Pairs of amino acids at the P- and A-sites of the ribosome predictably and causally modulate translation-elongation rates

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Variation in translation-elongation kinetics along a transcript's coding sequence plays an important role in the maintenance of cellular protein homeostasis by regulating co-translational protein folding, localization, and maturation. Translation-elongation speed is influenced by molecular factors within mRNA and protein sequences. For example, when proline is present in the ribosome's P- or A-site translation slows down, but the effect of other pairs of amino acids, in the context of all 400 possible pairs, has not been characterized. Here, we study *Saccharomyces cerevisiae* using a combination of mutational experiments, bioinformatics, and evolutionary analyses, and show that many different pairs of amino acids and their associated tRNA molecules predictably and causally encode translation rate information when these pairs are present in the A- and P-sites of the ribosome independent of other factors known to influence translation speed, including mRNA structure, wobble base pairing, tripeptide motifs, positively charged upstream nascent chain residues, and cognate tRNA concentration. The fast-translating pairs of amino acids that we identify are enriched seven-fold relative to the slow-translating pairs across *Saccharomyces cerevisiae*'s proteome, while the slow-translating pairs are enriched downstream of domain boundaries. Thus, the chemical identity of amino acid pairs contributes to variability in translation rates, elongation kinetics are causally encoded in the primary structure of proteins, and signatures of evolutionary selection indicate their potential role in co-translational processes.

INTRODUCTION

The rates associated with translation determine the time scales of protein synthesis [1,2], influence protein expression levels [3], and can affect the structure and function of the protein produced [4–6]. While the rate of translation initiation is a key kinetic parameter determining protein expression levels, the non-uniform rate of translation elongation across coding sequences can influence the fate of nascent proteins and the downstream cellular processes they take part in. Variation in translation-elongation kinetics influences protein homeostasis by modulating co-translational protein folding, localization, and maturation [6–8]. Hence, characterizing the molecular factors that determine the rate at which individual codon positions along a transcript are translated aids our understanding of how a functional proteome is regulated and produced *in vivo*.

The rate of translation elongation was originally thought to be determined only by the A-site codon's cognate tRNA concentration as it influences the rate of tRNA accommodation into the A-site [9,10]. More frequently used codons across a transcriptome were presumed to be translated at faster rates as they typically have a higher abundance of cognate tRNAs. Over the past decade some ten other molecular factors have been identified that can influence the rate of translation elongation [7,11] including features inherent to the mRNA sequence, such as mRNA structure [12–14], and features inherent to the protein sequence, such as the presence of particular tripeptide sequence motifs composed of one or more prolines [15–19] and positively charged nascent-chain residues within the negatively charged ribosome exit tunnel [20–22]. There are still unexplored molecular factors that have the potential to influence translation elongation rates. For example, a study that utilized Machine Learning indicated that features of the P-site correlate with translation speed [23]. Furthermore, the aforementioned protein-based factors suggest the intriguing possibility that the primary structures of proteins, beyond proline containing motifs [15], have the potential to causally encode translation-elongation rate information.

Since the ribosome catalyzes peptide bond formation between 400 unique amino acid pairs when they reside in the P- and A-sites of the ribosome, we hypothesized that the chemical identity of the P-site amino-acid, in the context of these pairs, could influence translation speed at the A-site in a predictable and causal way, beyond known effects arising from proline-containing pairs and cognate tRNA concentration. In this study, we utilize ribosome profiling data generated from *Saccharomyces cerevisiae* to test this hypothesis. We bioinformatically isolated the effect of the P-site amino acid and tRNA identities on translation at the A-site by keeping the A-site amino acid fixed, as this controls for cognate tRNA concentration and accommodation kinetics. We identified certain amino acids, that when present in the P-site, appear to either speed up or slow down the rate of translation when a particular amino acid is present in the A-site. We experimentally tested these predictions by mutating the P-site amino acid and detected the change in ribosome density, which is a function of translation speed, via ribosome profiling. While an amino acid effect has been well established for Proline, this is the first study to identify a large number of amino acid pairs for which the chemical identity of the P-site amino acid and tRNA systematically influences the translation elongation rate at the A-site. Finally, we demonstrate that across the *Saccharomyces cerevisiae* proteome there are signatures of evolutionary selection pressure on the fast- and slow-translating amino acid pairs we have identified that suggests these pairs might play a role in regulating the co-translational maturation of proteins.

RESULTS

Beyond proline, the identity of the P-site amino acid can influence the translation rate at the A-site. Ribosome profiling is a high-throughput technique that measures ribosome densities that are a function of the location and number of ribosomes translating different codon positions across a transcriptome [24]. The measured ribosome density ρ at a codon is inversely related to the speed at which ribosomes translate that codon [25]. A high ribosome density at a codon indicates that there is a slowdown of translation while a low ribosome density reflects faster than average translation. Thus, from one codon position to the next along a transcript a ribosome can be said to speed-up or slow-down its elongation speed.

We analyzed the translational profiles of 364 high-coverage transcripts (Data S1) measured in six independent, published data sets from four different labs [26–30] (Table S1). These datasets where chosen because they have high read coverage and do not use cycloheximide (CHX) pre-treatment, as CHX has been shown to artificially distort ribosome profiles [31]. The 364 transcripts were chosen after applying a filter of at least 3 reads per codon in the dataset with the highest coverage [27]. This high coverage filter allows us to minimize any sequencing noise and allows a more precise reporter of translation speed. This subset of transcripts is representative of the entire transcriptome as the sequence properties of these transcripts are similarly distributed as the transcriptome (see Fig. S8 of ref. 14).

