The multi-level phenotypic impact of synonymous substitutions: heterologous gene expression in human cells.

SHORT TITLE: Multi-level impact of synonymous substitutions in human cells.

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Funding Acquisition, Project Administration and Supervision : IGB; Methodology : IGB, AD, MALP; Investigation : MALP, FL, CC, AW, FB, JD, MD, AD; Data Curation : MALP; Formal Analysis : MALP, IGB; Visualization : MALP; Conceptualization and Writing : MALP, IGB.

ABSTRACT

Redundancy in the genetic code allows for differences in transcription and/or translation efficiency between sequences carrying synonymous polymorphisms, potentially leading to phenotypic changes. It is commonly admitted that the evolution codon usage bias ("CUB", the over-representation of certain codons in a genome, a gene or in positions along a gene) are driven by a combination of neutral and selective processes, but their relative contribution is a matter of debate, especially in mammals. Particularly, integrative studies quantifying the phenotypic impact of CUB at different molecular and cellular levels are lacking. Here we report a multiscale analysis of the effects of synonymous codon recoding during heterologous gene expression in human cells.

Six synonymous versions of the *shble* antibiotic resistance gene were generated, fused to a fluorescent reporter, and independently expressed in HEK293 cells. Multiscale phenotype was assessed by means of: i) mRNA-to-DNA and protein-to-mRNA ratios for each *shble* version; ii) cellular fluorescence, using flow cytometry, as a proxy for single-cell level expression; and iii) real-time cell proliferation in absence or presence of antibiotic, as a proxy for the cell fitness.

We show that differences in CUB strongly impact the molecular and the cellular phenotype: i) they result in large differences in mRNA and in protein levels, as well in mRNA-to-protein ratio; ii) they introduce splicing events not predicted by current algorithms; iii) they lead to reproducible phenotypic heterogeneity; iv) they lead to a trade-off between the benefit of antibiotic resistance and the burden of heterologous expression.

We interpret that CUB modulate mRNA availability and suitability for translation in human cells, leading to differences in protein levels and eventually eliciting phenotypic differences.

AUTHOR SUMMARY

The genetic code is redundant, with several codons encoding for the same amino acid. These synonymous codons are not used with equal frequencies. Instead, codon usage bias (CUB) varies between species, genes, and even positions along a gene. At each of these levels, CUB are shaped by the overall

balance between mutational biases and selection forces. To shed light on the molecular mechanisms underlying molecular and organism phenotypes, integrative studies quantifying the phenotypic impact of CUB at different levels of biological integration are necessary. Here, we monitored the multiscale changes induced by six synonymous versions of an antibiotic resistance gene independently expressed in a human cell line. We show that: 1. both mRNA levels, protein levels, and their ratios are affected by CUB; 2. potential effects on mRNA (splicing and availability for translation) seem to constitute the main level of action; 3. cell fitness is severely impacted by a trade-off between the burden of heterologous expression and the benefits of antibiotic resistance. This integrative study provides new insights on translation regulation and the associated phenotypic impact in human cells, associated to CU.

INTRODUCTION

The canonical scenario of gene expression posits that a DNA sequence is first transcribed into messenger RNAs (mRNAs) that are secondly translated into proteins, such as one given sequence of nucleotides encodes one predictable sequence of amino acids (1). The initial version of this scenario did not provide any explanation on how a unique set of genes could be associated with several cellular phenotypes. But, through the last decades, a large body of studies on gene expression have addressed this question and revealed multi-level regulation mechanisms increasing the diversity of the proteomic output from a given genome. The genetic code which establishes a correspondence between the DNA coding units (*i.e.* the codon, a triplet of nucleotides, 64 in total) and the protein building blocks (*i.e.* the amino acids, 20 in total) is degenerated: 18 of the amino acids can individually be encoded by two, three, four or six triplets, known as synonymous codons. In a first null hypothesis approach, one would expect synonymous codons to display similar frequencies. Instead, CUB (*i.e.* the uneven representation of synonymous codons (2)) have been reported in a multiplicity of organisms, and vary not only between species but also within a given genome or even along positions in a gene (3–8).

The origin and the contribution of the different neutral and/or selective forces shaping CUB constitute a classical research subject in evolutionary genetics. The hypothesis of translational selection proposes that differences in CUB result in gene expression variations that ultimately lead to phenotypic

differences, which could be subject to natural selection. And indeed, it has been established that variation in CUB might constitute an additional layer of gene expression modulation (9–11). Notably, genetic engineering has extensively resorted to CUB recoding for enhancing heterologous protein production, for its use in industrial applications or for vaccine design (12–15). Besides the plethora of successful gene recoding strategies, the interaction between CUB and the translation machinery has been well established, for instance in: i) the co-variation of genomic CUB and the tRNA content, from unicellular organisms (4,16,17) to metazoa (*Caenorhabditis elegans* (18), *Drosophila* (19–21), or humans (22); ii) the correspondence between CUB and expression level in bacteria (23) or in yeast (24,25); iii) the increase in translation efficiency in bacteria when supplementing *in trans* with rare tRNAs (26); iv) the changes in tumorigenic phenotype in mice when switching from rare to common codons in the sequence of a cancer-related GTPase (27).

