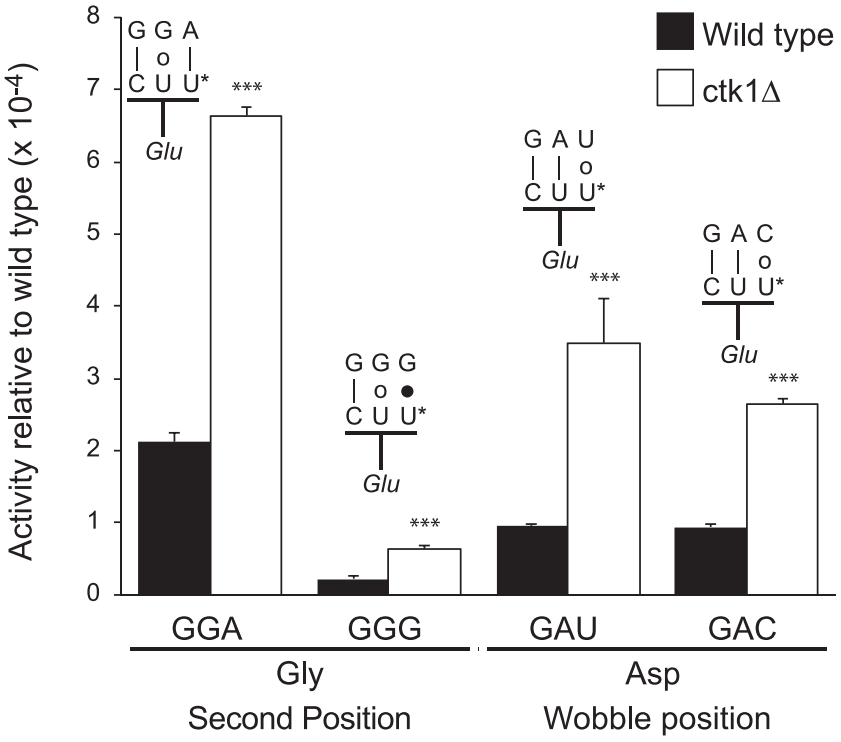


Figure 1. Loss of Ctk1 causes increased translational misreading errors *in vivo*. The relative activity of Glu537 mutants of β-galactosidase expressed in a *CTK1*$^+$ or *ctk1::KanMX* background. The relative activity is the ratio of activity of enzyme activity expressed from a gene with the indicated mutant form of codon 537 (e.g. GGA replacing GAA) to the activity expressed from a wild type gene (GAA codon), both forms expressed in the identical genetic background (*CTK1*$^+$ or *ctk1::KanMX*). Statistical significance of the effect of *ctk1::KanMX*, calculated using the Student's *t*-test to compare activities in the *ctk1::KanMX* to those in wild type (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001). For mutants showing a significant change the codon–anticodon complexes predicted for corresponding misreading events are shown (the upper line represents the codon, the lower the anticodon). Vertical lines represent Watson–Crick pairs, filled circles canonical wobble pairs and open circles non-Watson–Crick pairs that have been shown to mimic Watson–Crick geometry. Error bars show the standard error of the mean for the data.

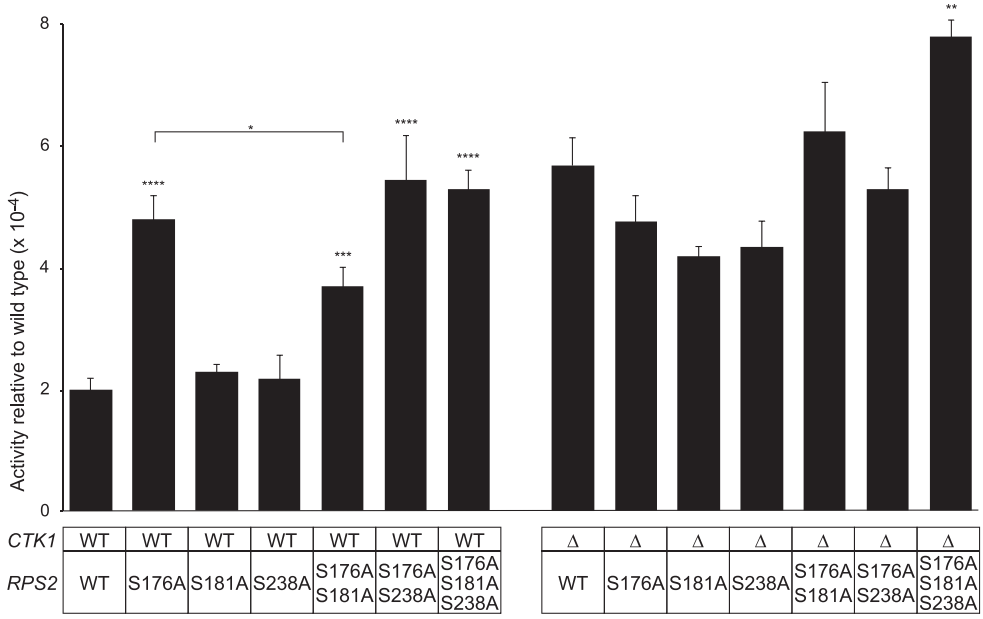


Figure 2. Ser176 but not Ser181 or Ser238 is necessary for Ctk1 dependent translational accuracy *in vivo*. The β-galactosidase activity expressed in single, double and triple Ser to Ala mutants of Ser176, Ser181 and Ser238 of uS5 (shown as in Figure 1). The values are shown for *CTK1*$^+$ strains (left) and *ctk1::KanMX* strains (right, labeled 'Δ'). Statistical significance of the *RPS2* mutations are indicated as in Figure 1 showing the significance compared to the *RPS2*$^+$ strain in each group, except for the comparison of *CTK1*$^+$ *rps2-S176A* to the *CTK1*$^+$ *rps2-S176A/S181A* double mutant.

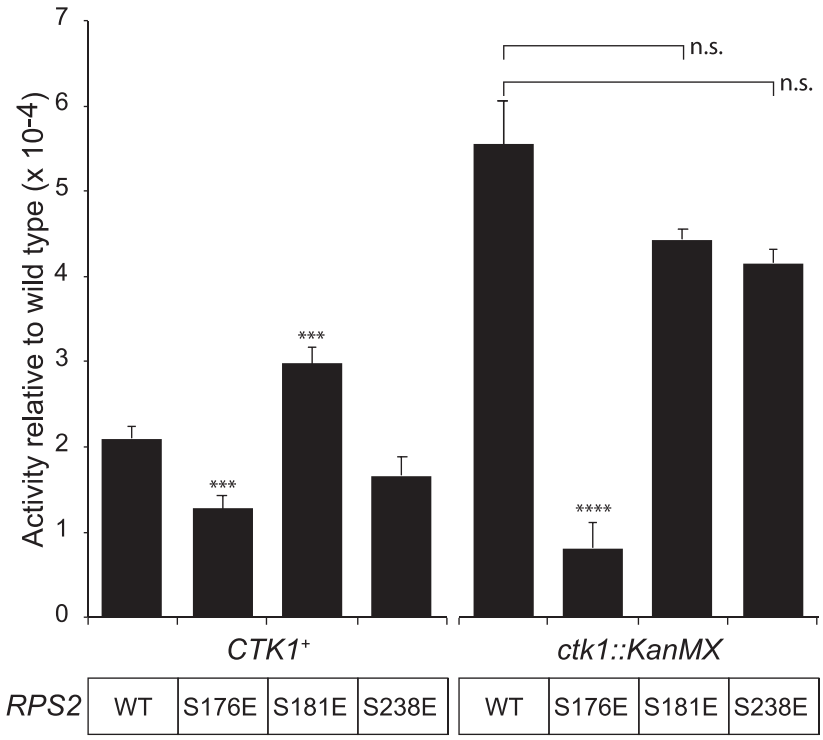


Figure 3. Phosphomimetic mutations demonstrate a role for phosphorylation of uS5 Ser176 in increasing translational accuracy. The β-galactosidase activity expressed in Ser to Glu mutants of Ser176, Ser181 and Ser238 of uS5 (shown as in Figure 1). Those values statistically different from the *CTK1⁺ RPS2⁺* wild type are indicated by asterisks, as in Figure 1, except where the lack of a significant difference is indicated the comparison of *ctk1::KanMX RPS2⁺* to *ctk1::KanMX rps2-S181E* or *ctk1::KanMX rps2-S238E*.

