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Specific Codons Control Cellular Resources and Fitness

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GLOSSARY

- **RSCU**: Relative synonymous codon usage
- **W_{ij}**: Relative adaptiveness (weight)
- **CAI**: Codon adaptation index
- **ENC**: Effective number of codons
- **CUB**: Codon usage bias
- **TAI**: tRNA adaptation index
- **sTAI**: Species-specific tRNA adaptation index
- **nTE**: Normalized translational efficiency
- **RFM**: Ribosome flow model
- **CFP**: Cyan fluorescent protein
- **YFP**: Yellow fluorescent protein
- **TxTL**: in vitro transcription-translation
- **UTR**: Untranslated region
- **AUC**: Area under the curve
- **Fitness**: Performance of induced culture ÷ Performance of uninduced culture
- **Growth Fitness**: AUC of growth curve (induced) ÷ AUC of growth curve (uninduced)
- **Co-Expression Fitness**: AUC of YFP fluorescence (with induced CFP or mCherry) ÷ AUC of YFP fluorescence (with uninduced CFP or mCherry)
- **Expression Level**: AUC of fluorescence from induced over-expressed protein (CFP or mCherry)
- **CHI (χ)**: Codon harmony index
- **MFE**: Mean free energy

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ABSTRACT:

As biotechnology research progresses from simply overexpressing proteins to creating intricate metabolic pathways, gene circuits, and complex phenotypes, harmonizing gene fitness in the context of a host organism has become essential. A significant amount of recent work has focused on decoupling gene expression from host resources to improve the outcome of synthetic biology and metabolic engineering efforts. While insightful, few of these studies have investigated the mechanistic underpinnings of resource allocation during translation elongation. There is a degeneracy in codons – but they are not equivalent. While there is an understanding that codon use is unequal in native genes, there is less knowledge of how this usage bias modulates the supply and demand of protein translation resources. Here we investigate how the partitioning of microbial translational resources, specifically through allocation of tRNA by incorporating dissimilar codon usage bias, can drastically alter expression of proteins and reduce the burden on the host resources. By isolating individual codons experimentally, we find heterologous gene expression can *trans*-regulate fitness of the host and other heterologous genes. Interestingly, specific codons drive profitable or catastrophic phenotypic outcomes. We correlate codon usage patterns with genetic fitness and empirically derive a novel coding scheme for multi-gene expression called Codon Harmony Index (CHI, χ). CHI enables the design of harmonious multi-gene expression systems while avoiding catastrophic cellular burden.

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INTRODUCTION:

The genetic code is degenerate with 61 codons and only 20 amino acids, creating an astronomically high level of mRNA sequence space for most protein coding genes. However, it is well accepted that synonymous codons are not equivalent[1,2], as numerous reports of *cis* and *trans* effects have been documented[3–11] – from mRNA structure and co-translational protein folding[12–14] to tRNA and ribosome competition[15–17]. Re-coding proteins typically proceeds through use of a codon adaptation index (CAI), which enables a gene to assume the codon usage bias (CUB) of a reference set, often a set of highly expressed genes[18]. This strategy may generally correlate CUB with protein expression, but it ignores the role CUB can play in partitioning translational resources such as tRNA and ribosomes. Several recent studies have demonstrated the ability of heterologous genetic CUB to *trans*-regulate host gene expression through translational resource completion[19,20], but there is little understanding of how specific CUB alters host fitness given that cellular resources are invariably limited. Re-coding strategies such as the tRNA adaptation index (tAI)[7,21] and normalized translational efficiency (nTE)[6] are attempts to address tRNA related translational supply-demand constraints, but they are limited by how predictive natural CUB and/or tRNA levels are for recombinant protein expression.

It is particularly important to consider translational resource competition in the context of multi-gene expression (e.g., in the case of metabolic engineering and synthetic biology), where the objective is often for global organism fitness in addition to high protein expression, and tradeoffs in protein expression can be highly consequential for pathway or genetic circuit function and robustness[22]. This area is currently underexplored, as most studies to date focus on feedback control mechanisms[23,24], resource partitioning[25,26], or attempt to draw inferences about elongation in larger genes from libraries limited to the 5’ sequence of a reporter[27,28], and experiments that do not isolate translation elongation from initiation effects[10]. As cellular engineering becomes increasingly complex, genetic resource competition can unravel designs and lead to unpredictable and undesirable phenotypes. While a role for CUB in the partitioning of cellular resources has been reported[29], identification of specific codons that present excess translational capacity could provide a novel avenue for harnessing underutilized resources that are insofar ignored.

In this study, we systematically isolate the role of codon choices during translational elongation and identify supply-demand constraints imposed on tRNA and ribosomal resources in *E. coli*. We demonstrate that tRNA limitations lead to competition between overexpressed genes as well as with the host’s demands. Select codons over-represented in native highly expressed genes are found to cause severe fitness costs when present in overexpressed protein sequences. While the traditional method of codon-optimization through maximizing CAI may promote use of these codons, our data reveal their demand and supply are delicately balanced. We define a new metric called "Codon Harmony Index” (CHI, χ) that quantitatively ranks codons by their capacity to remain orthogonal to host demands. We also posit using this metric as a new codon optimization scheme to mitigate competition with host demands and avoid growth defects. Genes characterized by high scores on this metric scheme demonstrate relatively high expression while minimizing the burden on the host cells, allowing effective multigene expression and cellular growth.

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RESULTS:

Fitness costs are incurred due to translation elongation limitation.

Genetic burden is frequently observed in microbial systems as a growth defect upon the overexpression of recombinant proteins[24]. While the cause of this effect varies, it is often attributed to resource competition at the level of mRNA translation[30]. In a fast-growing culture of *E. coli*, the availability of free ribosomes can limit mRNA translation, especially in a system with overexpressed protein[31] (**Figure 1a**). Elongation speed determines the rate at which free ribosomes are made available, hence sub-optimal mRNA transcripts that are poorly translated have higher ribosome occupancy. Such elongation limited mRNA sequences will sequester more ribosomes and return them to the free pool at a slower rate, thus reducing ribosome availability. Translational resource competition has been modeled in several ways[32], including the ribosome flow model (RFM)[33], which can be useful in examining translation rate as a function of elongation time that varies depending on the supply and demand of tRNAs in the cell. Applying a previously developed RFM[34] to the model gene cyan and yellow fluorescent proteins (CFP and YFP respectively) with high or low CAI values (where CAI is in reference to highly expressed *E. coli* genes) illustrates the increase in mRNA ribosome occupancy that occurs when codons with longer elongation times[35] are used, and indicates that elongation-limited sequences are less sensitive to changes in the rate of translation initiation (**Figure S1**).

We first sought to investigate the impact of translation elongation resource competition using an *in vitro* transcription-translation (TxTL) model. A significant challenge to investigating translational resource competition is the difficulty in isolating any single sequence parameter experimentally, as any synonymous mutation can have a multitude of effects on initiation, elongation, and mRNA structure[2]. A TxTL system allows for better physical control over the genetic expression environment by holding available resources (e.g., ribosomes, tRNAs, aminoacyl tRNA synthetases, RNA polymerase etc.) constant, and allowing precise titration of genes of interest in the reaction. We developed an assay for elongation limitation by leveraging the unique amino acid sequence similarity between CFP and YFP derived from a super-folder green fluorescent protein[36], which only differ by 2 amino acids[37], thus eliminating variability in protein structure and amino acid demand. The CFP-YFP pair permits the interrogation of competition between various sequence designs using effectively identical proteins, which should also be less susceptible to variation in co-translational protein folding due to their high stability. We also include mCherry in the study, which is <30% identical to CFP-YFP and serves as a comparison point to find trends independent of amino acid sequence (see supplementary data for sequences). The TxTL kit is based off the *E. coli* MRE600 strain, which has a nearly identical CUB as K12 MG1655 and is therefore assumed to be a good proxy for the tRNA profile in a K12 strain used subsequently (**Figure S2**). Reactions were driven by a T7 promoter using a bicistronic domain (BCD) in place of a traditional ribosome binding site to minimize interactions between the 5' untranslated region and gene of interest that could lead to differential expression[38]. To further isolate translation elongation as the primary variable in sequence design, we chose to keep the 5' and 3' untranslated regions (UTRs) as well as the first 51 base pairs (17 codons) constant to mitigate any effect sequence changes may have on translation initiation (**Figure 1b**).