To isolate the effect of the P-site on translation at the A-site we compare the ribosome density when a particular amino acid is present in the P-site versus when it is not. Specifically, for each of the 400 unique pairs of amino acids that can reside in the P- and A-sites — which for a given pair we denote as (X, Z), where X is the amino acid in the P-site and Z is the amino acid in the A-site — we first determined the normalized ribosome density distribution, [$\rho(X, Z)$], arising from all instances of the pair (X, Z) in the data set versus the distribution [$\rho(\sim X, Z)$] arising from all instances of Z being in the A-site but X not being present in the P-site. For example, for the pair denoted (N, R), N is in the P-site and R is in the A-site, while (~N, R) corresponds to the 19 other naturally occurring amino acids that can be in the P-site when R is in the A-site (Fig. 1a). We then calculated the percent change (Eq. 1) in the median of [$\rho(X, Z)$] relative to the median of [$\rho(\sim X, Z)$], as this measures whether the identity of the P-site amino acid tends to lead to faster or slower translation relative to when any other amino acid is present in the P-site.

$$Percent\ change = \frac{Median[\rho(X,Z)] - Median[\rho(\sim X,Z)]}{Median[\rho(\sim X,Z)]} * 100\ \% \qquad [1]$$

We use the median of these [$\rho(X, Z)$] distributions, as opposed to the average, because the median is less sensitive to outlier, long-tail effects in right-skewed distributions typically found in ribosome profiling (Fig. 1c). Critically, this analysis approach controls for confounding cognate tRNA concentration effects and accommodation kinetics at the A-site because the A-site amino acid is held fixed. Applying this analysis to each of the six published datasets we obtained six matrices reporting the percent change (Eq. 1) in ribosome density when a particular amino acid pair is present in the P- and A-sites (Fig. S1).

Given the well-known variability in ribosome profiling results between different experiments [32] we focus on highly robust, reproducible results by (*i*) only drawing conclusions from the sign of the percent change, which indicates either a speed up or slow down between different pairs of

amino acids, and by (*ii*) taking the intersection of those pairs that exhibit a consistent sign change in all six datasets and have a percent change that statistically different than zero in at least four of the datasets. We found 84 pairs in which the presence of a particular P-site amino acid is correlated with faster translation (green-shifted colors in Fig. 1b) and 73 pairs in which the identity of the P-site amino acid is correlated with slower translation (red-shifted colors in Fig. 1b). The results for the remaining pairs are not significant or are not consistent across the datasets (gray boxes in Fig. 1b). An important internal control in these data is proline, which is known from biochemical studies to tend to slow down translation when present in the P-site. Consistent with those findings, we observe a vertical strip of warm colors in Fig. 1b when Pro is in the P-site, indicating that when paired with almost any other amino acid in the A-site, proline tends to increase ribosome density, *i.e.*, slow translation down relative to any other amino acid being in the P-site. These results suggest that the identity of the P-site amino acid in 157 pairs of amino acids can, on average, predictably speeding up or slowing down translation relative to the average speed when any other amino acid is in the P-site.

Factors known to modulate translation speed do not explain these results. To test whether the potentially confounding factors of tripeptide motifs [15], positively charged upstream residues [20,21], downstream mRNA structure [13,33] or cognate tRNA concentration [29,34] explain the direction of the speed changes in Fig. 1b, we controlled for each of these factors separately and found that even in their absence the sign of the speed change is preserved in 156 of the 157 pairs (Fig. S2).

A recent study [35] identified 17 very rare codon pairs in *S. cerevisiae* that when present in the P- and A-sites inhibit translation due to the interaction of their predominantly inosine-modified, wobble-decoding tRNAs. To test whether this mechanism explains our slow-translating pairs of amino acids we removed all instances of codon pairs decoded by wobble base pairing and found that 72 out of 73 of our slow-translating pairs remained slow (Fig. S3). Thus, wobble base pairing does not explain our observations.

In summary, while these molecular factors undoubtedly contribute to codon translation rates, they do not explain the pairs that we observe tend to lead to faster or slower translation elongation.

Mutating the P-site amino acid is predicted to alter the translation rate at the A-site. Figure 1b predicts that by keeping the A-site amino acid fixed and mutating the P-site amino acid it is possible to speed up or slow down translation elongation (*i.e.*, change the sign of the percent change in Eq. 1). For example, when comparing the amino acid pairs (N,R) to (S,R), where R is the amino acid in the A-site, we find (N,R) tends to have 53% more ribosome density than (S,R) (Fig. 1c). Hence, we predict that the codon encoding R in the (S,R) pair will be translated faster than the codon encoding R in the (N,R) pair. We predict that for the 7,980 possible P-site mutations in amino acid pairs where the A-site is fixed there will be a systematic change in translation rate for a majority of them (Fig. 1d and Data S2). Because we are dealing with overlapping ribosome density distributions (Fig. 1c) it is most appropriate to think probabilistically, in terms of the likelihood that a mutation will speed up or slow down translation. We can calculate the odds (Eq. S3) that a mutation at the P-site will speed up or slow down translation. For example, for the mutation (N,R) to (S,R) we calculate translation will speed up with 3-to-1 odds. We

calculated these odds for each of the possible 7,980 mutations and found a broad distribution (Fig. 1e). With odds of 5.7-to-1, mutating (W,G) to (P,G) will slow down translation, while with odds of 1-to-1, mutating (V,W) to (H,W) is as equally likely to speed up translation as it is to slow down translation when Val is mutated to His in the different instances of (V,W) across the proteome (Data S2). In summary, this bioinformatics analysis predicts which P-site amino acid mutations will result in higher or lower translation elongation rates relative to the wild-type protein sequence.

Mutational experiments are consistent with amino acid identity influencing translation elongation rates. To experimentally test these predictions, we introduced 12 non-synonymous mutations into various positions of five non-essential *S. cerevisiae* genes that are not involved in translation, and no mutations were made at functional sites of the encoded proteins [36] (Table S2). Five of the mutations we bioinformatically predicted will speed up translation, five we predicted will slow down translation, and two we predicted to have minimal effects on translation speed when the mutated residue is present in the P-site.

To ensure precise measurements at codon resolution we performed ribosome profiling experiments at unconventionally high read depths, having an average of 86 million mapped exome reads per sample after removing reads mapped to rRNA genes, and totaling 1.7 billion mapped exome reads across samples (Table S3). The resulting ribosome profiles exhibit strong 3-nt periodicity, 87% of mapped reads are in frame zero at a fragment size of 28 nt, and there is a very strong correlation between ribosome profiles for the same gene across samples (Pearson r=0.96, Figs. S4 and S5). These results indicate technical biases are minimal in these experiments, and any such biases that exist will likely cancel out when we carry out the relative comparison between wild type and mutant results.