racy effect of phosphoryl-Ser176 is that it forms a salt bridge with one or more positively charged amino acids in uS4 (encoded by *RPS9A* and *RPS9B*). Loss of this additional stabilizing energy could be used to increase selectivity for correct aminoacyl-tRNAs (14). To test the credibility of this model, we modeled changing Ser176 to Glu using the molecular modeling program UCSF Chimera (53) and available structural data for the *S. cerevisiae* 80S ribosome lacking bound tRNAs in the decoding center (54), which corresponds to the bacterial 'open' complex. We identified a structurally favored conformation of Glu176 allowing a 3.0 Å salt bridge between the negatively charged O^{δ} of uS5 Glu176 and the N^{ζ} of uS4 Arg57 (Figure 4A). Also shown in the figure, no salt bridge is possible with uS4 Arg53 because it is both too distant (4.5Å) from uS5 Glu176 and instead is involved in an H-bond with the backbone carbonyl of uS5 Arg174. The length of the predicted bond between uS5 Glu176 and uS4 Arg57 (3.0 Å) and the angle of the bond (156°) are consistent with formation of a salt bridge (55). We further modeled the effect of exchanging the Glu and Arg between these two residues to create a charge inversion and found conformations allowing an equivalent flipped salt bridge (Figure 4B). We also modeled the formation of the salt bridge in a second structure with a tRNA in the P/E hybrid state but before elongation factor G-induced ratcheting, corresponding to the 'closed' complex. In this conformation, the distance between O^{δ} of uS5 Glu176 and N^{ζ} of uS4 Arg57 has increased by 3.0–5.8 Å (Figure 4C), which should either preclude formation of the salt bridge or greatly decrease the energy of interaction (56). The increased stabilizing energy of the putative salt bridge under this model would increase selectivity in the ribosomal A site.

To test the reality of these salt bridges, we generated mutants of uS5 Ser176, uS4 Arg53 and uS4 Arg57 that exchanged the Arg and Glu residues between uS4 and uS5. Because uS4 is encoded by duplicated genes *RPS9A/RPS9B*, these mutations were introduced into *RPS9B* in a strain in which *RPS9A* was removed by insertion of a drug resistance cassette, *HphMX6*, as described in Materials and Methods. Reporter enzyme activity was compared to a *RPS9B* wild type strain lacking *RPS9A*. We performed analysis in the *ctk1::KanMX* strain lacking Ctk1 to exclude confusion from any potential phosphorylation by Ctk1. Our analysis of the effect of the single or combined mutations uS5 Ser176Arg, uS4 Arg53Glu and uS4 Arg57Glu confirmed the predicted charge inversion effect (Figure 5). The *ctk1::KanMX* strains carrying these mutations singly all produced misreading levels indistinguishable from *ctk1::KanMX* background and very significantly increased from *CTK1⁺* wild type. This demonstrates that all three residues are necessary for maximal Ctk1-dependent accuracy. The uS5 Ser176Arg or uS4 Arg57Glu mutants would disrupt the predicted salt bridge and the uS4 Arg53Glu would eliminate the H-bond to the carbonyl of uS4 Arg54. Either of these effects could be expected to weaken the uS4•uS5 interface. However, a double mutation combining uS5 Ser176Arg and uS4 Arg57Glu produced enzyme activity indistinguishable from the *CTK1⁺* wild type, indicating that it restores accuracy to wild type levels, and supporting the existence of a single salt bridge between uS5 pSer176 and uS4 Arg57 that is required for the Ctk1-dependent accuracy phenotype.

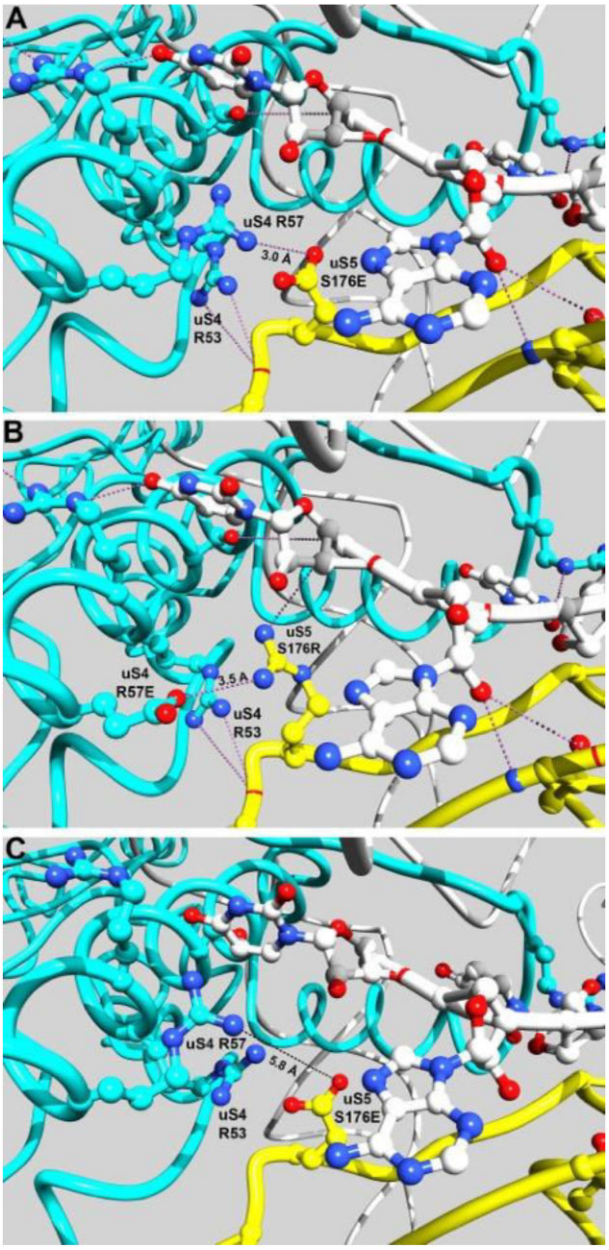


Figure 4. Molecular models of the uS4•uS5 interface. (**A**) A molecular model of the uS4•uS5 interface of an 80S ribosomal open complex involving the uS5 mutant protein in which the Glu replaces Ser176 showing a predicted salt bridge to uS4 Arg57 (pink dotted line). The length of the predicted bond is shown in Ångstroms. The ribbons represent uS4 (cyan), uS5 (yellow) and the 5′ end of 18S rRNA (white). All residues in the region shown thought to make intermolecular contacts are represented in ball and stick cartoon form. (**B**) A similar open complex model but including a charge reversal mutation involving uS5 Ser176Arg and uS4 Arg57Glu. (**C**) A model of a closed complex with cognate aminoacyl-tRNA in the ribosomal A site involving the uS5 Ser176Glu mutant. Note the bond between the Glu and Arg residues exceeds the maximum for a salt bridge.