Utilizing the idealized TxTL competition assay, we evaluated baseline expression rates from CFP, YFP, and mCherry re-codes with extreme CAI values (0.96, 0.25, or 0.16) (**Figure 1c**). We find that identical sequence pairs for CFP-YFP behave very similarly in terms of relative expression, and that protein expression rates for CFP, YFP, and mCherry correspond well with CAI value. This supports that TxTL recapitulates translation elongation limitation – i.e., genes with lower CAI that use lower abundance tRNAs show lower protein synthesis rates. Next, we examined competition between different pairs of genes. As in the RFM, we expected elongation-limited sequences with lower CAI to disrupt expression of other genes through the sequestration of free ribosomes. We titrated CFP template DNA against constant YFP or mCherry DNA using re-codes with either very high or very low CAI (**Figure 1d–g**). For instances of two identically re-coded sequences with any CAI tested, YFP and mCherry synthesis rates are inversely correlated with CFP DNA concentration (**Figure 1d–e**), irrespective of their baseline expression, indicating strong competition for limiting resources (i.e., tRNA). This indicates that while an excess protein synthesis capacity exists in the TxTL system, sequences with lower CAI are still resource-limited, likely due to lower availability of tRNA.

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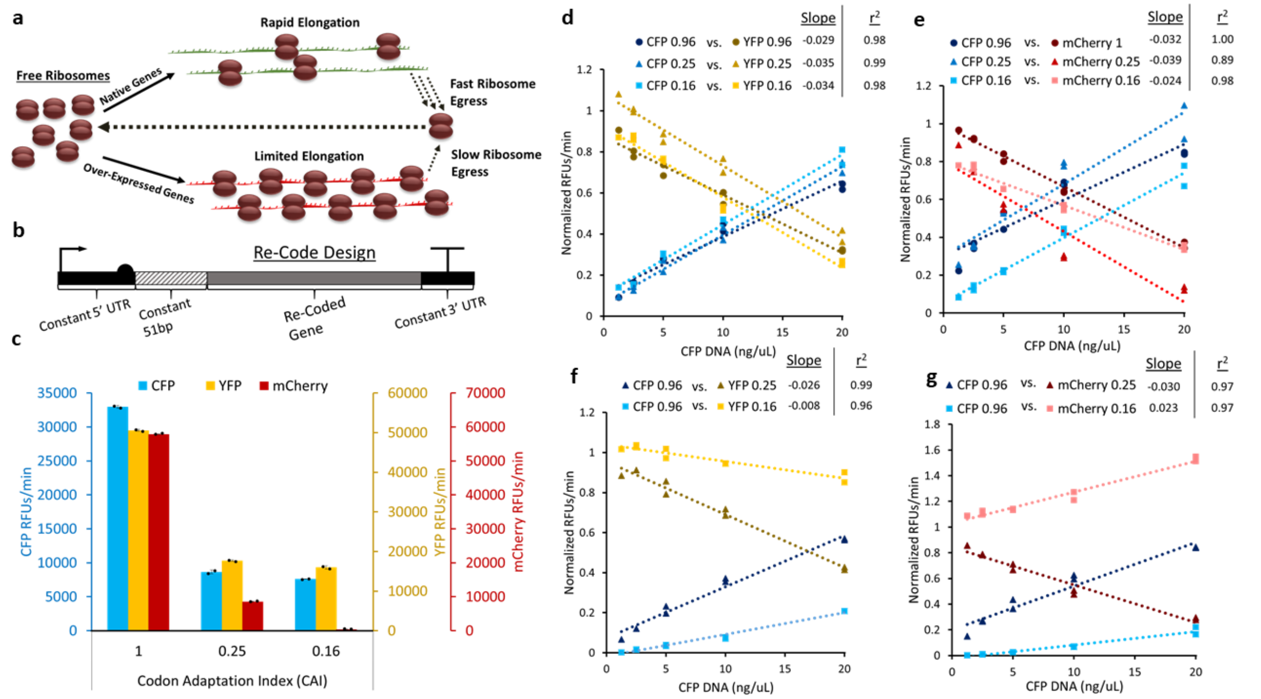

Figure 1: Elongation-limited TxTL system exhibits direct competition for tRNAs and/or ribosomes. a. Schematic of two actively translated genes competing for ribosomes. Overexpressed genes with low adaptation to tRNA use are expected to exhibit higher ribosomal occupancy and sequester excessive translational resources. **b.** Gene design for expression in TxTL assay. A T7 promoter followed by a strong RBS (BCD7) drives expression of re-coded genes, where the 1st 51 nucleotides are constant, as well as the 5′ and 3′ UTR. **c.** CFP, YFP, mCherry raw quantified expression rates *in vitro* in isolation with a TxTL assay with individual data points displayed over means (n=2). Individual expression cassettes (DNA) were each added to 20 ng/μL. Lower CAI values generally reduce protein synthesis rate across all genes. **d–g.** Competitive *in vitro* TxTL results between pairs of CFP and YFP or mCherry. The gene and corresponding CAI value are indicated in the figure legend. Protein synthesis rates are normalized to those in isolation. YFP and mCherry expression cassettes (DNA) were each added to 10 ng/μL whereas the CFP cassette (DNA) was titrated from 1.25 – 20 ng/μL. Panels d–e exhibit competition between sequences with identical codon usage, while panels f–g are between dissimilar sequences. Elongation-limited low CAI sequences are less affected by high CAI sequences but can cause catastrophic reductions in system-wide gene expression. Negative slopes for YFP and mCherry indicate competition for protein expression resources.

More interesting observations are seen when dissimilar CAI re-codes are under competition upon co-expression (**Figure 1e–f**). Low CAI YFP and mCherry synthesis rates are not very sensitive to increasing resource demand by high CAI CFP synthesis. Conversely, the relative CFP expression is much lower than we observed either in isolation or when competing with a high CAI sequence. The observed results appear to be consistent across different sequence pairs, indicating that this phenomenon is independent of protein sequence. When examined in the context of an RFM, we deduce that the rare codon enriched YFP and mCherry sequences sequester ribosomes to such a degree that even excess CFP template DNA does not yield high synthesis rates. On the other hand, YFP and mCherry are not affected due to severe elongation limitation. This model is further supported by our observation that YFP and mCherry rates are reduced when competing with similarly re-coded low CAI CFP sequences, which is a likely consequence of competition for scarce tRNAs. Overall, our data indicates that proteins coded with similar CAI (high or low) are strongly competitive due to demand for the same tRNA pool. Conversely, genes coded under distinct CAI regimes are constrained by the availability free ribosomes, which are in turn limited due to slow/stalled translation from scarce tRNA resources. Our TxTL data strongly support the argument that translation

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elongation limitation could play an important role in cellular resource competition and highlights the impact to global translational resources (e.g., free ribosomes, tRNA) in multigene expression environments.