Comparing the normalized ribosome densities between the wild-type and mutant strains (Fig. 2) we find that in all cases the direction of change in ribosome density at the A-site is consistent with the predictions from Fig. 1b. Two of these ten mutations include a proline in the P-site, for which we observe a speedup when the pair (P,G) is mutated to (E,G) in the gene *YMR122W-A* (Fig. 2a), while mutating (Q,D) to (P,D) in *YOL109W* leads to a slowdown of translation (Fig. 2b). These two mutations serve as positive controls because proline has previously been shown to slow down translation [16,19].

Two additional mutations were incorporated as negative controls and are predicted to cause little change in the rate of translation (*i.e.*, mutations that switch between gray boxes in Fig. 1b). We found that while the normalized ribosome densities of these mutants are statistically different from that of the wild type (Fig. 2c) but the effect size is much smaller. The median effect size on translation speed was 2.5-fold lower than what we observed for the other 10 mutations (Fig. 2d). That the negative controls exhibit minor changes in translation speed should be expected, as previously discussed we are dealing with overlapping ribosome density and speed distributions (Figs. 1c,e), and there is an associated odds of seeing some speed up or slow down. In summary, the results from these mutational experiments are consistent with the hypothesis that the P-site amino acid can predictably alter the translation rate at the A-site.

Translation rates are affected by both the identity of pair of amino acids and the tRNAs they are attached to by varying degrees. The amino acid mutations we introduced also change the identity of the tRNA molecule at the P-site. Therefore, this change in tRNA identity could be

an alternative explanation for the cause of the altered translation speeds (Fig. 2). To address this, we carried out a bioinformatic analysis and experiments to estimate the source of the change in translation speed.

To bioinformatically estimate the relative contributions of amino acid versus tRNA identity to changes in speed, we projected the normalized ribosome density on to the 42 unique tRNA molecules that can reside in the P- and A-sites. Based on a comparison of these distributions, we can estimate the contributions of tRNA and amino acids to the change in translation speed (Eq. S4, and see Methods). Briefly, Eq. S4 assess whether different tRNA molecules attached to the same P-site amino acid correlate with similar or different A-site translation rates. If, for the same amino acid mutation in the P-site, we observe a significant change of speed at the A-site for one tRNA encoding that amino acid but not the others, then we can infer that tRNA identity is a major contributor to the change in translation speed. Applying Eq. S4 to the Ribo-seq data we find that the contribution of these two factors is a continuum for different pairs of amino acids (Fig. 3a and Data S3). For some pairs, such as when (E,N) is mutated to (H,N), we estimate that the change in speed is driven entirely by the amino acid identity, while other pairs, such as (S,G) mutated to (E,G), are driven entirely by tRNA identity; many pairs are driven by a mixture of these two factors (Fig. 3a).

To experimentally estimate the relative contribution of amino acid identity we took the three mutations that we previously incorporated into the gene *YOL109W* (Table S2) and created a new gene construct with the same three amino acid mutations but used synonymous codons that are decoded by different tRNA molecules (Table S4). For the mutation (N,R) to (S,R), for example, we previously used the codon UCC to mutate N to S. In the new strain, we used the synonymous codon UCG, which is decoded by a different tRNA molecule [37] (Fig. 3b). For the mutants (G,G) to (S,G) and (Q,D) to (P,D), there was a change in normalized ribosome density that was in the same direction and similar in magnitude regardless of the tRNA molecule used (Figs. 3c, d). This indicates that for these mutations, the change in amino acid identity in the P-site is the primary cause of the change in translation rate in the A-site (Fig. 3c, d). In contrast, for the mutation (N,R) to (S,R), we observed a change in ribosome density when one tRNA molecule was used but no change in ribosome density when another tRNA molecule was used (Fig. 3e), indicating that the tRNA identity was the primary cause. Our bioinformatic analysis predicted that for the mutations (G,G) to (S,G), and (Q,D) to (P,D), the amino acid change is the major contributor to the observed change in speed (Data S3). For the mutation (N,R) to (S,R), involving different tRNAs, we were unable to make a bioinformatic prediction because the sample size was too small ($n < 10$) to apply any statistical test. Thus, the bioinformatic predictions (Data S3) and experimental results are qualitatively consistent, indicating that in some cases it is the amino acid identity that causes the change in speed and in others it is the tRNA identity.

***Signatures of evolutionary selection for fast and slow translating amino acid pairs*.** If evolutionary selection pressures have acted to encode translation rate information in the primary structures of proteins, then there should be a non-random distribution of fast and slow-translating pairs of amino acids across the proteome. To test this hypothesis, we calculated the enrichment and depletion of all 400 pairs of amino acids across the *S. cerevisiae* proteome relative to the occurrence expected from a random pairing. We selected the top 20% of the amino acid pairs that were enriched across the proteome and the bottom 20% that were depleted and determined

how many of the 84 fast-translating and 73 slow-translating amino acid pairs were present in either of these quintiles. The odds ratio of fast-translating pairs being enriched across the proteome and slow-translating pairs being depleted was 7.5 (Eq. S6, $p = 0.0011$, Fischer's exact test), indicating that selection pressures have indeed selected for the presence of fast-translating pairs and selected against slow-translating pairs (Fig. 4a) across the proteome. This result is consistent with the hypothesis [8,38] that evolution selects for molecular factors that increase the efficiency of translation, which is one of the most energy consuming cellular processes [39].

Despite the preference for fast-translating pairs, we found that the slow-translating amino acid pairs were locally enriched by 15% (95% CI: [6%, 23%], p<0.0001, n=170 domains, random permutation test) in protein segments that are translated after domains have emerged from the ribosome exit tunnel. (Figs. 4b,c). For simplicity, we refer to these segments as linkers. In those linker regions that start 30 residues downstream of domain boundaries, one-fifth of the amino acid pairs, on average, are slow-translating pairs, and in the extreme cases of genes *YDR432W* and *YGL203C*, there are 18 slow pairs in a 30 residue stretch. Codon usage does not explain this enrichment of slow-translating pairs, as we found no difference in the frequency of non-optimal codon usage between linker and domain regions (Fig. S6). These results indicate that a number of slow-translating pairs exist in linker regions that can cumulatively lead to a slowdown of translation as domains have fully emerged from the ribosome exit tunnel, which may aid in co-translational folding by providing more time for domains to fold outside of the ribosome exit tunnel.