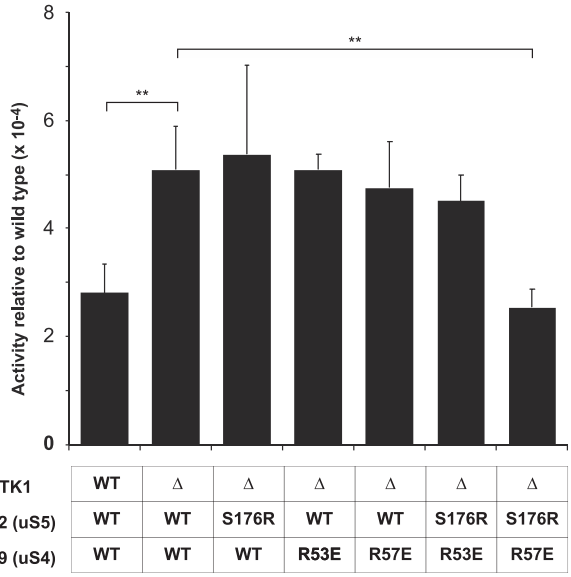


Figure 5. Genetic evidence for the putative uS4–uS5 salt bridge involving uS5 Ser176. The β-galactosidase activity of single and double mutants including uS5 Ser176Arg, uS4 Arg53Glu and uS4 Arg57Glu. The increased activity compared to wild type of the single mutants indicates that each is error prone. Wild type accuracy is restored by the double mutant of uS5 Ser176Arg with uS4 Arg57Glu but not with uS4 Arg53Glu (the significance of this effect shown as in Figure 1), supporting the salt bridge modeled in Figure 4B.

TORC1 and Pkc1 also control translational accuracy and the effect requires uS5 Ser176.

Our data clearly suggests that phosphorylation of uS5 Ser176 modulates translational accuracy. Phosphorylation, because it is reversible, implies physiological control and various lines of evidence suggest accuracy can be regulated. Misreading is known to be stimulated to cope with various physiological challenges (57–62) implying that the uS5–Ser176 system might modulate accuracy in response to some physiological change. Given the known connection between misreading and oxidative stress (58), we tested the effect of sub-lethal concentrations of paraquat (750 μM) or hydrogen peroxide (H_2O_2, 1.5 mM), both known to induce oxidative stress *in vivo* in yeast (63). Oxidative stress had no effect on the level of misreading of the E537G (GGA) mutant of β-galactosidase in either the *CTK1*$^+$ *RPS2*$^+$ or *CTK1*$^+$ *rps2-S176A* strains (Figure 6). However, the error-prone phenotype of *ctk1::KanMX* was reversed to normal accuracy by oxidative stress (Figure 6). In effect, oxidative stress bypasses the requirement for Ctk1 to maintain the normal level of accuracy. Importantly, oxidative stress failed to restore the accuracy of the *ctk1::KanMX rps2-S176A* strain, indicating that this restoration requires the putative phosphorylation target, Ser176. This is the first indication that a kinase other than Ctk1 might directly phosphorylate Ser176, suggesting that Ctk1 might control Ser176 phosphorylation indirectly.

Oxidative stress activates the MAP kinase Hog1 (64) which, in *S. cerevisiae*, inhibits TORC1 (26). The *S. cerevisiae* protein kinase C, Pkc1 (27), is a second part of this oxidative stress response pathway as a phosphorylation target of TORC1 (65). TORC1 and Pkc1 are known to stimulate translational efficiency by phosphorylating translation factors and ribosomal proteins (66,67), and Pkc1 binds to the small subunit ribosomal protein, RACK1 (68,69) near to the uS4–uS5 interface. These proteins make attractive candidates for parts of a pathway connecting oxidative stress to translational accuracy. Both TORC1 and Pkc1 are essential kinases in yeast but inhibitors are available to reduce their activity, rapamycin for TORC1 (70) and cercosporamide for Pkc1 (71). We used

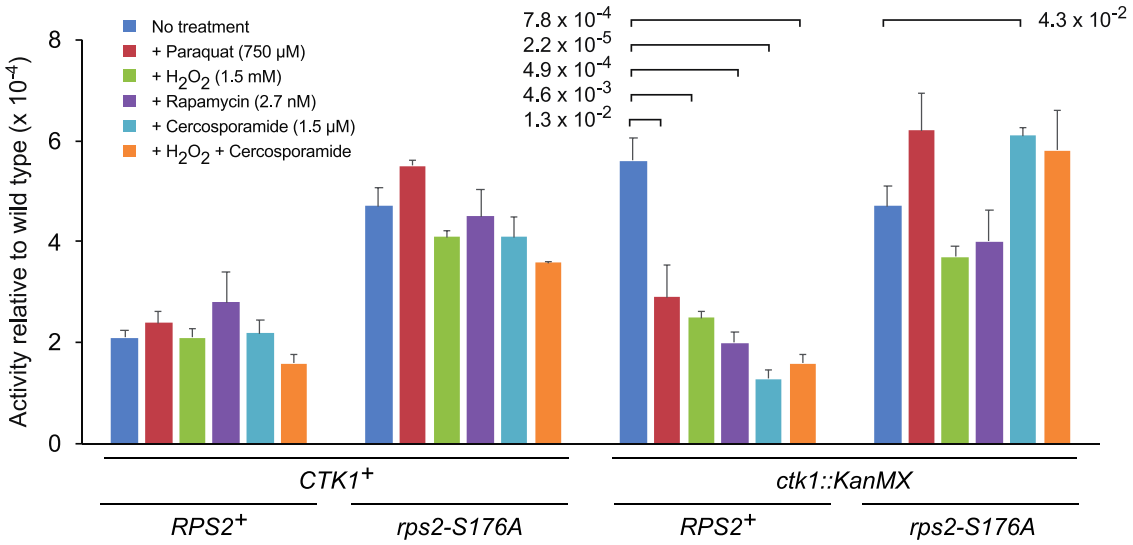


Figure 6. Oxidative stress or inactivation of TORC1 or Pkc1 induced increased translational accuracy depends on Ser176. The effect of exposure of wild type and *ctk1::KanMX* strains to oxidative stress or inhibitors of a TORC1/Pkc1 pathway. Cells were treated with concentrations of oxidative stress inducers (paraquat or H_2O_2) or inhibitors of TORC1 (rapamycin) or Pkc1 (cercosporamide) about 2-fold less than would induce a significant decrease in cell growth rate. The effect of these treatments relative to the untreated wild type control on the β-galactosidase Glu537 system is shown as in Figure 1.

these inhibitors to test the role of each enzyme on translational accuracy.

Exposure to sublethal concentrations of either rapamycin or cercosporamide had the same effect as oxidative stress, having no effect on misreading in either *CTK1*$^+$ *RPS2*$^+$, *CTK1*$^+$ *rps2-S176A* or *ctk1::KanMX rps2-S176A*, but causing a strong increase in accuracy in *ctk1::KanMX RPS2*$^+$ (Figure 6 and Supplementary Table S4). This indicates that the accuracy resulting from inactivating TORC1 by rapamycin or Pkc1 by cercosporamide requires Ser176 but not Ctk1. To determine if oxidative stress, TORC1 and Pkc1 all function in the same or parallel pathways, we combined treatments to look for additive or synergistic effects. In the *ctk1::KanMX RPS2*$^+$ strain treatment with both H_2O_2 and cercosporamide resulted in no significant difference from either single treatment (Figure 6 and Supplementary Table S4), suggesting that the treatments affect a common pathway. Our results are consistent with a Hog1-TORC1-Pkc1 kinase pathway that controls phosphorylation of uS5 Ser176. We found that Pkc1 inactivation by cercosporamide caused increased accuracy, consistent with increased phosphorylation of uS5, which suggests that Pkc1 cannot be the Ser176 kinase but instead that Pkc1 in some way blocks Ser176 phosphorylation.