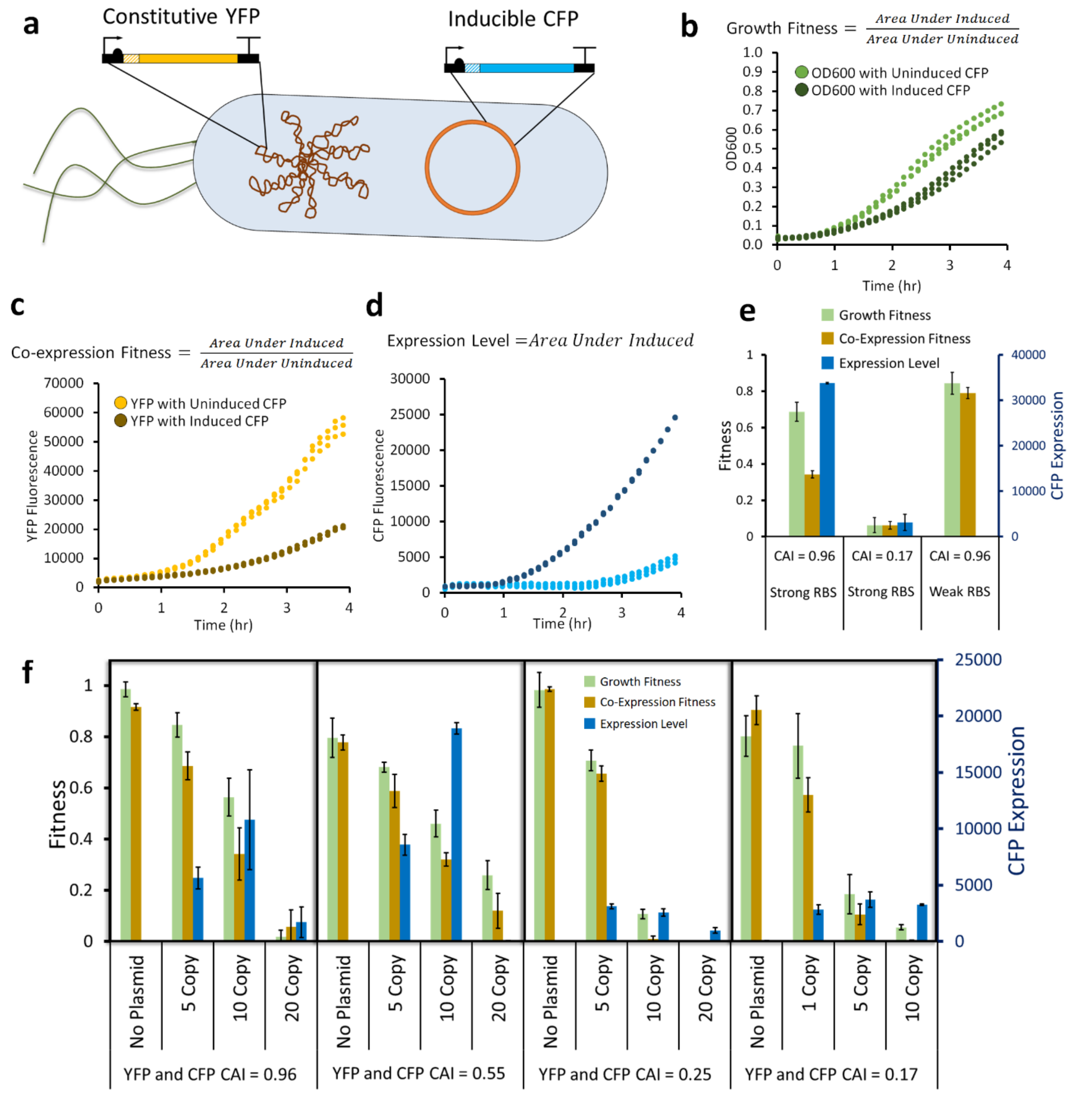

Figure 2: Optimization of *in vivo* fitness assay. a. Schematic of in vivo system, with constitutive YFP integrated into *E. coli* chromosome, and inducible CFP expressed from plasmid. 5' and 3' UTRs along with the 1st 51 bp are held constant for all re-codes. **b.** OD_{600} data with a growth fitness cost due to CFP induced burden. **c.** YFP fluorescence data with a Co-expression Fitness cost due to induced CFP protein burden. **d.** CFP fluorescence data used to determine Expression Level. **e.** Analysis of fitness and Expression Level for control CFP sequences reveals burden created by gene expression is dependent on translation (n=3). CFP is expressed from a 10-copy vector with indicated varying CAI, whereas YFP (CAI = 0.96) is constitutive and chromosomally integrated. **f.** Fitness and expression data for re-coded CFP-YFP pairs with co-varying CAI, where CFP is expressed from different copy number plasmids (n=3). Matching CFP and YFP CAI as indicated in figure. Higher copy plasmids and/or lower CAI re-codes impose a higher burden. Copy #s: 1 = f1 origin, 5 = pSC101 origin, 10 = p15A origin, 20 = pBR322 origin.

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We next set out to optimize an in vivo system for *E. coli* expression to efficiently interrogate the effect alternative recoding designs have on gene expression and host fitness. Our system generally consists of a strong constitutively expressed YFP reporter gene (CAI = 0.96) integrated into the *E. coli* chromosome paired with an inducible CFP on a plasmid driven by the inducible promoter P_{trc} with a strong RBS (**Figure 2a**). As before, we held the 1^{st} 51 bases and the 5' and 3' UTRs constant for all re-codes. Cells grown in rich medium with a common pre-culture were passaged under inducing or non-inducing conditions. The area under the curve (AUC) is used to measure each of the 3 signals (growth and 2 fluorescent proteins), which captures the aggregate effects of different lag phases and expression rates (**Figure S3**). We define fitness as the ratio of AUC induced vs. uninduced, which ranges from 0 to 1 for low and high fitness, respectively (or conversely, high and low burden). Fitness can be in terms of Growth Fitness based on OD_{600}, or Co-expression Fitness based on YFP fluorescence (chromosomal reporter), while Expression Level is based on CFP or mCherry fluorescence (i.e., the overexpressed protein) (**Figure 2b-d**). We generally observed a reduction in both growth and YFP fluorescence upon CFP induction. Examining several controls expressed from a p15A origin in **Figure 2e**, a "codon-optimized" high CAI CFP gene expresses well but elicits a significant fitness cost in terms of Growth Fitness and Co-expression Fitness. For a CFP recoded with rare codons, the result is catastrophic, and cultures are unable to grow at all. The effect also seems mediated by translation (not transcription) since the codon-optimized CFP with a very weak RBS, but intact promoter neither synthesizes protein nor demonstrates much fitness cost. Upon varying plasmid copy number with several pairs of CFP and YFP with different CAI levels, we found that fitness costs (Co-expression and Growth) were strongly dependent on copy number that is further exacerbated by low CAI (**Figure 2f**). Interestingly, the CFP Expression Level was not very correlated with CAI nor copy number. Based on these results, we picked the 10-copy vector (p15A origin) with the YFP CAI = 0.96 chromosomal reporter as the platform for further studies to investigate re-coding schemes that may reduce fitness costs.

Systematic analysis of codon use reveals supply and demand constraints in tRNA resources.

Prior to designing novel re-coded genes that moderate translation elongation resources, we first investigated CUB in the *E. coli* transcriptome. CAI calculations are typically based on the natural CUB in highly expressed genes. CUB can be represented as a 64-dimensional space (total number of codons) using RSCU values (observed vs. expected frequency) for each protein coding gene. Initial analysis revealed that groups of genes within the *E coli* transcriptome cluster according to distinct CUB schemes (**Figure S4**). We focused on a consolidated set of this sequence space by analyzing all operons with at least 2 protein coding genes, given that functionally related genes that naturally cluster have similar CUB (**Figure S5**). The resulting 64 dimensions of codon usage across 773 operons can be represented in 2 dimensions accounting for 41.2% of total variance (**Figure S6**) using principal component analysis (PCA) as shown in **Figure 3a**. The loading vectors mapped onto the plot represent the 10 codons that contribute most significantly to codon bias across the 773 operons.

This analysis captures the CUB naturally observed in the *E. coli* transcriptome and highlights a positive correlation between CAI and expression. This is expected because here CAI is calculated by optimizing towards CUB in highly expressed genes[18] (see methods) (**Figure S7**). Consistent with previous studies, we corroborate that genes in the most extremely biased CUB space are some of the most highly expressed genes in the *E. coli* proteome that often serve essential functions (**Figure 3b).** The natural bias leading to the CAI scale is very well explained by PC1 (**Figure 3c**). Despite the apparent correlation between CAI and expression, studies have reported that CAI often does not predict higher gene expression[10]. Importantly, the CAI paradigm of re-coding proteins to match the CUB of highly expressed genes ignores potential resource competition that can occur at the tRNA level. For 18 of 20 amino acids, multiple codons exist, and 10 of 18 of those can be coded to use different tRNAs in *E. coli* K12 MG1655 (**Figure S8**). Upon examining the PCA loadings, there are clearly particular codons that are very overrepresented in highly expressed proteins (e.g., arg CGT, leu CTG, and pro CCG). For such high-demand codons, using alternative codon/tRNA pairs, or even codons that recruit tRNAs with weaker affinity, have the potential to reduce translation elongation-based resource competition between overexpressed proteins and native essential and/or highly expressed genes.