When the Hsp70 chaperone Ssb is bound to ribosome-nascent chain complexes translation is faster than when Ssb is not bound, possibly because chaperone binding prevents nascent chain folding and hence allows translation to become uncoupled from folding and to proceed faster [40]. We examined if the fast-translating amino acid pairs we identified contributed to this speedup. We found that the fast-translating amino acid pairs were enriched by at least 4% (95% CI: [2.3%, 6.1%], p=0.0001, n=425, random permutation test) in regions translated while Ssb is bound, suggesting that these pairs do make a contribution (Fig. S7). Taken together, these results indicate that across the primary structures of proteins, evolutionary pressures have selected for amino acid pairs that exhibit faster translation, and along a transcript, fast- and slow-translating pairs are enriched locally in regions that are associated with co-translational folding and chaperone binding.

DISCUSSION

We have demonstrated that independent of known confounding factors the chemical identity of some pairs of amino acids and tRNA molecules, when present in the P- and A-sites, can predictably and causally result in a speedup or slowdown of translation in the A-site of *S. cerevisiae* ribosomes. An essential and unique feature of our analyses of ribosome profiling data is Eq. 1, which holds fixed the amino acid identity in the A-site while varying the amino acid in the P-site. This approach controls for variation in tRNA concentration and accommodation rates in the A-site, thereby allowing us to isolate the effect of the P-site amino acid on the overall translation rate at the A-site. In total, we identified 157 amino acid pairs that exhibit above average or below average translation speeds (Fig. 1b) and verified these predictions experimentally for 10 of these pairs. These speed changes are not explained by alternative hypotheses concerning the presence of mRNA structure, wobble base pairing, tripeptide motifs, positively charged upstream nascent chain residues, or variation in cognate tRNA concentration (Figs. S2 and S3). As an

important internal control, these results are consistent with the commonly seen translational slow-down effect of proline [16,41] evidenced by the vertical stripe of red-shifted colors in Fig. 1b.

Over- or under-representation of particular pairs of codons, compared to random chance, occurs across the transcriptome of many organisms and in a phenomenon referred to as “codon pair utilization bias” [42]. A biochemical study found that synonymously changing five of these codon pairs resulted in a change in protein expression level, which was interpreted as arising from alterations in elongation speed [43]. These, and other studies [44–46], hypothesized that synonymous codon pairs translate at different speeds due to differences in the molecular interactions between neighboring tRNAs when they are co-located at the A- and P-sites of the ribosome. The results of this study find that indeed, a change in translation speed upon a change in codon pair in some cases is entirely due to a change in the identity of the tRNA at the P-site, but also that there are many other cases where the change of translation speed is entirely due to the change in identity of the amino-acid at the P-site (Fig. 3). Thus, the results from this study indicate that there is a greater richness of molecular mechanisms that can contribute to codon pair utilization bias, and identify a large number of amino-acid pairs where systematic speed differences are observed.

Most studies on the relationship between translation kinetics and nascent peptide sequence features at the A- and P-site have involved the presence of proline, often in the context of ‘*X*PP*Z*’ peptide motifs [15,47,48], where *X* and *Z* can be any amino acid. To observe an effect of these motifs on translation, engineered bacterial cells must be used that lack elongation-factor P because its presence eliminates proline-induced stalling [17,18,49]. *In vitro* enzymology studies involving proline have focused only on the kinetics of the tRNA accommodation and peptide bond formation sub-steps of the translation cycle [16,41]. In contrast, this study used wild-type *S. cerevisiae* cells in which we discovered predictable changes in speed in 128 pairs of amino acids that do not involve proline. The Ribo-seq experimental signal we analyze is proportional to the dwell time of the ribosome at the A-site [24,50], and thus the effects we observe reflect what happens to the overall rate of one cycle of translation elongation rather than just the effect on individual steps of accommodation, peptide bond formation, or translocation. Thus, in contrast to an *in vitro* enzymology study that concluded that although the P-site amino acid can alter the peptide bond formation rate it has little effect on elongation due to the slower tRNA accommodation step [51], we observe wide spread P-site identity effects on overall codon translation rates *in vivo*. This suggests the possibility that for some of these pairs accommodation is not always rate limiting *in vivo* and that the effects of peptide bond formation rates may constitute a larger proportion of the dwell of a ribosome than previously thought. One promising approach to measure this proportion comes from the use of three different antibiotics that can trap *S. cerevisiae* ribosomes in different states of elongation [52]. The depth of coverage in the datasets from that study, with 7 million mapped reads on average compared to 87 million mapped reads in this study, are too sparse for us to test this hypothesis.

We observe consistent and robust influences of the amino-acid at the P-site. The effect of the P-site amino acid on translation speed tends to be consistent in either speeding up/slowing down translation, or having no effect at all, regardless of the amino acid in the A-site. Out the twenty amino acids that can reside in the P-site, nine (A,E,F,I,M,S,T,V,Y) speed up translation (green-shifted colors in Fig. 1b) or have little effect (gray colors); six (D,G,H,K,N,P) slow down translation (red-shifted colors) or have little effect; and three (L,R,W) can either speed up, slow

down, or have no effect relative to the average translation speed. For example, Ala in the P-site speeds up translation when A, G, P or Q is in the A-site, Lys in the P-site slows down translation when A, D, E, or Q is in the A-site, whereas Leu in the P-site slows down translation when G or Q is in the A-site but speeds up translation when D, K or N is in the A-site. Thus, 15-out-of-20 amino acids, when present in the P-site, consistently change translation speed in one direction. Pro, Glu, Ser, Gly and Asp are the most robust in terms of changing speed for the largest number of possible A-site amino acids, as evidenced by their vertical stripes of colors in Fig. 1b. Consistent with our findings, an *in vitro* enzymology study [51] that measured peptide bond formation rates of puromycin with eight different types of amino acids in the P-site found that Asp was the second slowest amino acid to form a peptide bond with puromycin after proline, and serine was the second fastest.