Ypk2 kinase is downstream of the TORC1/Pkc1 kinase pathway and required for phosphorylation of uS5 Ser176

Any kinase downstream of the Ctk1 and TORC1/Pkc1 pathways should recognize the PKA consensus site motif of the Ser176 context. We used a candidate approach to identify the putative Ser176 kinase. Several PKA-related A or AGC kinases are known or suspected to be regulated by Tor kinase as part of TORC1 or TORC2 (reviewed by (72) including Tpk1/Tpk2/Tpk3, the redundant yeast protein kinase A (73), Sch9, yeast protein kinase B (74,75), Rim11, yeast glycogen synthase kinase 3β (76), and the closely related A kinases, Ypk3, the yeast ribosomal protein S6 kinase (23), and Ypk1/Ypk2, paralogous proteins similar to serum and glucocorticoid regulated kinase (Sgk-like) (28). Viable single null mutants of each of these kinases and a double mutant lacking both Ypk2 and Ypk3 were tested for an effect on accuracy *in vivo* using the E537G (GGA) reporter system. If any of these were the Ser176 kinase, the phenotype of a null mutation would be loss of phosphorylation, which would result in reduced accuracy.

As shown in Figure 7, loss of any of four of these kinases (Rim11, Sch9, Ste11 and Ypk1) showed no statistically significant effect on accuracy as compared with the wild type control. Mutants lacking three others (Tpk1, Tpk2 or Ypk3) showed statistically significant but small effects on accuracy. None of these mutants phenocopied the effect of the *ctk1::KanMX* mutant suggesting that Ctk1 does not exert its effect entirely through them. The only mutants with phenotypes indistinguishable from the *ctk1::KanMX* mutant were those lacking Ypk2 or the double mutant lacking Ypk2 and Ypk3. The effect of lack of Ypk2 was not significantly different than loss of both kinases, suggesting that Ypk2 is primarily responsible for uS5 Ser176 phosphorylation. If Ypk2 is the direct Ser176 kinase then lack of that kinase should eliminate the effect of the Hog1-TORC1-Pkc1 pathway. We tested this by exposing the ypk2Δ ypk3Δ mutant strain to rapamycin, finding that there was no significant difference in activity, as predicted (Supplementary Table S4). Also, if Ypk2 targets uS5 Ser176 then the error-prone phenotype caused by the lack of Ypk2 we would expect the phenotype to be reversed by the uS4-Ser176Glu phosphomimetic mutation. We introduced this phosphomimetic mutation into the ypk2Δ ypk3Δ background and found that the phenotype was reversed to as accurate as the wild type (Figure 7). These data suggest that Ypk2 functions downstream of Ctk1 in phosphorylating Ser176. Ctk1 is not known to phosphorylate Ypk2. TORC2, however, does activate Ypk2 by phosphorylating its C-terminal region (77) and Ctk1 interacts with the TORC2 subunit Tsc11 (78), the yeast homologue of mammalian Rictor (79). Rictor plays a role in substrate selectivity that can be blocked by phos-

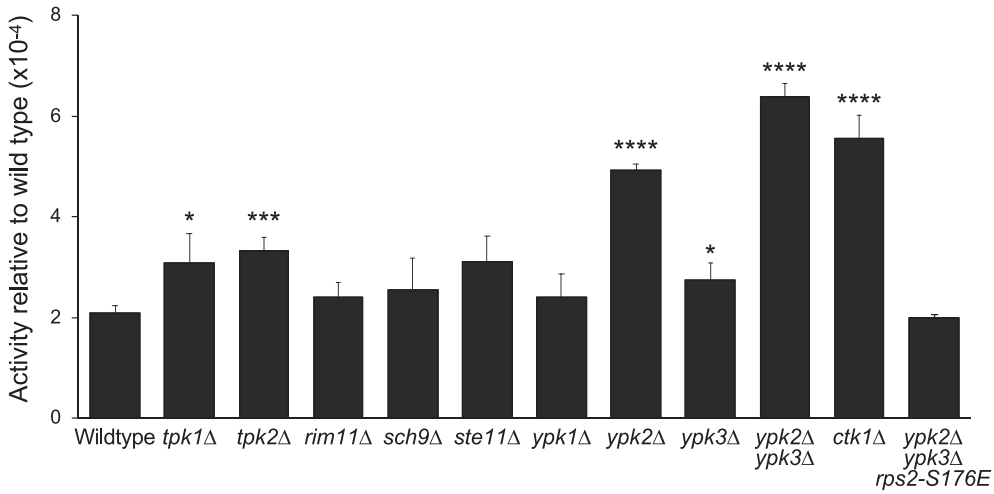


Figure 7. Only Ypk2 is required to maintain maximal accuracy. The effect of singly or multiply deleting each of ten kinases known or suspected to be regulated by a TORC1 pathway that are predicted to be specific for sites like that of uS5 Ser176. Note that the only mutants to phenocopy the loss of Ctk1 kinase involve the loss of Ypk2 alone or in combination with Ypk3 and that the *rps2-S176E* phosphomimetic mutant completely reverses the error phenotype of the *ypk2Δ ypk3Δ* strain. The effect of these mutations on the β-galactosidase Glu537 system and the statistical significance of the data are shown as in Figure 1.

phorylation (80). Therefore, a similar pathway from Ctk1 to TORC2 may activate Ypk2.

Discussion

We have confirmed that *S. cerevisiae* Ctk1, the catalytic subunit of RNA polymerase II C-terminal kinase, regulates translational accuracy by targeting phosphorylation of ribosomal protein uS5, encoded by *RPS2* (16). Contrary to previous work, we demonstrate that the regulation occurs by phosphorylation of uS5 Ser176. This conclusion is based, first, on a mutation replacing uS5 Ser176 with Ala mutant (*rps2-S176A*) completely phenocopying a *ctk1::KanMX* null mutation. Similar replacements of two other tested putative cyclin dependent kinase targets, Ser181 and Ser238, did not affect accuracy. Ser238 had previously been implicated as the targeted residue (16); the inability of the Ser181Ala mutant to phenocopy the loss of Ctk1 rules it out as the target of the regulation. From these data we conclude that Ser176 is necessary for the Ctk1 accuracy effect, and the others are dispensable. Second, we show that replacing Ser176 with a phosphomimetic amino acid, Glu, causes hyperaccuracy in the absence of Ctk1, but similar replacements of Ser181 and Ser238 do not. This shows that the presence of a negative charge in place of the polar Ser176 is sufficient for the Ctk1 accuracy effect. Third, the identification of uS5 Ser176 as the true target supports a molecular explanation of the accuracy effect. Ser176 is located at the interface between uS5 and uS4, the site of canonical translational accuracy mutations both in bacterial (11,12,81–83) and yeast (13) ribosomes. The strength of the interface interaction is thought to ensure the selection of correct cognate aminoacyl-tRNAs in the ribosomal A site (14,15). This suggests that phosphoserine at uS5 Ser176 might strengthen the uS4•uS5 interface to reduce misreading. Molecular modeling suggested that uS5 Ser176 is close enough to uS4 Arg57 to form a phosphoserine•arginine salt bridge. The existence of this interaction *in vivo* was supported by the fact that a double mutation exchanging the two charged residues, either of which singly causes an error prone phenotype, restores normal wild type levels of accuracy. This is most consistent with the double mutant recreating a charge reversed salt bridge that restores normal function.