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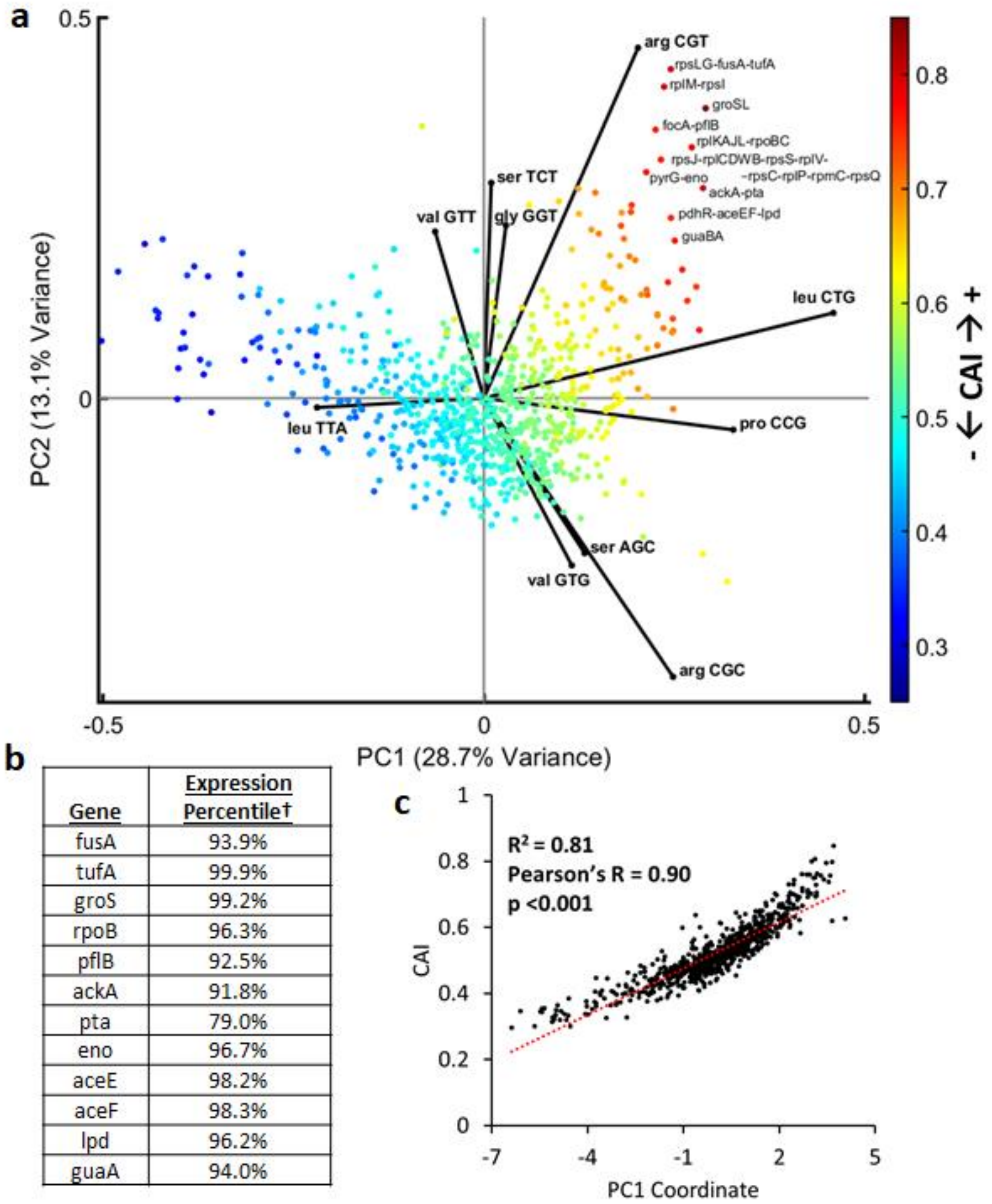

Gene	Expression Percentile†
fusA	93.9%
tufA	99.9%
groS	99.2%
rpoB	96.3%
pflB	92.5%
ackA	91.8%
pta	79.0%
eno	96.7%
aceE	98.2%
aceF	98.3%
lpd	96.2%
guaA	94.0%

Figure 3: Codon usage bias in highly expressed *E. coli* genes. a. PCA analysis of RSCU in 773 *E. coli* operons with loadings mapped for the 10 codons with the highest contribution to variance. CAI is mapped onto individual operons and indicated in the figure legend. **b.** Select genes from the most extremely biased operons and their expression percentile. [†] Expression data from Taniguchi et al.[39] **c.** PC1 is largely explained by CAI with a very strong Pearson correlation.

Using our optimized in vivo assay, we sought to experimentally determine the contribution of individual codons to gene Expression Level and Co-Expression Fitness. The synonymous codon sequence space that could be explored in even a small gene such as CFP is experimentally intractable. Holding the first 51 bp constant and co-varying all possible synonymous codons would produce a massive library size of 1.8×10^{104}. While a more constrained codon library is possible, we chose a focused experimental approach by interrogating individual codon contribution to

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gene Expression Level and Co-Expression Fitness. Starting with a CFP or mCherry sequence having a high CAI (0.96 – 1.0) and using a single codon for each amino acid where the effective number of codons (ENC) = 20 (for details on ENC, see methods), for each amino acid we re-coded every instance to another synonymous codon, resulting in a total of 41 possible re-coded sequences (= 64 possible codons – 20 high CAI codons already in use – 3 stop codons) (**Figure 4a**). Results were normalized in terms of both Expression Level and Co-expression Fitness (defined in **Figure 2b**) relative to the high CAI parent control (**Figure 4b**) and indicate wide ranging benefits or costs. In several instances, alternative codons provide a significant improvement in Co-Expression Fitness across both mCherry and CFP. Variations in phenotypes could in part be due to different amino acid composition between mCherry and CFP, as the number of re-coded amino acids was not held constant between genes (**Figure S9**). We chose to re-code all instances of each amino acid so as not to limit the number of altered codons to the amino acid with the fewest instances. Most of the re-codes do not improve expression (**Figure 4c**), which is expected since they were derived from (and normalized to) high CAI sequences that emulate highly expressed genes. CFP and mCherry re-codes are also less consistent in Expression Level than Co-Expression Fitness, reflecting a higher degree of variability between genes in *cis* compared *trans* effects. Notably, there are several alternative codons for leucine, proline, and one for arginine, which robustly improve Co-expression Fitness, suggesting that dissimilar codon use could be a means to generally reducing heterologous gene burden. Expression Level and Co-expression Fitness do not correlate well for mCherry or CFP re-codes (**Figure 4d–e**), indicating that while there may be general tradeoffs between expression and fitness, there are many instances where specific codon/tRNA pairs possess excess translational capacity.

Novel recoding scheme yields genes with robustly improved fitness.

Next, we developed a new recoding index derived from Co-expression Fitness values for individual codons in **Figure 4b**. We chose to focus on fitness rather than expression since our primary aim was to investigate how re-coding schemes can modulate resource competition during translation elongation. To convert the Co-expression Fitness data for CFP and mCherry re-codes into generalized codon weights, we took the Euclidean distance from the origin to the coordinates of each data point shown in **Figure 4b** as a raw score for each sequence, where each parent codon held a normalized coordinate value of (1,1). Similar to calculating CAI, relative adaptiveness (W_i) scores were then determined by normalizing the raw weights from each amino acid codon set to the codon with the highest fitness (see methods and **Data S1**). We refer to this new metric as the Codon Harmony Index (CHI or χ).

A comparative analysis between CUB in the overall *E. coli* genome, CAI (using highly expressed genes as a reference), and χ reveals that χ favors very different codon use than CAI and discourages use of codons enriched in highly expressed genes (**Figure 5a**), notably for Arg CGT, Leu CTG, and Pro CCG. There are instances where χ and CAI do correspond well (e.g., Gly GGA, GGC, GGG), but many codons show inverse trends between the two scales. Generally, amino acids with multiple available tRNAs (including Arg, Leu, and Pro) correspond with larger differences between expected RSCU values calculated for CAI and χ (and shown in **Figure 5a**), suggesting that recruitment of different tRNAs is playing a role in determining Co-Expression Fitness (**Figure S10**). Interestingly, χ favored codons do not always correspond to amino acids with multiple available tRNAs, indicating tRNA abundance may not alone account for the observed effect, which could also be in part due to different translation efficiencies created by favorable interactions of tRNA codon-anticodon pairs.