We find variable contributions of tRNA and amino acid identity to the change in translation speed when the P-site amino acid is mutated. Pairs whose effect we estimate arise primarily due to tRNA identity (reported in Dataset S3) could influence steps in the translation-elongation cycle other than peptide bond formation, including hybrid state formation and translocation as these steps structurally alter the intermolecular interactions between tRNA molecules, and between tRNA molecules and the ribosome. Disentangling the detailed molecular causes of the speed changes identified in Fig. 1b is likely to be a fruitful area of future research.

Our evolutionary analyses show an overall selection for fast-translating amino acid pairs, potentially to increase the efficiency of energy-intensive process of translation while locally enriching slow-translation pairs that might aid in co-translational processes important for a protein to attain its structural and functional form. Evolutionary selection pressures select only against phenotypic traits, not genotype. Therefore, the enrichment of fast-translating amino acid pairs across the *S. cerevisiae* transcriptome and the clusters of slow- and fast-translating pairs along transcripts that are correlated with co-translational processes suggest that the elongation kinetics encoded by these pairs influence organismal phenotype and fitness. More speculatively, these results open up the possibility that there may exist disease-causing amino acid mutations that do not alter the final folded structures of proteins but instead alter the co-translational behavior and processing of the nascent proteins via altered elongation kinetics.

In summary, separate from other molecular factors known to influence translation speed, elongation kinetics are causally and predictably encoded in protein primary structures through the identity of particular pairs of amino acids and the tRNAs they are attached to, with broad implications for protein and mRNA sequence evolution, and translational control of gene expression.

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AUTHOR CONTRIBUTIONS

E.P.O. conceived the study. U.F., G.K. and B.B. carried out experiments to generate *S. cerevisiae* mutant strains and Ribosome profiling. N.A. analyzed the data. N.A., P.S., P.C. contributed to analysis methods of published Ribo-Seq data and annotation of domain boundaries in *S. cerevisiae*. N.A, P.S., U.F., G.K. and E.P.O. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AVAILABILITY

Mutant ribosome profiling sequencing data is available on NCBI GEO database with accession number GSE133370.

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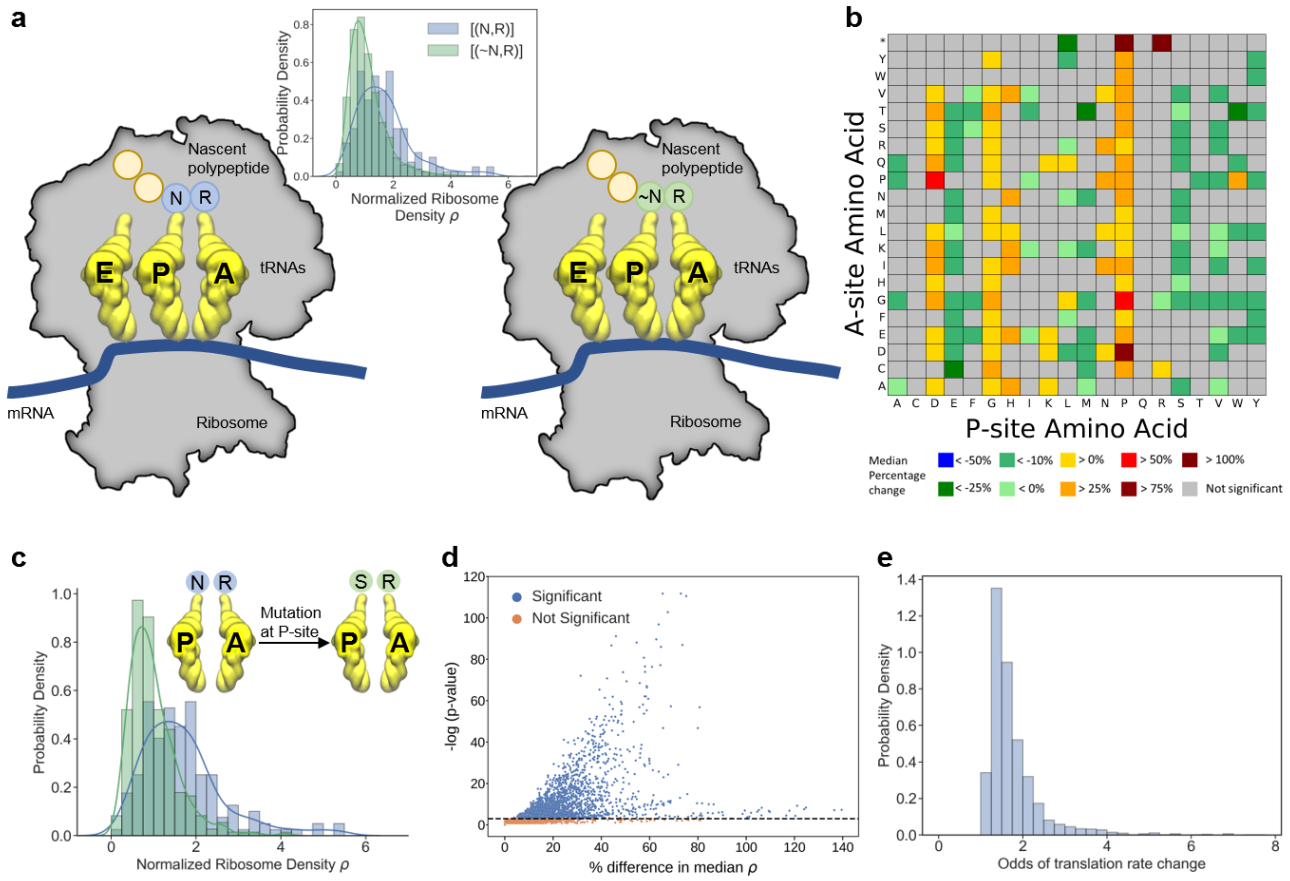