The fact that normal accuracy requires phosphorylation dependent on Ctk1 implies physiological regulation of accuracy. There is evidence of the opposite response to stress in which, in the absence of active Ctk1, a second pathway regulating accuracy dependent on uS5 Ser176 phosphorylation, TORC1/PkC1, responds to reactive oxygen stress by inducing higher accuracy. Because inhibition of TORC1 or Pkc1 has the same effect of increasing accuracy in the absence of Ctk1, and there is no synergy between Pkc1 inhibition and oxidative stress, we presume that oxidative stress also inhibits the TORC1/Pkc1 pathway. Consistent with this idea, oxidative stress activates Hog1 MAP kinase which inhibits TORC1 activity (26) and in turn blocks downstream activation of protein kinase C, Pkc1 (27). We know that all these effects were eliminated in a uS5 Ser176Ala error prone mutant, showing that Ser176, and presumably its phosphorylation, is required for the accuracy phenotype. The sequence context of uS5 Ser176 is a consensus for an A kinase and not a cyclin dependent kinase like Ctk1. This and the fact that accuracy can be restored in the absence of Ctk1 by inactivating the TORC1/Pkc1 pathway implies the existence of one or more other kinases that directly phosphorylate Ser176. Our evidence implicates the A kinase Ypk2 in maintaining accuracy. The critical evidence is that Ypk2 genetically acts downstream of the two kinase pathways based on lack of Ypk2 reversing the accuracy phenotype either in the presence of Ctk1 or the absence of both Ctk1 and TORC1/Pkc1 pathways. We are investigating the link between these pathways and Ypk2. TORC2 can activate Ypk2 by phosphorylating its C-terminal region (77), which may stimulate Ypk2 to phosphorylate uS5 Ser176. We envision biochemical analysis necessary to demonstrate this pathway and genetic and biochemical analysis to clarify how Ctk1 may modulate the interaction.

The trans-acting factors that are required for this regulatory system are widely conserved among eukaryotic species but that does not mean that the system is also widely conserved.

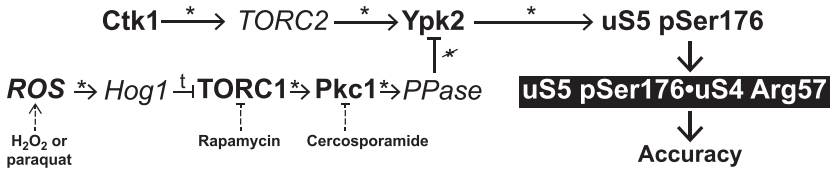


Figure 8. A proposed pathway to regulate phosphorylation of uS5 Ser176. We propose a provisional pathway to explain our data on the regulation of phosphorylation of uS5 Ser176. The bold, roman lettering indicates parts of the proposed pathway that we have demonstrated whereas the italic lettering indicates predicted intermediates in the pathway for which direct evidence has not been provided. Arrows indicate positive interactions and blunt arrows indicate negative interactions. For example, Ypk2 is proposed to promote phosphorylation of uS5 Ser176 and Hog1 is proposed to inhibit TORC1. The arrows should not be interpreted as indicating direct actions since intermediate steps may be missing. Asterisks indicate phosphorylation by a kinase (e.g. Ypk2 promoting phosphorylation of uS5 Ser176). A 't' on an arrow indicates transcriptional activation of the downstream target gene. Dotted arrows indicate drug or small molecule activation (arrow) or inhibition (blunt arrow) of the indicated protein.

To determine how phylogenetically widespread this regulatory system is we compared the primary sequences across the regions of uS4 and uS5 in the protein-protein interface to determine if the putative uS5 pSer176-uS4 Arg57 salt bridge mechanism could be phylogenetically conserved. As expected, these ribosomal proteins overall are highly conserved as are specific residues implicated in the regulation. Ser or Thr are widely present at uS5 positions 176, 181 and 238 across all eukaryotic taxa, although Ser176 is replaced by Ala in over half of plants (Supplementary Table S5). Similarly, the uS4 Arg57 implicated in the salt bridge with uS5 pSer176 is highly conserved in all eukaryotic taxa (Supplementary Table S6).

The argument for conservation of the system is weakened by the general conservation of these proteins across eukaryotes, but there is evidence of selection for loss of the system in specific taxa that indirectly supports its general conservation. In the subphylum Saccharomycotina (budding yeast), u5 Ser176 is widely present but replaced by Lys in five clades, creating a charge reversal from pSer; note this replacement in *Debaryomyces hansenii* and *Candida dubliniensis* in Supplementary Table S5. This replacement, similar to the uS5 Ser176Arg, is predicted to disrupt the proposed salt bridge. The largest of these clade is made up predominately by species in which CUG is frequently decoded both as Ser and as the canonical Leu in varying ratios, as was first discovered in *Candida albicans* (84). This 'CUG-Ser1 Clade' species are mainly pathogenic and exhibit Ser/Leu ambiguity of the canonical Leu codon CUG; this ambiguity in encoded proteins induces the general stress response, which has been proposed to be advantageous for life in the human body (85). The coincidence of CUG redefinition and the presumed error-prone mutation to Lys176 suggest inaccuracy resulting from loss of the uS5 pSer176–uS4 Arg57 salt bridge was selected to increase misreading error generally to aid in inducing the stress response important for survival of members of the clade. The Ser to Lys substitution in multiple budding yeast lineages and widespread substitution of Ala for Ser in plant lineages may reflect a selective loss of the accuracy system as well.

Röther and Strässer (16) showed that human Cdk9, a distant relative of yeast Ctk1, associates with the polysomes, consistent with a role in regulating translation; the closest homologue of human *CDK9*, however, is the yeast *BUR1*, which encodes a distinct RNA polymerase CTD cyclin dependent kinase, whereas *CTK1* is more closely related to human *CDK12* and *CDK13* (86). Our preliminary evidence shows that loss of Cdk9 in mammalian cells results in a large increase in misreading suggesting that human Cdk9, like yeast Ctk1, controls an accuracy system in mammals (G.W. and P.J.F., unpublished). Experiments are in progress to determine how similar that system may be to the Ctk1-dependent system in budding yeast.

Phosphorylation is used to regulate translation extensively, mainly targeting translation factors. The best characterized existing paradigms are the phosphorylation of eIF4E by Mnk1/Mnk2 (87) and of the eIF4E binding protein (4E-BP) by TORC1 (88). Both events stimulate translation initiation by increasing the activity of eIF4E directly or indirectly. Recent progress in understanding the mechanism of these effects has shown that in each case phosphorylation stabilizes the structure of an intrinsically disordered region of the targeted protein, a common regulatory mechanism (89). For eIF4E, phosphoSer209 forms intra-protein salt bridges to Lys159 and Lys162, both of which lie in the cap-binding interface (90). The effect is to both stabilize the normally dynamic C-terminus of the protein that includes Ser209 and to alter electrostatic and hydrophobic features of the cap-binding pocket. The phosphorylated form has lower affinity for the cap based on electrostatic repulsion; this may increase the off rate from the cap, perhaps increasing the turnover number of the factor. For 4E-BP, phosphorylation also causes an intrinsically disordered region to fold into a four-stranded β domain in which a normally α-helical eIF4E recognition signal is buried in one of the β strands (91).