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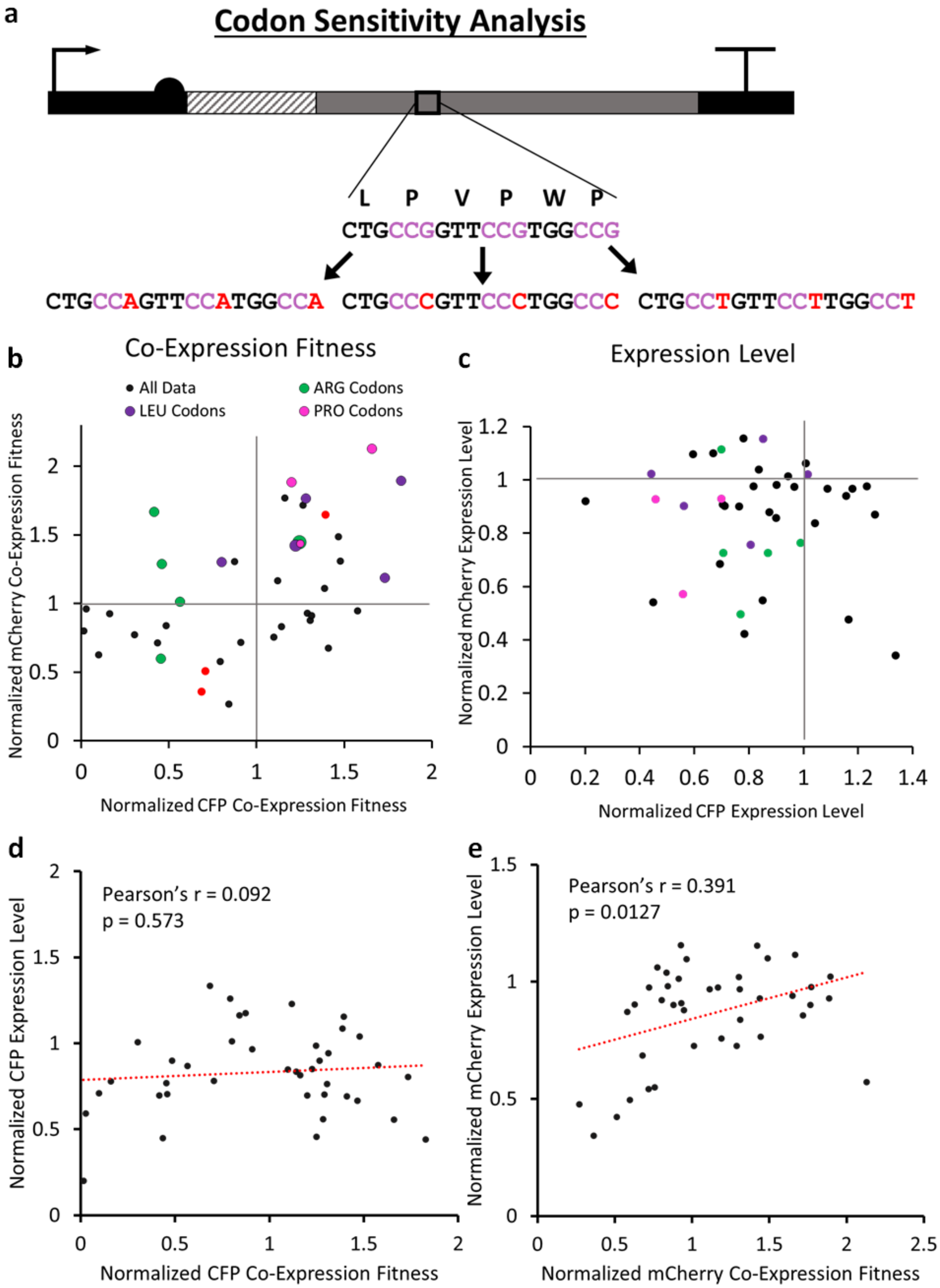

Figure 4: Systematic codon sensitivity analysis. a. Schematic of how genes are recoded for every amino acid. Starting with the highest CAI weighted codon for every instance of each amino acid, they are recoded to alternative synonymous codons. Example shown is for Proline. **b.** Mean fold change in (YFP) Co-expression Fitness upon CFP or mCherry co-expression, normalized to the parental (high CAI) control. **c.** Mean fold change in CFP or mCherry Expression Level relative to parental control. **d–e.** Poor correlations (Pearson's r) between fold change in Expression Level of CFP or mCherry re-codes with Co-expression Fitness.

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Utilizing the new χ weights, we next created several CFP and mCherry sequences that were optimized to varying degrees on the new χ scale (**Figure 5b**). Specifically, we created a χ = 1, ENC = 20 sequence, along with 4 sets of 3 different sequences each holding χ constant at 0.95, 0.85, 0.75, and 0.65 for both CFP and mCherry by using a greedy algorithm (**Figure S11**). The lower end of the χ scale for the CFP/mCherry genes was approximately 0.6, which is dictated by the protein sequence, and lowest W_{ij} values for each set of codons (see methods). Thus, the effective working range of χ is ~ 0.6 – 1.0 compared to CAI that operates from ~ 0.2 – 1.0. When the χ recoded sequences were assayed for fitness and expression (**Figure 5c–e**), there was a very strong positive correlation between CFP and mCherry analogous re-codes for fitness and expression, indicating that these synonymous coding schemes are a primary determinant for how a gene performs regardless of amino acid sequence. Remarkably, we also observe a strong positive correlation between χ and, both, Growth Fitness and Co-Expression Fitness—indicating that the weights derived from the individual codon assay are additive to improve the fitness of various globally-recoded sequences (**Figure S12**). High χ sequences clearly provide reduced competition for host resources and improved fitness. The χ scale is less predictive of expression, which is expected as it was not part of the criteria used to create the codon weights. Despite this, there is a good correlation between CFP and mCherry re-coded sequences in terms of Expression Level, indicating that codon usage bias does generally predict expression. Importantly, there are several sequences with reduced burden that retain relatively high expression, which represents an excess translational capacity for sequences re-coded using high χ values.

To investigate which codon usage bias patterns have the greatest contribution to Co-expression Fitness, we analyzed RSCU across all variable 59 codon dimensions (excluding stop, Trp, and Met codons) for each of the CFP and mCherry re-coded sequences (as seen in **Figure 5b**) using PCA (**Figure 6**). We were able to represent 46.7% of the total sequence variation in the first 3 dimensions (**Figure S13**) when analyzing the CFP and mCherry re-codes' RSCU along with 773 *E. coli* operons. Here again PC1 and PC2 primarily explain variation across *E. coli* sequences, but intriguingly we see a new highly orthogonal dimension in PC3 that explains variation in the χ sequences, and PC1 vs. PC3 best differentiate the χ re-coded sequences from natural *E. coli* operons. The χ sequences generally have intermediate to low values on the CAI scale with low overall CAI variation, meaning they would not have been predicted to express well using CAI (**Figure 6a**). This is somewhat surprising given that many of the re-codes with moderate to high χ (0.8–0.95) still exhibit relatively high expression compared with the high CAI control as demonstrated in **Figure 5e**. When mapping χ values to the data, we see that χ describes variation along PC3 very well (**Figure 6b, Figure S14**). *E. coli* operon sequences do not vary significantly on the χ scale, implying that the re-coded sequences explore novel coding schemes orthogonal to natural sequence space. Examining the loadings for the 3 most biased natural codons, we find that the high χ sequences are using synonymous variations for Arg, Leu, and Pro that differ as expected from highly expressed genes. We conclude that competition for tRNA isoacceptors in high demand by highly expressed essential genes primarily drives competition for translation elongation resources and avoiding specific codons that are over-represented in such native genes provides a novel strategy to improve the Co-Expression Fitness of heterologous genes.

Given the breadth of existing knowledge regarding codon optimization, we also evaluated how χ compares with other reported CUB strategies such as the tRNA adaptation index (tAI)[7] and normalized translation efficiency (nTE)[6]. These approaches weight codons based on their co-adaptation to the tRNA pool or the tRNA supply vs. codon demand respectively. We calculated the expected RSCU of a perfectly adapted gene sequence using these various scales to assess their degree of similarity (**Figure S15**), and found that stAI (species specific TAI using *E. coli* specific weights)[21] correlates the closest with χ (Pearson's r = 0.393, p = 0.002), but does not provide as much differentiation between codons available for each amino acid. We suspect the primary differentiator of the χ re-coding strategy relative to tAI or nTE is that it provides empirical insight into which specific codons have excess capacity for translation as opposed to an approach relying solely on genomic statistics and approximations. Further analysis of the χ re-coded sequences did not reveal any consistent correlation with secondary structure or GC content between CFP and mCherry re-codes, supporting the notion that specific codon use is likely driving sequence behavior (**Figure S16**). We also re-coded 10 random genes with 3 free commercial re-coding algorithms to analyze whether any of them exhibit exploration of χ related CUB strategies and found that they generally vary along classical *E. coli* CUB and seek to adapt to host codon use without optimizing in the χ sequence space (**Figure S17**).