Figure 1. Bioinformatic analyses of ribosome profiling data indicate that the identity of amino acids in the P- and A-sites can predictably alter the translation speed of the A-site codon. (a) A ribosome with the amino acids N and R in the P- and A-sites, respectively. From ribosome profiling data, we calculated the distribution of ribosome densities in the A-site from all instances of (N, R) in our dataset and compared the result to the distributions of all other instances of R in the A-site when N is not present in the P-site, denoted [(~N, R)] (top panel). **(b)** Each box in the matrix indicates, for a pair of amino acids in the P- and A-sites, the percent change in median normalized ribosome density ρ when that particular amino acid is in P-site compared to any other amino acid in the P-site, keeping the A-site amino acid unchanged (Eq. 1). The sign of the percent change must be consistent in all 6 analyzed ribosome profiling datasets and statistically significant in at least 4 out of the 6 datasets, otherwise the box is colored gray. * corresponds to any of the stop codons being present in the A-site. **(c)** Comparison of distributions of amino acid pairs where R is kept constant at the A-site while the P-site is mutated from N to S. The distributions of normalized ribosome densities for P- and A-site pairs (N, R) and (S, R), which differ significantly from each other, are shown (Mann-Whitney U test, $p = 4.45 \times 10^{-17}$). The median normalized ribosome densities of the two distributions differ by 53.4%, and the odds of a change in translation speed when (N, R) is mutated to (S, R) or vice versa is 2.98 (Eq. S3). **(d)** The estimated percent difference values for all 7,980 mutations of amino acid pairs with a constant A-site are plotted with respect to the statistical significance of the difference between the distributions (see Methods). We estimate that mutating the P-site will lead to significant changes in translation speed in 4,254 (53%) of these mutations. **(e)** For the significant combinations of amino acids pairs, the distribution of the odds of mutating any instance of the pair resulting in a change in speed is plotted.