The data presented here establish another paradigm for phosphoregulation of protein synthesis in eukaryotes. The uS4•uS5 interface is a highly structured complex combining surfaces of the two proteins and the rRNA sequence from the extreme 5′ end of 18S rRNA (Supplementary Figure S2A). This structure is predicted to involve H-bond and salt bridge contacts with the rRNA from both uS4 and uS5 and direct contacts between uS5 pSer176 and uS4 Arg57, between the main chain of uS5 Arg174 and uS4 Arg53 and between uS5 pSer181 and uS4 Arg17. This region has a critical function in regulating accuracy of aminoacyl-tRNA recruitment whether in the high or lower accuracy form. All but the interaction by uS4 Arg17 with pSer181 and rRNA nucleotides U3 and C4 are predicted to be absent in the closed complex formed when a cognate aminoacyl-tRNA occupies the ribosomal A site (Supplementary Figure S2B). Rather than phosphorylation converting between a disordered and highly ordered state, phosphoserine at uS5 176 alters the kinetics of this transient dissociation of the interface during cognate tRNA recruitment. A pSer•Arg salt bridge is especially strong (92) and has been described as 'covalent like' (93) but a Glu•Arg bridge is somewhat weaker; the hyperaccuracy seen with the Ser to Glu mutants, we suspect, results from the conversion of a substoichiometric pSer to stoichiometric Glu at the site. We are

not aware of a similar control mechanism in any other enzyme. The proposed pSer176 is appreciably larger than Ser (89) and, if present, might be evident in structural data. This suggests that pSer is lost in preparation of ribosomes for structural analysis. Similar instances of pSer•Arg salt bridges may have been missed for lack of structural or genetic evidence.

In summary, our data support the existence of two opposing kinase pathways that control accuracy through phosphorylation of uS5 Ser176; the phosphorylation increases the stability of the uS4–uS5 interface to increase accuracy (Figure 8). The phosphorylation state of uS5 Ser176 should be confirmed by direct mass spectrometric or other biochemical characterization and many other details of the system remain to be determined including the identity of other members of kinase/phosphatase pathways that control the level of phosphorylation of Ser176. Having identified these enzymes should provide more targets for manipulating translational accuracy. The ability to control translational accuracy, both to increase and decrease accuracy, may provide a means to treat human diseases that are exacerbated by either abnormally high or low translational accuracy, potentially including neurodegenerative or neuromuscular disorders and cancer.

Data availability

The data underlying this article are available in the Dryad Digital Repository at DOI: 10.5061/dryad.jdfn2z3gs.

Supplementary data

Supplementary Data are available at NAR Online.

Acknowledgements

This work was supported by grants from the National Institute of General Medical Sciences (GM29480) and the National Science Foundation (MCB 1645795, CBET 1805139).

Funding

National Institute of General Medical Sciences [GM29480]; National Science Foundation [CBET 1805139, MCB 1645795]. Funding for open access charge: NSF [CBET 1805139].

Conflict of interest statement

None declared.

References

1. Warner,J.R. (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci*, **24**, 437–440.
2. Volarevic,S., Stewart,M.J., Ledermann,B., Zilberman,F., Terracciano,L., Montini,E., Grompe,M., Kozma,S.C. and Thomas,G. (2000) Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science*, **288**, 2045–2047.
3. Parker,J. (1989) Errors and alternatives in reading the universal genetic code. *Microbiol. Rev.*, **53**, 273–298.
4. Kramer,E. and Farabaugh,P. (2007) The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. *RNA*, **13**, 87–96.
5. Drummond,D.A. and Wilke,C.O. (2008) Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. *Cell*, **134**, 341–352.
6. Valastyan,J.S. and Lindquist,S. (2014) Mechanisms of protein-folding diseases at a glance. *Dis Model Mech*, **7**, 9–14.
7. Klaips,C.L., Jayaraj,G.G. and Hartl,F.U. (2018) Pathways of cellular proteostasis in aging and disease. *J. Cell Biol.*, **217**, 51–63.
8. Francisco,S., Ferreira,M., Moura,G., Soares,A.R. and Santos,M.A.S. (2020) Does proteostasis get lost in translation? Implications for protein aggregation across the lifespan. *Ageing Res. Rev.*, **62**, 101119.
9. Gorini,L. (1974) In: Nomura,M., Tissièrres,A. and Lengyel,P. (eds.) *Ribosomes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 791–803.
10. Ozaki,M., Mizushima,S. and Nomura,M. (1969) Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in E. coli. *Nature*, **222**, 333–339.
11. Deusser,E., Stoffler,G. and Wittmann,H.G. (1970) Ribosomal proteins. XVI. Altered S4 proteins in Escherichia coli revertants from streptomycin dependence to independence. *Mol. Gen. Genet.*, **109**, 298–302.
12. Stoffler,G., Deusser,E., Wittmann,H.G. and Apirion,D. (1971) Ribosomal proteins. XIX. Altered S5 ribosomal protein in an Escherichia coli revertant from strptomycin dependence to independence. *Mol. Gen. Genet.*, **111**, 334–341.
13. Alksne,L.E., Anthony,R.A., Liebman,S.W. and Warner,J.R. (1993) An accuracy center in the ribosome conserved over 2 billion years. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9538–9541.
14. Ogle,J.M., Murphy,F.V., Tarry,M.J. and Ramakrishnan,V. (2002) Selection of tRNA by the ribosome requires a transition from an open to a closed form. *Cell*, **111**, 721–732.
15. Zaher,H.S. and Green,R. (2010) Hyperaccurate and error-prone ribosomes exploit distinct mechanisms during tRNA selection. *Mol. Cell*, **39**, 110–120.
16. Rother,S. and Strasser,K. (2007) The RNA polymerase II CTD kinase Ctk1 functions in translation elongation. *Genes Dev.*, **21**, 1409–1421.
17. Patturajan,M., Conrad,N.K., Bregman,D.B. and Corden,J.L. (1999) Yeast carboxyl-terminal domain kinase I positively and negatively regulates RNA polymerase II carboxyl-terminal domain phosphorylation. *J. Biol. Chem.*, **274**, 27823–27828.
18. Roux,P.P. and Topisirovic,I. (2018) Signaling pathways involved in the regulation of mRNA translation. *Mol. Cell. Biol.*, **38**, e00070-18
19. Spriggs,K.A., Bushell,M. and Willis,A.E. (2010) Translational regulation of gene expression during conditions of cell stress. *Mol. Cell*, **40**, 228–237.
20. Hinnebusch,A.G. (2005) Translational regulation of GCN4 and the general amino acid control of yeast. *Annu. Rev. Microbiol.*, **59**, 407–450.
21. Fonseca,B.D., Smith,E.M., Yelle,N., Alain,T., Bushell,M. and Pause,A. (2014) The ever-evolving role of mTOR in translation. *Semin. Cell Dev. Biol.*, **36**, 102–112.
22. Fenton,T.R. and Gout,I.T. (2011) Functions and regulation of the 70kDa ribosomal S6 kinases. *Int. J. Biochem. Cell Biol.*, **43**, 47–59.
23. Gonzalez,A., Shimobayashi,M., Eisenberg,T., Merle,D.A., Pendl,T., Hall,M.N. and Moustafa,T. (2015) TORC1 promotes phosphorylation of ribosomal protein S6 via the AGC kinase Ypk3 in Saccharomyces cerevisiae. *PLoS One*, **10**, e0120250.
24. Sonenberg,N. and Hinnebusch,A.G. (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell*, **136**, 731–745.
25. Migliaccio,N., Martucci,N.M., Ruggiero,I., Sanges,C., Ohkubo,S., Lamberti,A., Agostinelli,E. and Arcari,P. (2016) Ser/Thr kinases and polyamines in the regulation of non-canonical functions of elongation factor 1A. *Amino Acids*, **48**, 2339–2352.
26. Hughes Hallett,J.E., Luo,X. and Capaldi,A.P. (2014) State transitions in the TORC1 signaling pathway and information processing in Saccharomyces cerevisiae. *Genetics*, **198**, 773–786.