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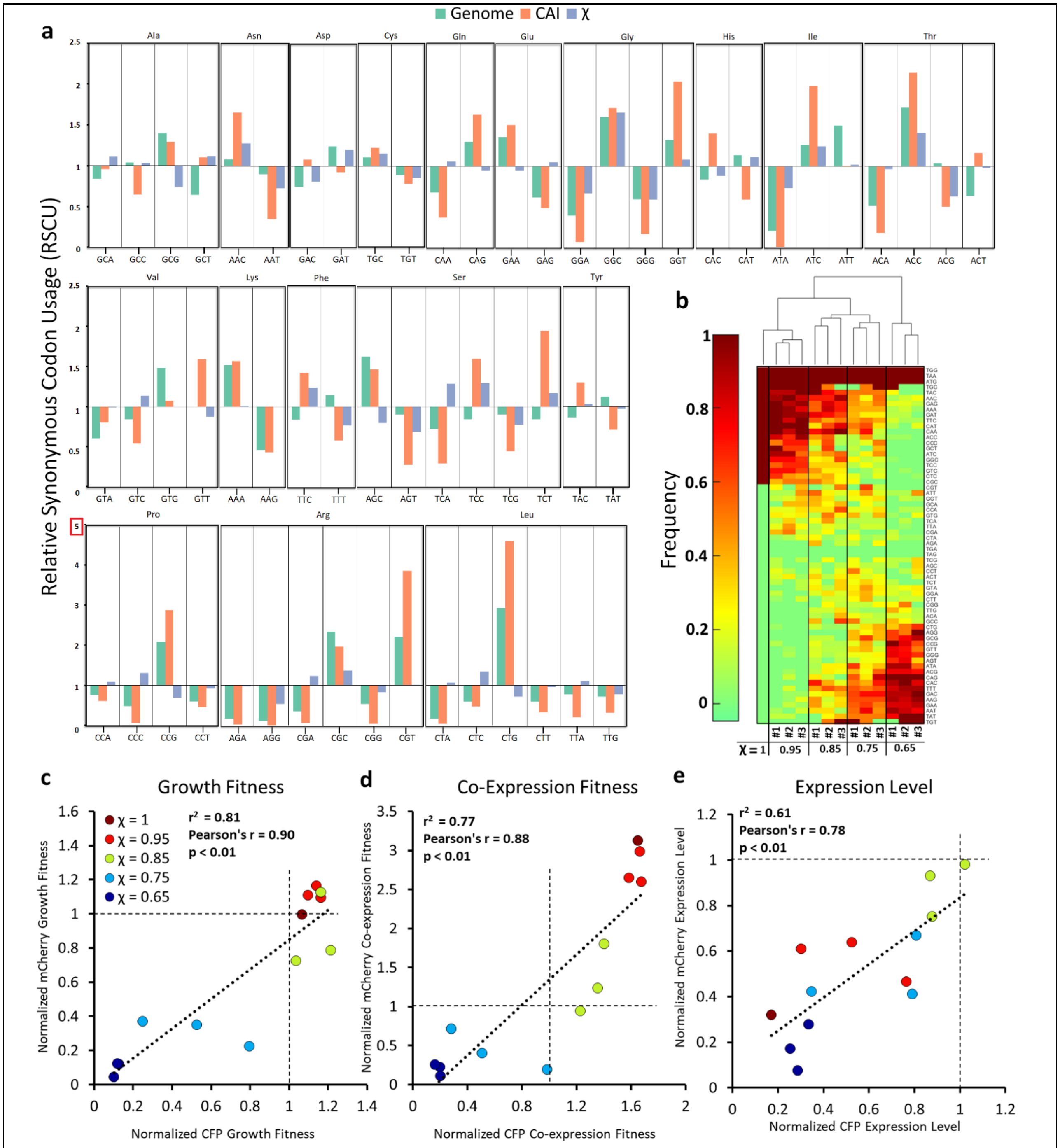

Figure 5: Codon Harmony Index (CHI, χ) used to design and test sequences for CFP and mCherry. a. Relative synonymous codon usage (RSCU) observed in the *E. coli* genome or calculated for weighted CAI and χ scales. **b.** Codon frequency of CFP or mCherry re-coded sequences using variable χ values illustrated on a clustered heat map. **c–e.** Growth Fitness, Co-expression Fitness, and Expression Level data for CFP and mCherry re-coded using χ. Note that for χ values range from ~ 0.6 – 1.0, unlike CAI that ranges from ~ 0.2 – 1.0.

In theory, χ could also correlate with CUB in phages that infect *E. coli* and have co-adapted to maximize gene expression without overwhelming host resources. There have been reports of not only co-adaptation to tRNA pools[40,41], but also translational selection for CUB dissimilarity between viruses and hosts to avoid excessive

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competition for tRNAs[42]. We examined codon usage in 12 common coliphages known to infect *E. coli* to examine whether CUB in such parasitic viruses may have evolved to harmonize with bacterial hosts as a means to allow better co-utilization of shared translational resources (**Figure S18**). Our analysis indicates that phage genes generally tend to avoid CUB at high values of CAI (> 0.7) and exhibit a slightly higher mean χ than *E. coli* genes. This suggests that it may be more productive in the phage life cycle to avoid excessive similarity and competition with their host, but there is another unique aspect of the CUB in χ that was not strongly selected for in phages. It is possible that the translational resource demand from an overexpressed protein on a multi-copy vector is higher than natural genes have encountered and is thus under a higher level of translational selection resulting in novel types of advantageous CUB reflected by χ that cannot be inferred from natural sequence space.

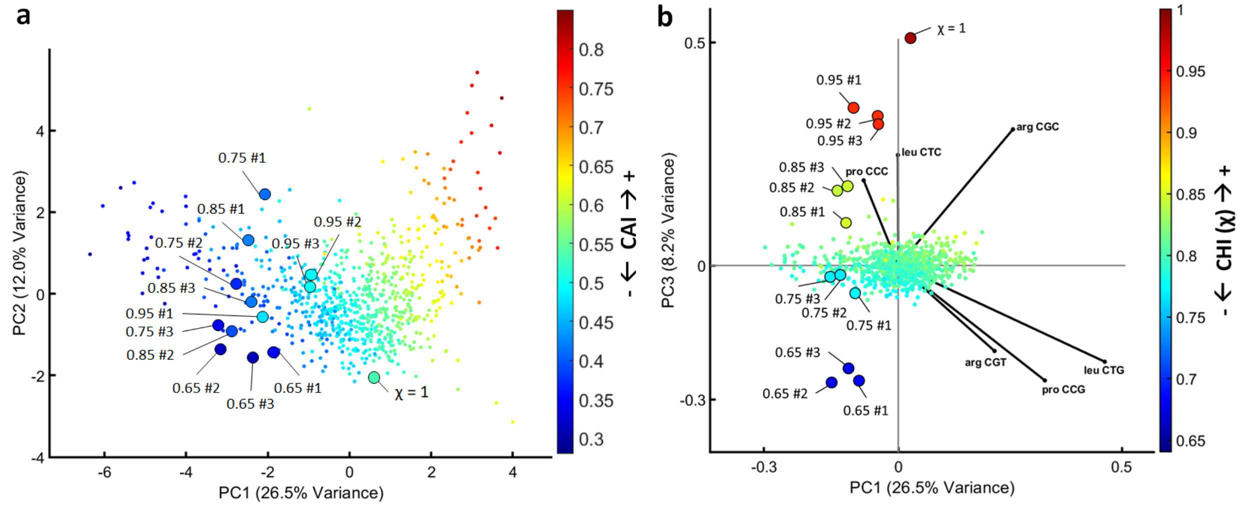

Figure 6: PCA analysis of χ and CAI metrics on *E. coli* and CFP/mCherry re-codes. a. PCA of 773 *E. coli* operons as well as 13 χ re-coded sequences with CAI value mapped to individual points showing PC1 vs. PC2. The χ re-codes all fall at low-modest CAI values (< 0.55). **b.** Same PCA with χ mapped to individual points instead of CAI showing PC1 vs. PC3. All *E. coli* genes map to modest χ values (~0.8). Both coding schemes are largely orthogonal.