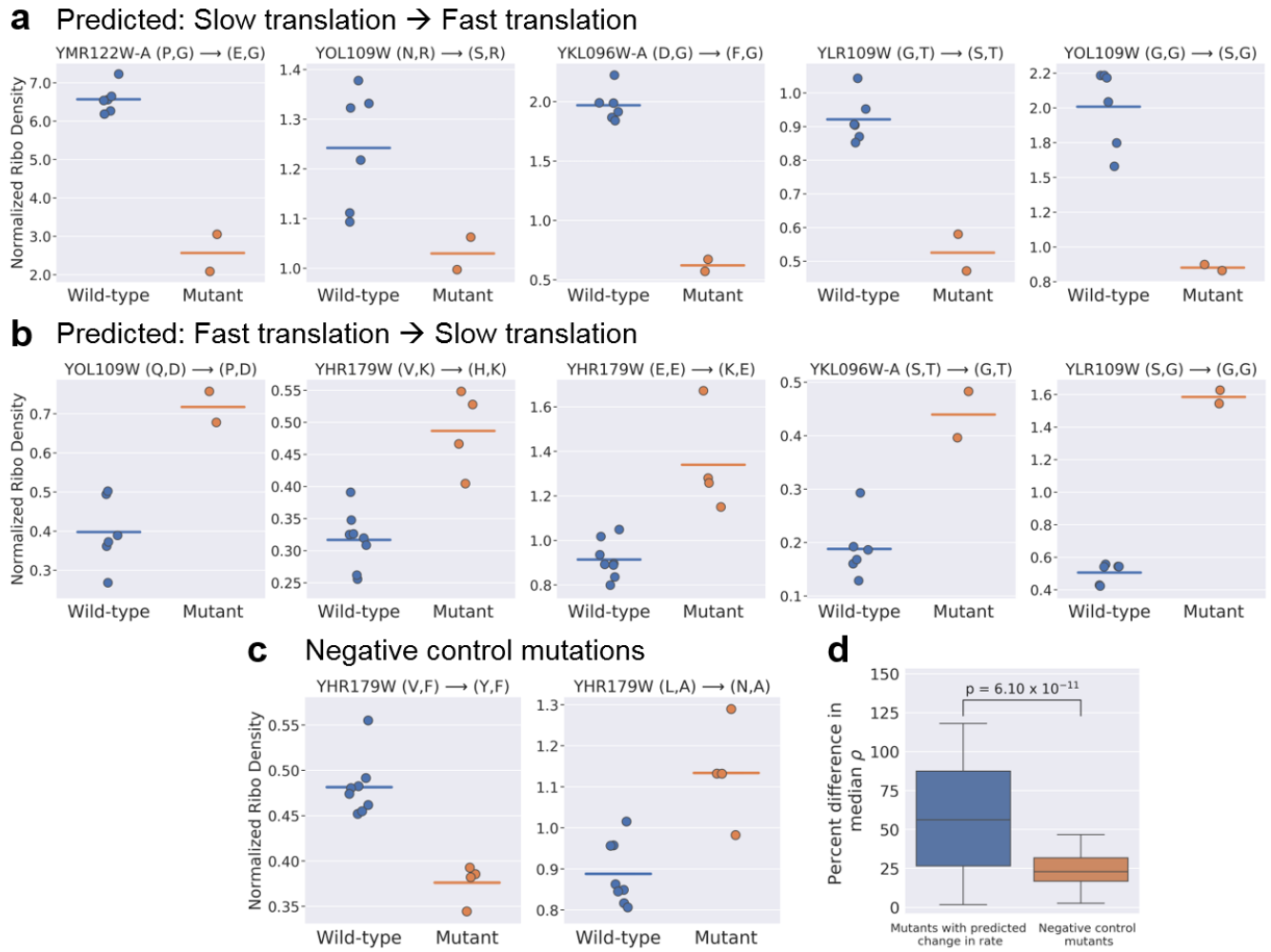


Figure 2: Experiments demonstrate that the identity of amino acids in the P- and A-sites can predictably alter the translation speed of the A-site codon, consistent with the predictions from Fig. 1b. Normalized ribosome density (Eq. S1) upon mutation at five pairs of residues that are predicted to slow down translation **(a)**, five other pairs that are predicted to speed up translation **(b)**, and two negative control mutations that are predicted to have little to no effect on translation speed **(c)** were measured in *S. cerevisiae*. The gene name and pair of amino acids before and after mutation are listed above the panels in (a) through (c). Full details concerning the mutations are provided in Table S2. In each panel, the normalized ribosome density measured at the A-site residue is reported for the wild-type sequence transcript (blue data points) and mutated sequence (orange data points). Each data point corresponds to one biological replicate; the horizontal bar indicates the mean value. The difference between the medians in each panel is statistically significant (Fisher-Pitman permutation test, $p = 0.036$ for all subpanels in (a) and for mutations in YOL*, YKL* and YLR* in (b). $p = 0.002$ for two mutations in YHR* in (b). $p = 0.002$ and $p = 0.004$ for the two subpanels in (c), respectively). The distribution of percent differences in ribosome density between the mutant and wild-type sequences for the data in panels (a) and (b) is shown as a blue box plot in panel **(d)**, and for the negative control sequences in panel (c), the distribution is shown as an orange box plot in panel (d). The mutations in the negative controls do show a statistically significant difference in normalized densities compared to wild-type (Fisher-Pitman permutation test, $p = 0.002$ and $p = 0.004$)**.** However, these mutations exhibit a 2.5-fold reduction in effect size (d), consistent with the predictions from Fig. 1b.

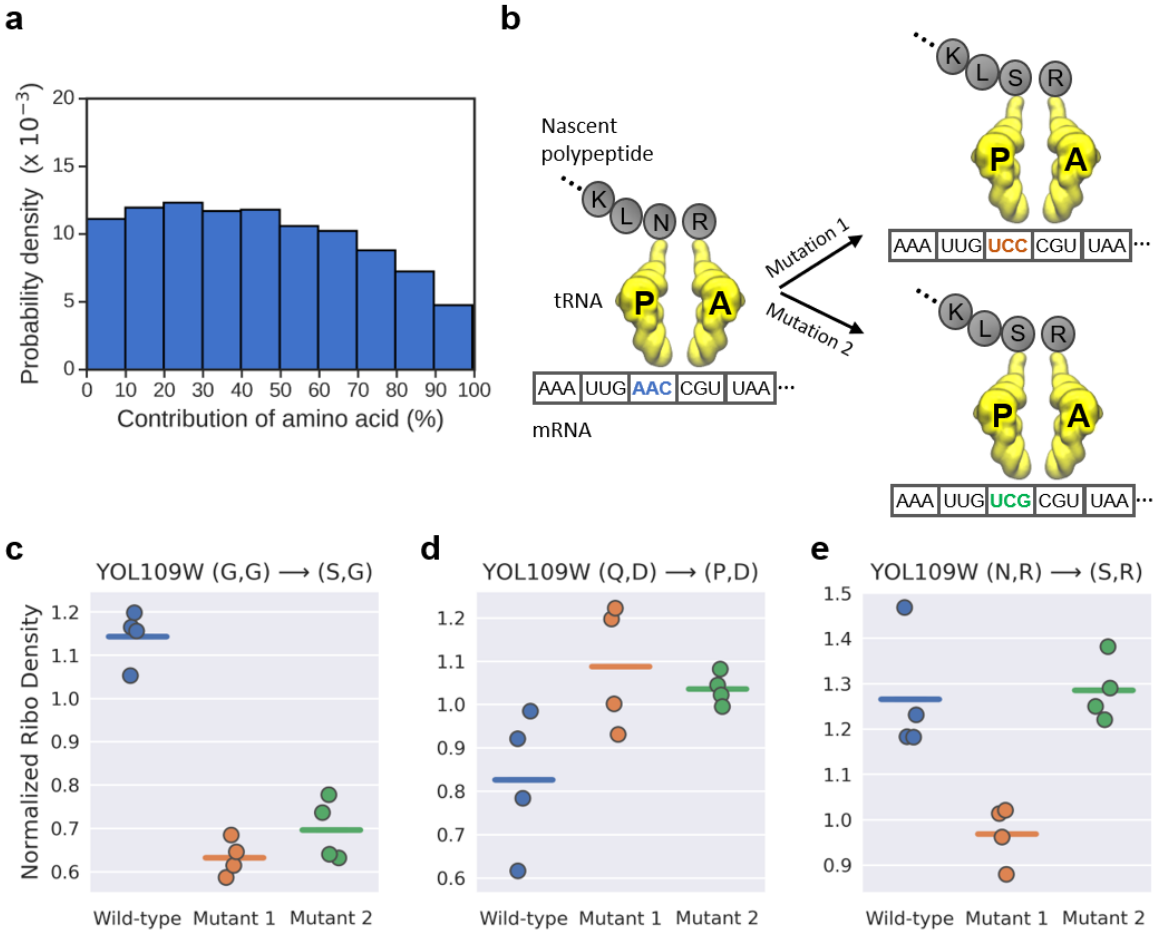
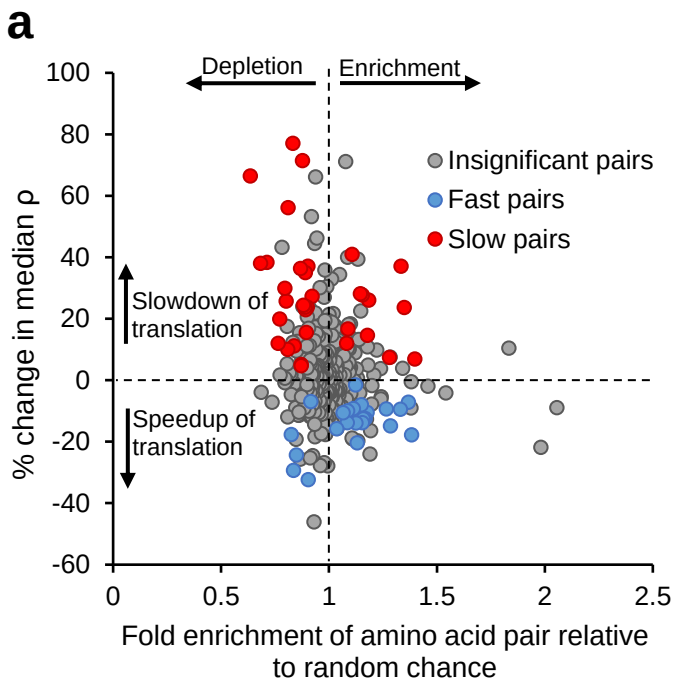
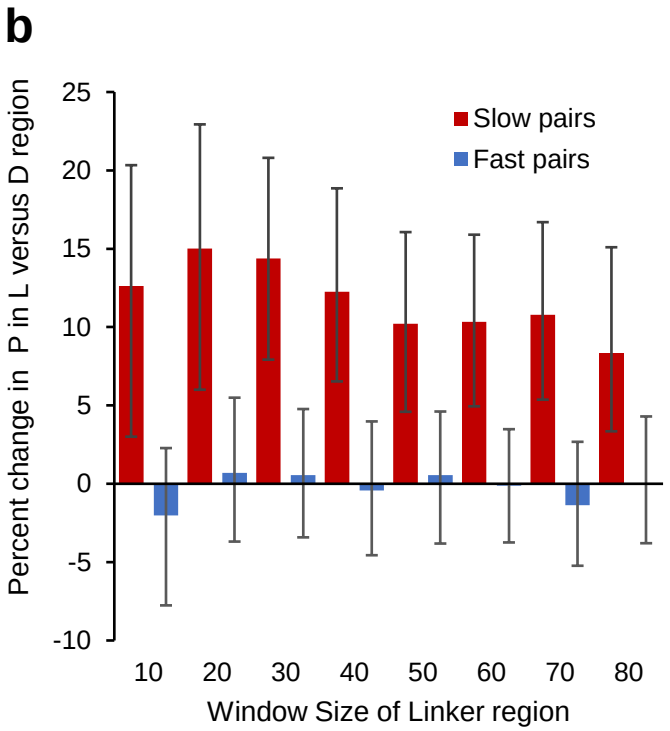