27. Watanabe,M., Chen,C.Y. and Levin,D.E. (1994) Saccharomyces cerevisiae PKC1 encodes a protein kinase C (PKC) homolog with a substrate specificity similar to that of mammalian PKC. *J. Biol. Chem.*, **269**, 16829–16836.
28. Casamayor,A., Torrance,P.D., Kobayashi,T., Thorner,J. and Alessi,D.R. (1999) Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr. Biol.*, **9**, 186–197.
29. Schmelzle,T., Helliwell,S.B. and Hall,M.N. (2002) Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. *Mol. Cell. Biol.*, **22**, 1329–1339.
30. Taylor,R.G., Walker,D.C. and McInnes,R.R. (1993) E. coli host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. *Nucleic Acids Res.*, **21**, 1677–1678.
31. Brachmann,C.B., Davies,A., Cost,G.J., Caputo,E., Li,J., Hieter,P. and Boeke,J.D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*, **14**, 115–132.
32. Guthrie,C. and Fink,G. (1991) In: *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego.
33. Gietz,R.D. and Schiestl,R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.*, **2**, 31–34.
34. Joshi,K., Bhatt,M.J. and Farabaugh,P.J. (2018) Codon-specific effects of tRNA anticodon loop modifications on translational misreading errors in the yeast Saccharomyces cerevisiae. *Nucleic Acids Res.*, **46**, 10331–10339.
35. Manickam,N., Joshi,K., Bhatt,M.J. and Farabaugh,P.J. (2016) Effects of tRNA modification on translational accuracy depend on intrinsic codon-anticodon strength. *Nucleic Acids Res.*, **44**, 1871–1881.
36. Rothstein,R.J. (1983) One-step gene disruption in yeast. *Methods Enzymol.*, **101**, 202–211.
37. Noguchi,C., Garabedian,M.V., Malik,M. and Noguchi,E. (2008) A vector system for genomic FLAG epitope-tagging in Schizosaccharomyces pombe. *Biotechnol. J.*, **3**, 1280–1285.
38. Hoffman,C.S. and Winston,F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. *Gene*, **57**, 267–272.
39. Berman,H.M., Westbrook,J., Feng,Z., Gilliland,G., Bhat,T.N., Weissig,H., Shindyalov,I.N. and Bourne,P.E. (2000) The Protein Data Bank. *Nucleic Acids Res.*, **28**, 235–242.
40. Pettersen,E.F., Goddard,T.D., Huang,C.C., Couch,G.S., Greenblatt,D.M., Meng,E.C. and Ferrin,T.E. (2004) UCSF Chimera–a visualization system for exploratory research and analysis. *J. Comput. Chem.*, **25**, 1605–1612.
41. Kramer,E.B., Vallabhaneni,H., Mayer,L.M. and Farabaugh,P.J. (2010) A comprehensive analysis of translational missense errors in the yeast Saccharomyces cerevisiae. *RNA*, **16**, 1797–1808.
42. Manickam,N., Nag,N., Abbasi,A., Patel,K. and Farabaugh,P.J. (2014) Studies of translational misreading in vivo show that the ribosome very efficiently discriminates against most potential errors. *RNA*, **20**, 9–15.
43. Gebler,J.C., Aebersold,R. and Withers,S.G. (1992) Glu-537, not Glu-461, is the nucleophile in the active site of (lac Z) beta-galactosidase from Escherichia coli. *J. Biol. Chem.*, **267**, 11126–11130.
44. Lee,J.M. and Greenleaf,A.L. (1991) CTD kinase large subunit is encoded by CTK1, a gene required for normal growth of Saccharomyces cerevisiae. *Gene Expr.*, **1**, 149–167.
45. Lee,J.M. and Greenleaf,A.L. (1997) Modulation of RNA polymerase II elongation efficiency by C-terminal heptapeptide repeat domain kinase I. *J. Biol. Chem.*, **272**, 10990–10993.
46. Holt,L.J., Tuch,B.B., Villen,J., Johnson,A.D., Gygi,S.P. and Morgan,D.O. (2009) Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science*, **325**, 1682–1686.
47. Swaney,D.L., Beltrao,P., Starita,L., Guo,A., Rush,J., Fields,S., Krogan,N.J. and Villen,J. (2013) Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. *Nat. Methods*, **10**, 676–682.
48. Songyang,Z., Blechner,S., Hoagland,N., Hoekstra,M.F., Piwnica-Worms,H. and Cantley,L.C. (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr. Biol.*, **4**, 973–982.
49. Kennelly,P.J. and Krebs,E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.*, **266**, 15555–15558.
50. Bodenmiller,B., Wanka,S., Kraft,C., Urban,J., Campbell,D., Pedrioli,P.G., Gerrits,B., Picotti,P., Lam,H., Vitek,O., *et al.* (2010) Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Sci. Signal*, **3**, rs4.
51. Schuwirth,B.S., Borovinskaya,M.A., Hau,C.W., Zhang,W., Vila-Sanjurjo,A., Holton,J.M. and Cate,J.H. (2005) Structures of the bacterial ribosome at 3.5 A resolution. *Science*, **310**, 827–834.
52. Svidritskiy,E., Brilot,A.F., Koh,C.S., Grigorieff,N. and Korostelev,A.A. (2014) Structures of yeast 80S ribosome-tRNA complexes in the rotated and nonrotated conformations. *Structure*, **22**, 1210–1218.
53. Petterson,E., Goddard,T., Huang,C., Couch,G., Greenblatt,D., Meng,E. and Ferrin,T. (2004) UCSF Chimera–a visualization system for exploratory research and analysis. *J. Comput. Chem.*, **25**, 1605–1602.
54. Ben-Shem,A., Garreau de Loubresse,N., Melnikov,S., Jenner,L., Yusupova,G. and Yusupov,M. (2011) The structure of the eukaryotic ribosome at 3.0 A resolution. *Science*, **334**, 1524–1529.
55. Kumar,S. and Nussinov,R. (2002) Close-range electrostatic interactions in proteins. *ChemBioChem*, **3**, 604–617.
56. Kumar,S. and Nussinov,R. (2002) Relationship between ion pair geometries and electrostatic strengths in proteins. *Biophys. J.*, **83**, 1595–1612.
57. Netzer,N., Goodenbour,J.M., David,A., Dittmar,K.A., Jones,R.B., Schneider,J.R., Boone,D., Eves,E.M., Rosner,M.R., Gibbs,J.S., *et al.* (2009) Innate immune and chemically triggered oxidative stress modifies translational fidelity. *Nature*, **462**, 522–526.
58. Ling,J. and Soll,D. (2010) Severe oxidative stress induces protein mistranslation through impairment of an aminoacyl-tRNA synthetase editing site. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 4028–4033.
59. Wiltrout,E., Goodenbour,J.M., Frechin,M. and Pan,T. (2012) Misacylation of tRNA with methionine in Saccharomyces cerevisiae. *Nucleic Acids Res.*, **40**, 10494–10506.
60. Javid,B., Sorrentino,F., Toosky,M., Zheng,W., Pinkham,J.T., Jain,N., Pan,M., Deighan,P. and Rubin,E.J. (2014) Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proc. Natl. Acad. Sci. U.S.A.*, **111**, 1132–1137.
61. Meyerovich,M., Mamou,G. and Ben-Yehuda,S. (2010) Visualizing high error levels during gene expression in living bacterial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 11543–11548.
62. Ribas de Pouplana,L., Santos,M.A., Zhu,J.H., Farabaugh,P.J. and Javid,B. (2014) Protein mistranslation: friend or foe?*Trends Biochem. Sci*, **39**, 355–362.
63. Kuge,S. and Jones,N. (1994) YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. *EMBO J.*, **13**, 655–664.
64. Bilsland,E., Molin,C., Swaminathan,S., Ramne,A. and Sunnerhagen,P. (2004) Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. *Mol. Microbiol.*, **53**, 1743–1756.
65. Vilella,F., Herrero,E., Torres,J. and de la Torre-Ruiz,M.A. (2005) Pkc1 and the upstream elements of the cell integrity pathway in Saccharomyces cerevisiae, Rom2 and Mtl1, are required for cellular responses to oxidative stress. *J. Biol. Chem.*, **280**, 9149–9159.