DISCUSSION:

Protein translation is one of the most resource intensive cellular processes, which has yielded significant CUB observed in nature, especially in single cellular microorganisms often used as expression hosts[43]. Most conventional codon optimization strategies operate under the key assumption that translational selection in naturally evolved systems provides CUB that is relevant for the overexpression of heterologous genes. This may be partially true, but realistically, the overexpression of genes can push host resource demand beyond levels required for native gene expression[44], resulting in translational selective pressures that organisms haven't evolved with. Protein expression must also be considered in the context of increasingly complicated engineered systems, and often in synthetic biology and metabolic engineering efforts, overexpression is not nearly as important as reliable and predictable gene expression and host fitness[45]. Here we have revealed both in vitro and in an *E. coli* model that translation elongation can limit protein expression, and often has profitable or catastrophic consequences on system-wide resource availability.

In our TxTL assay, we found that proteins coded with similar CAI compete for the same tRNA supply, and re-coded genes can reduce such competition. Consequently, high CAI sequences are ribosome-limited, demonstrating reduced synthesis rates that are also highly sensitive to competition. In certain cases, low CAI genes are monopolistic or anti-competitive with free ribosomes and are thus insensitive to increased demand from high CAI sequences, albeit at the expense of overall resources. Theoretical frameworks have been well established to explain how resource limited translation can lead to the sequestration of ribosomes, but these studies generally

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rely on ribosome footprinting data[35] and tRNA copy number[6,7] to infer codon elongation times, which are indirect measurements of ribosome flux on a given mRNA.

Our novel experimental approach using an *E. coli* model demonstrates the sensitivity of system resources at individual codon resolution and reveals key differences between the optimal CUB for highly expressed native genes vs. overexpressed proteins. Several previous studies have investigated CUB using randomized libraries that fail to thoroughly explore the vast sequence space available when re-coding a gene[46]. Such randomized sequences will generally regress to intermediate RSCU values for each codon, and rarely sample the extremities of the sequence space available (**Figure S19**). By systematically re-coding individual amino acids to each alternate codon in multiple proteins, we have methodically investigated how individual codons contribute to gene Expression Level and Co-Expression Fitness at further extremities of the theoretical design space than have been previously explored. The avoidance of codons with very high CUB in native essential genes (e.g., for Arg/Leu/Pro) is a novel driver of reduced genetic burden.

We used individual codon sensitivity data to create a new re-coding strategy that optimizes for fitness (CHI or χ) and demonstrate how the new codon weighting method enables the creation of unique CUB strategies that are not represented naturally in *E. coli*. Using PCA for dimensional reduction, our methodology reveals how sequences with identical CAI scores can still exhibit distinct variations in CUB that result in different phenotypes, namely improvements in Co-Expression Fitness. Remarkably, globally re-coded sequences were found to have predictable phenotypes informed from the additive effects of individual codon use, allowing us to leverage a relatively small dataset to predict phenotypes in a vast sequence space. While global sequence characteristics including GC content, structure, and a variety of sequence motifs are all known to contribute to protein expression[2], our results suggest that codon bias is a strong predictor of both protein expression and fitness and can be optimized independently of the UTRs or 5′ coding sequence. An analysis of *E. coli* phage CUB reveals that while parasitic organisms may avoid over-use of preferred host codons, a concept that has been recently suggested[42], the demands of heterologous gene over-expression and resulting selective pressures are likely to have different resource demands than those of viruses, and thus may have overlapping yet still largely distinct CUB fitness landscapes.

The data-informed strategy in this study represents an approach that could be extended to other microbes including eukaryotic systems, where ongoing controversy over the impact CUB has on host-gene fitness has been unresolved[47–51]. While our study included 2 proteins (CFP and mCherry) with very different amino acid sequences, measuring Expression Level and Co-Expression Fitness for additional proteins could further refine χ, and provide additional insight for maximizing expression and fitness together. The new χ metric is more predictive of *trans* effects (Co-expression Fitness) than *cis* effects (Expression Level), thus further optimization of translation initiation and CUB that maximizes both expression and fitness is an interesting future objective. The observation that there are several sequences with relatively high expression and high fitness illustrates there are solutions to co-optimize both genetic traits. In practice, re-coding genes with high CAI will often lead to higher expression with low overall fitness, but re-coding with high χ values (between 0.9−0.95) should provide reasonably high expression with more orthogonal resource demands. Similar data sets could also be collected for any organism where protein expression is feasible, which could also provide insights into how species differ in the role CUB plays regarding resource allocation. It is possible that with more inter-species data, organism specific χ weights could be predicted *a priori* based on the avoidance of codons overrepresented in host genes. Practically, this study should improve the predictability and robustness of genetic engineering by enabling the co-optimization of gene expression and fitness, especially for multi-gene expression systems.

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MATERIALS AND METHODS:

Equations used to assess codon usage bias.

We calculated codon adaptation following the classical method reported originally by Sharp and Li[18]. This method relies on first calculating relative synonymous codon usage (**RSCU**) in a genetic sequence, which is defined by **Equation 1**:

$$RSCU_{ij} = \frac{X_{ij}}{\frac{1}{n_i}\sum_{j=1}^{n_i} X_{ij}} \qquad (1)$$

RSCU calculates the observed frequency of codon **j** belonging to amino acid **i** divided by expected frequency, where **X** is the number of occurrences for codon **j** in a given sequence. The expected frequency is simply the number of occurrences for any codon belonging to amino acid **i**, divided by the number of codons (**n**) available for that particular amino acid. RSCU is used instead of raw frequency values to normalize observed codon frequency based on the total codons available. An RSCU value < 1 indicates bias against the codon, while an RSCU value > 1 indicates a bias toward the codon, and RSCU = 1 indicates no bias. The RSCU values for each codon can be used to calculate relative adaptiveness (**W**), which is defined by **Equation 2:**

$$W_{ij} = {}^{RSCU_{ij}}\!/_{RSCU_{imax}} \qquad (2)$$

Relative adaptiveness is the RSCU for a codon **j** belonging to amino acid **i** divided by the RSCU for the codon in the set for amino acid **i** with the highest RSCU value (imax). In other words, W gives a value of 1 for codons in a target sequence that match the frequency of the most common codon in a reference sequence. W values are used in calculating the codon adaptation index (**CAI**) defined by **Equation 3:**

$$CAI = \left(\prod_{k=1}^{L} w_k\right)^{1/L} \qquad (3)$$

CAI is the geometric mean of the W values for each codon in a given sequence containing L codons. Importantly, the reference sequence(s) and calculated RSCU values that W values are derived from can be from any source. Unless otherwise indicated, in this study, CAI refers to W values for a set of highly expressed set of E. coli genes. Alternatively, CAI can be computed based on W values for CUB across the entire genome, sTAI weights[21], or χ weights (**See Data S2 for W values used in various calculations**). Normalized translational efficiency (nTE) was calculated as previously described[6] by taking the ratio of species specific TAI weights for E. coli[21] (supply) vs. the codon use across the E. coli transcriptome (demand) defined by **Equation 4**:

$$nTE_{ij} = {}^{sTAI_{ij}}\!/_{Frequency_{ij}} \qquad (4)$$

The nTE_{ij} values are analogous to W_{ij} values for the calculation of nTE, which proceeds the same as for CAI by taking the geometric mean across a sequence (as in equation 3). In this study, nTE was calculated using genomic codon frequency as opposed to codon use (originally defined as codon occurrence multiplied by RNA transcript abundance), as the two were found to be highly correlated (**Figure S20**). Lastly, the effective number of codons (**ENC**) is often used as a measure of codon bias in a sequence, and is calculated using **Equation 5:**

$$ENC = 2 + \frac{9}{F_2} + \frac{1}{F_3} + \frac{5}{F_4} + \frac{3}{F_6} \qquad (5)$$

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ENC can take a value from 20, in the case of extreme bias where one codon is exclusively used for each amino acid, to 61 when the use of alternative synonymous codons is equally likely. The value F is the average probability that two randomly selected codons for an amino acid with n number of synonymous codons will be identical[52].

Data sources used in analysis.