Figure 3: Depending on the amino acid pair, translation speed is influenced by either the identity of the tRNA pair, the amino acid pair, or both. (a) A bioinformatic analysis that models all 7,980 possible mutations to the P-site residue for a given starting amino acid pair (Eq. S4 and Methods section) estimates that speed changes are caused by a combination of the change in tRNA identity and amino acid identity. Plotted is the probability density of the estimated contribution of the change in amino acid identity to the speed change. For some pairs, upon mutation, the speed change is entirely due to the amino acid change (*i.e.*, data near 100%), for some, the speed change is entirely due to the tRNA molecule change (*i.e.*, data near 0%), and for others, the speed change is due to a combination of the two changes. **(b)** For the same amino acid mutation, two mutants are created using synonymous codons decoded by different tRNAs. **(c-e)** To experimentally measure the contribution of amino acid identity, a given non-synonymous mutation in the P-site was encoded using two different synonymous codons, each resulting in the same amino acid mutation but decoded by different tRNA molecules (see (b)). For the mutations *YOL109W* (G,G)→(S,G) (c) and *YOL109W* (Q,D)→(P,D) (d), the change in ribosome density from wild-type was similar for both synonymous mutants (Mutant 1 vs Mutant 2, Fisher-Pitman permutation test, $p = 0.1857$ and $p = 0.7714$, respectively), and hence, the amino acid is the predominant cause for the change in translation speed. For the mutation *YOL109W* (N,R)→ (S,R) (e), the speedup was seen for only one mutant while the other mutant exhibits a normalized ribosome density indistinguishable from that of the wild-type (Wild type *vs*. Mutant 2, Fisher-Pitman permutation test, $p = 0.1857$), indicating in this case that the tRNA identity is the predominant cause for the change in speed


a
Depletion
Enrichment
Insignificant pairs
Fast pairs
Slow pairs
Slowdown of translation
Speedup of translation
% change in median ρ
100
80
60
40
20
0
-20
-40
-60
0
0.5
1
1.5
2
2.5
Fold enrichment of amino acid pair relative to random chance


b
Slow pairs
Fast pairs
Percent change in P in L versus D region
25
20
15
10
5
0
-5
-10
10
20
30
40
50
60
70
80
Window Size of Linker region

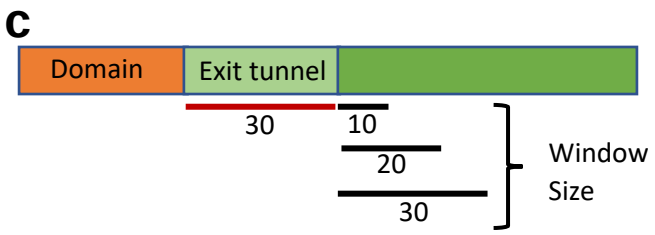

c
Domain
Exit tunnel
30
10
20
30
Window Size

Figure 4: Evolution selects for fast-translating pairs across the proteome but enriches slow-translating pairs across interdomain linker regions. (a) The enrichment and depletion of amino acid pairs across the *S. cerevisiae* proteome is plotted against the percent change in median normalized ribosome densities (ρ) of amino acid pairs taken from Fig. 1b. Among the top 20% enriched and top 20% depleted set, the odds ratio of fast-translating pairs being enriched and slow-translating pairs being depleted is 7.5 (Eq. S6, Fisher's exact test, $p = 0.0011$). **(b)** The enrichment of fast- and slow-translating pairs in linker (L) regions relative to domain (D) regions. The percent change is calculated as $\frac{f(X,L)-f(X,D)}{f(X,D)} * 100\%$, where $f(X,L)$ is the fraction of either slow or fast pairs in the linker region, and $f(X,D)$ is the fraction of either slow or fast pairs in the domain regions. A positive percent change would indicate an enrichment in the linker region, while a negative value would indicate a depletion. As a test of robustness, $f(X,L)$ was computed over different window sizes in the linker region, discarding the first 30 residues after the domain to account for those residues being in the ribosome exit tunnel, as illustrated in panel **(c).** $n = 170$ for a window size of 30 residues. For all window sizes, the percent change was significant ($p < 0.05$) for slow-translating pairs and insignificant ($p > 0.05$) for fast-translating pairs. p-values were computed using the random permutation test, and error bars in (b) represent 95% CI calculated from bootstrapping.