66. Saxton,R.A. and Sabatini,D.M. (2017) mTOR signaling in growth, metabolism, and disease. *Cell*, **168**, 960–976.
67. Tomioka,M., Shimobayashi,M., Kitabatake,M., Ohno,M., Kozutsumi,Y., Oka,S. and Takematsu,H. (2018) Ribosomal protein uS7/Rps5 serine-223 in protein kinase-mediated phosphorylation and ribosomal small subunit maturation. *Sci. Rep.*, **8**, 1244.
68. Ron,D., Chen,C.H., Caldwell,J., Jamieson,L., Orr,E. and Mochly-Rosen,D. (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 839–843.
69. Gerbasi,V.R., Weaver,C.M., Hill,S., Friedman,D.B. and Link,A.J. (2004) Yeast Asc1p and mammalian RACK1 are functionally orthologous core 40S ribosomal proteins that repress gene expression. *Mol. Cell. Biol.*, **24**, 8276–8287.
70. Loewith,R., Jacinto,E., Wullschleger,S., Lorberg,A., Crespo,J.L., Bonenfant,D., Oppliger,W., Jenoe,P. and Hall,M.N. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell*, **10**, 457–468.
71. Sussman,A., Huss,K., Chio,L.C., Heidler,S., Shaw,M., Ma,D., Zhu,G., Campbell,R.M., Park,T.S., Kulanthaivel,P., *et al.* (2004) Discovery of cercosporamide, a known antifungal natural product, as a selective Pkc1 kinase inhibitor through high-throughput screening. *Euk. Cell*, **3**, 932–943.
72. Jacinto,E. and Lorberg,A. (2008) TOR regulation of AGC kinases in yeast and mammals. *Biochem. J.*, **410**, 19–37.
73. Schmelzle,T., Beck,T., Martin,D.E. and Hall,M.N. (2004) Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol. Cell. Biol.*, **24**, 338–351.
74. Urban,J., Soulard,A., Huber,A., Lippman,S., Mukhopadhyay,D., Deloche,O., Wanke,V., Anrather,D., Ammerer,G., Riezman,H., *et al.* (2007) Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. *Mol. Cell*, **26**, 663–674.
75. Sobko,A. (2006) Systems biology of AGC kinases in fungi. *Sci. STKE*, **2006**, re9.
76. Shin,S., Wolgamott,L., Yu,Y., Blenis,J. and Yoon,S.O. (2011) Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, E1204–E1213.
77. Kamada,Y., Fujioka,Y., Suzuki,N.N., Inagaki,F., Wullschleger,S., Loewith,R., Hall,M.N. and Ohsumi,Y. (2005) Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol. Cell. Biol.*, **25**, 7239–7248.
78. Costanzo,M., VanderSluis,B., Koch,E.N., Baryshnikova,A., Pons,C., Tan,G., Wang,W., Usaj,M., Hanchard,J., Lee,S.D., *et al.* (2016) A global genetic interaction network maps a wiring diagram of cellular function. *Science*, **353**,aaf1420-1–aaf1420-14.
79. Sarbassov,D.D., Ali,S.M., Kim,D.H., Guertin,D.A., Latek,R.R., Erdjument-Bromage,H., Tempst,P. and Sabatini,D.M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.*, **14**, 1296–1302.
80. Chen,C.H., Shaikenov,T., Peterson,T.R., Aimbetov,R., Bissenbaev,A.K., Lee,S.W., Wu,J., Lin,H.K. and Sarbassov dos,D. (2011) ER stress inhibits mTORC2 and Akt signaling through GSK-3beta-mediated phosphorylation of rictor. *Sci. Signal*, **4**, ra10.
81. Birge,E.A. and Kurland,C.G. (1970) Reversion of a streptomycin-dependent strain of Escherichia coli. *Mol. Gen. Genet.*, **109**, 356–369.
82. Kreider,G. and Brownstein,B.L. (1971) A mutation suppressing streptomycin dependence. II. An altered protein on the 30 s ribosomal subunit. *J. Mol. Biol.*, **61**, 135–142.
83. Donner,D. and Kurland,C.G. (1972) Changes in the primary structure of a mutationally altered ribosomal protein S4 of Escherichia coli. *Mol. Gen. Genet.*, **115**, 49–53.
84. Santos,M.A. and Tuite,M.F. (1995) The CUG codon is decoded in vivo as serine and not leucine in Candida albicans. *Nucleic Acids Res.*, **23**, 1481–1486.
85. Santos,M.A., Cheesman,C., Costa,V., Moradas-Ferreira,P. and Tuite,M.F. (1999) Selective advantages created by codon ambiguity allowed for the evolution of an alternative genetic code in Candida spp. *Mol. Microbiol.*, **31**, 937–947.
86. Corden,J.L. (2013) RNA polymerase II C-terminal domain: tethering transcription to transcript and template. *Chem. Rev.*, **113**, 8423–8455.
87. Buxade,M., Parra-Palau,J.L. and Proud,C.G. (2008) The Mnks: MAP kinase-interacting kinases (MAP kinase signal-integrating kinases). *Front. Biosci.*, **13**, 5359–5373.
88. Gingras,A.C., Gygi,S.P., Raught,B., Polakiewicz,R.D., Abraham,R.T., Hoekstra,M.F., Aebersold,R. and Sonenberg,N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.*, **13**, 1422–1437.
89. Newcombe,E.A., Delaforge,E., Hartmann-Petersen,R., Skriver,K. and Kragelund,B.B. (2022) How phosphorylation impacts intrinsically disordered proteins and their function. *Essays Biochem.*, **66**, 901–913.
90. Lama,D. and Verma,C.S. (2020) Deciphering the mechanistic effects of eIF4E phosphorylation on mRNA-cap recognition. *Protein Sci.*, **29**, 1373–1386.
91. Bah,A., Vernon,R.M., Siddiqui,Z., Krzeminski,M., Muhandiram,R., Zhao,C., Sonenberg,N., Kay,L.E. and Forman-Kay,J.D. (2015) Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch. *Nature*, **519**, 106–109.
92. Mandell,D.J., Chorny,I., Groban,E.S., Wong,S.E., Levine,E., Rapp,C.S. and Jacobson,M.P. (2007) Strengths of hydrogen bonds involving phosphorylated amino acid side chains. *J. Am. Chem. Soc.*, **129**, 820–827.
93. Woods,A.S. and Ferre,S. (2005) Amazing stability of the arginine-phosphate electrostatic interaction. *J. Proteome Res.*, **4**, 1397–1402.

Received: August 22, 2023. **Revised:** January 8, 2024. **Editorial Decision:** January 9, 2024. **Accepted:** February 8, 2024