Genomic codon usage for *E. coli K12* MG1655 and *E. coli* MRE600 were assessed by analyzing codon bias from published annotated genomes obtained from NCBI under the accession numbers NC_000913.3 and CP014197.1 respectively using MATLAB. Phage analysis was done with annotated phage genomes from NCBI, and accession numbers are listed in **Figure S18**. Exact codon frequencies and relative adaptiveness values (W) used in this study for calculating CAI in reference to highly expressed genes CUB, entire genome CUB, sTAI, or nTE, can be found in **Data S2**. The W values for χ and associated information from the study can be found in **Data S1**. W values for highly expressed genes were originally downloaded online from GenScript, and were cross referenced to published values[53]. The sTAI codon weights were downloaded online from a publically available database (http://tau-tai.azurewebsites.net/)[21]. The tRNA copy numbers referenced in this study (**Figure S8**) were downloaded from the Genomic tRNA Database (http://gtrnadb.ucsc.edu/)[54].

Ribosome flow model.

The implemented ribosome flow model (RFM) (**Figure S1**) was adapted from Zur et al. using open source Matlab® code[34]. In this model, an mRNA is divided into n number of chunks, where each chunk is 9 codons (27 bases), approximately the footprint of an E. coli ribosome. Translation time of each chunk is based on local λ, which is a sum of the individual times it takes to translate each codon in a chunk. Codon times used are available in **Data S3**. Ribosome collisions are also accounted for in the model as a function of the ribosome density in adjacent positions. In this model, the protein production rate is the rate of translation of the final position on the mRNA. For this application, steady state ribosome densities were computed for CFP and YFP re-coded to use preferred (high CAI) or rare (low CAI) codons. To demonstrate the relationship between initiation rate and translation rate for different sequences, steady state protein production rates are calculated for different initiation rates.

Gene design and re-coding.

All genetic re-coding designs and analysis were executed in Matlab® using custom functions. Code is made available online at https://github.com/nair-lab. A full list of amino acid and DNA sequences used in this study can be found in **Data S4.** CFP and YFP were initially cloned through site directed mutagenesis of an existing super-folder GFP protein based on previously reported sequences.[37,55] For the systematic analysis of codon use design, CFP or mCherry were re-coded starting from highly biased sequences using the most preferred codon for each amino acid (CAI = 1 and ENC = 20), not taking into account the first 17 codons. The first 17 codons were held constant for all re-codes and were based on previously used sequences that functionally expressed well. A Matlab® script was then used to systematically design sequences where every instance of an amino acid was mutated to a single alternate synonymous codon. In the design of sequences with novel re-coding schemes, a greedy algorithm was used (**Figure S11**), that functions by randomly mutating a codon to a synonymous alternative, then evaluating whether the new sequence is closer to the target CAI (or in this specific instance χ value). To re-code CFP and mCherry to a desired χ value, a starting sequence was first randomized to ensure there was no initial bias, and then the algorithm was followed to the target χ value. We generated several unique output sequences with the same χ value but different coding sequences, then selected 3 sequences for each value of χ tested making sure they were substantially different from each other based on hierarchal clustering done in Matlab®.

Plasmids and strain construction.

All plasmids were cloned from existing vectors with restriction enzyme sites already present (**figure S21, S23, Data S4**), which also contained 5' and 3' UTRs. Genes were all custom ordered synthesized as full length double

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stranded DNA fragments with AarI restriction sites on the 5' and 3' termini. A type IIS restriction enzyme cloning approach with AarI was used to insert synthesized double stranded DNA gene fragments into the desired vector. All constructs were sequence verified from clonally pure DNA using Sanger sequencing across the gene and UTRs. The screening strain used to assess Co-Expression fitness was engineered from *E. coli* K12 MG1655 (CGSC#: 6300). The YFP reporter was integrated in an intergeneic region (~3,938,000 bp) between the rsmG-atpI genes using λ-Red based homologous recombination of the YFP CAI = 0.96 sequence, which was under the control of a strong constitutive promoter (FAB46) and RBS (BCD7) based on a previous study,[38] and a 5' insulator and 3' terminator (**Figure S22**, **Data S4**). The method of integration and marker excision method has been previously reported (Datsenko and Wanner).[56] Briefly, a linear cassette consisting of the gene, UTRs, and an attached kanamycin resistance marker was amplified by PCR with ~500bp of homology to the desired locus on either end. Chromosomally integrated clones were identified by colony PCR and sequence verified via Sanger sequencing of the PCR product including several hundred bases of chromosomal DNA and the entire integrated heterologous expression cassette. Sequence verified clones had the integrated kanamycin marker removed through the previously described FLP-FRT site specific recombinase method and were again Sanger sequenced for final verification.

in vitro transcription-translation (TxTL) assay.

The TxTL assay was carried out using the NEB PURExpress® kit (E6800). This assay relies on T7 polymerase, and consists of purified reconstituted components. Accordingly, CFP, YFP, and mCherry expression cassettes were first cloned into a pBAC vector with a T7 promoter and strong RBS (BCD7) (**Figure S23 a–b, Data S4**). The genes were also flanked by an insulator and terminator sequence on the 5' and 3' UTR respectively. Once clonally pure and sequence verified, expression cassettes were amplified by PCR (from the beginning of the insulator to end of the terminator) and normalized in concentration using UV-vis spectroscopy at λ = 260nm. A master mix was first prepared according to the PURExpress® published protocol, which was kept on ice until use. Reactions were scaled down to 5 µL final volume and carried out in Corning® low volume 384-well white flat bottom polystyrene TC-treated microplates (part # 3826). Reactions were initiated by the addition of DNA using a multi-channel pipette (n=2 per condition), followed by immediate transfer to a Tecan Infinite® M1000 microplate reader. A DNA concentration of 20ng/µL each was found to generally maximize competition between two genetic cassettes (**Figure S23 c-d**). Assays were run for 6hr. at 37°C with fluorescent reads every 5 minutes of each protein being analyzed (CFP: Ex. 435nm, Em. 470nm, YFP: Ex. 510nm, Em. 530nm, mCherry: Ex. 585nm, Em. 612nm). Reported reaction rates reflect the maximum rate observed.

in vivo fitness and expression assay.

To assess Co-Expression Fitness, Growth Fitness, and Expression Level, sequence verified plasmid constructs were transformed into *E. coli* K12 MG1655 with the chromosomally integrated YFP reporter. Unless noted otherwise, overexpressed proteins were under control of the Trc promoter with a strong RBS (BCD7) (**Data S4)**. 3 individual transformants were isolated and grown overnight in 400µL LB broth (BD Difco^{TM}) with selective antibiotic at 37°C in 96 deep well plates (Greiner Bio-One MASTERBLOCK®, 96 Well, 2 ML Item: 780270) for 24 hr. Cultures were then split and diluted 1:40 into LB broth with selective antibiotic and with or without 500µM inducer (IPTG) in black 96 well clear bottom micro-titer plates (Thermo product: 165305). Plates were incubated for 8 hours with shaking at 37°C in a Tecan Infinite® M1000 microplate reader with monitoring every 5 minutes for OD600, as well as fluorescence (CFP: Ex. 435nm, Em. 470nm, YFP: Ex. 510nm, Em. 530nm, mCherry: Ex. 585nm, Em. 612nm). Data were analyzed by comparing induced vs. uninduced cultures in terms of fluorescence and growth. To account for lag phase and differences in rates within a single term, the background subtracted area under the curve (AUC) was used for each respective signal using a Matlab® numerical integrator. The timespan evaluated was bounded by the time it took any sample to reach the upper limit of detection for fluorescence, which often took between 4-6 hours.

Additional data analysis.

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Linear regression, correlation analysis, dimensional reduction, and associated statistics were calculating using built in functions in Matlab®. Principal component analysis and hierarchal clustering were always carried out on an m x n matrix of RSCU values with codons in 61 rows and n number of gene sequences in columns. For RNA folding calculations, the minimum free energy was calculated for sequences using the Vienna RNAfold Version 2.5.1 software.[57]

COMPETING INTERESTS:

The authors declare no competing or conflicting interests.

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ASSOCIATED CONTENT:

There are 24 figures included in the Supplemental Information, and there are 4 supplementary data files.

DATA AVAILABILITY STATEMENT:

Additional data are available upon request. Additional supplementary Matlab® code can be found at https://github.com/nair-lab/CHI.

AUTHOR CONTRIBUTIONS:

A.M.L. performed the experimental work and data analysis. A.M.L. and N.U.N. conceived the study, planned the experiments, and wrote/edited the manuscript.

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