In contrast, a number of studies have communicated the lack of covariation between CUB and gene expression (in bacteria, yeast, or human) (28–31); or even a negative impact of a presupposed "optimization", which may in fact decrease the expression or the activity of the protein product (32,33). To address these conflicting results, it is important to tease apart the underlying mechanisms through which CUB can impact the molecular, cellular and/or organismal phenotype. It has hitherto been established that CUB can impact: 1. mRNA localisation, stability and decay (34–38), 2. translation initiation (31,39–41), 3. translation efficiency (20,42–55); 4. co-translational protein folding (56–58). But, fueling the controversy, the respective contribution of each mechanism, if any, depends on the studied system (*e.g.* in which organism, whether the expressed gene is autologous or heterologous gene, whether it has been recoded or not).

In this study, we aim at providing an integrated view of the molecular and cellular impact of alternative CUB of a heterologous gene in human cells. We designed six synonymous version of the *shble* antibiotic resistance gene with distinct CUB coupled them to a *egfp* reporter, and transfected them into cultured cells. By combining transcriptomics, proteomics, fluorescence analysis and cell growth evaluation, we attempt to describe qualitatively, and to quantify as far as possible, the impact of CUB, and associated sequence composition, on the molecular and cellular phenotype of human cells in culture.

RESULTS

1. Alternative CUB of the *shble* gene resulted in differences in mRNA abundance, and splicing.

The expected transcript was a 1,602 base pair (bp) long mRNA encompassing a 1,182bp coding sequence (CDS). The CDS spanned an *AU1*-tag sequence in 5', a *shble* CDS, a *P2A* peptide sequence inducing ribosomal skipping, and an *EGFP* reporter CDS (Sup. Fig. 1). Only the *shble* CDS differed between constructs, and was characterized by distinct degrees of similarity to the average human CUB (estimated using the COdon Usage Similarity INdex, i.e. COUSIN) (59), GC composition at the third nucleotide of codons (GC3), and CpG dinucleotide frequency (CpG) (Table 1). Modifications in the *shble* sequence also entailed variations on the mRNA folding energy (Table 1). All these four parameters allowed for a good discrimination of all constructs (Sup. Fig. 2), partly reflecting sequence similarities (Sup. Table 1).

Table 1. Experimental conditions: the different constructs, and their sequence composition variables.

Condition	**Description**	**COUSIN of the *shble* sequence**	**%GC3 of the *shble* sequence**	**%CpG of the *shble* sequence**	**Folding energy of the total transcript (kcal/mol)**
shble#1	The most common codons in the human genome	2.93	93.08	18.46	-649.34
shble#2	The GC-richest among the two most common codons	2.982	99.23	22.56	-673.07
shble#3	The AT-richest among the two most common codons	-0.414	20.00	4.62	-581.47
shble#4	The rarest codons in the human genome	-1.651	33.85	20.51	-613.49
shble#5	The GC-richest among the two rarest codons	0.973	91.54	35.90	-687.76
shble#6	The AT-richest among the two rarest codons	-0.924	9.23	0.51	-543.50
#empty	No *shble* but only *EGFP* CDS	n.a.	n.a.	n.a.	n.a.
#superempty	Neither *shble* nor *EGFP* CDS	n.a.	n.a.	n.a.	n.a.
mock	No plasmid	n.a.	n.a.	n.a.	n.a.

Transcriptomic analysis (RNA-seq), through the observation of the read distribution along the plasmid sequence, revealed the presence of splicing events for the two constructs with the lowest similarity to the human average CUB, namely shble#4 (construct using the rarest codon for each amino acid) and

shble#6 (using rare and AT-rich codons) (Sup. Fig. 3). The shble#6 transcript presented two spliced forms, using the same 5' donor position and differing in three nucleotides at the 3' acceptor position. The shble#4 transcript presented one spliced form, with donor and acceptor positions in the precise same location than observed for shble#6, despite the lack of identity in the intron-exon boundaries. In all cases the spliced intron (either 306 or 309 nucleotides long) was fully comprised within the 396 bp long *shble* sequence (Sup. Fig. 4), and the event did not involve any frameshift. Thus, *shble* splicing resulted in the ablation of the SHBLE protein coding potential without affecting the EGFP coding potential. It is important to state that none of these alternative splicing events was predicted by the HSF (Human Splicing Finder) (60) nor the SPLM (61) splice detection algorithms used for sequence scanning during design.

The mRNA abundances, expressed as transcript per millions (TPM), showed that the spliced isoform 1 (shared by both affected conditions) represented about 30% of the heterologous transcripts for shble#4, and 56% for shble#6. The spliced isoform 2, exclusively found in condition shble#6, corresponded to 22% of the heterologous transcripts (Figure 1). The full-length mRNA, albeit present in all conditions, was differentially represented, as follows: (i) the highest values were found in shble#3 (using the AT-richest among common codons); (ii) the variance was largest in shble#5 (using the GC-richest among rare codons); and (iii) shble#4 and shble#6 displayed the lowest mRNA abundance even when considering the sum of all isoforms (Figure 1, Sup. Table 2). We further verified that variations in transcript levels were not related to variations in transfection efficiency, by correcting the TPM values with the plasmid DNA levels in each sample as estimated by qPCR. After this normalisation, the above described pattern remained unchanged (Sup. Fig. 5). This suggests that variations in mRNA levels are not due to differences in the DNA level, and may instead be linked to the differentially recoded *shble* sequences.

In order to allow further comparison between mRNA and protein levels, while accounting for the differential splice events, we have taken into account that the SHBLE protein was exclusively encoded by the full-length mRNA, while the EGFP protein could be translated from any of the three transcript isoforms. Hence, we used the ratio full-length mRNA over total transcripts (*i.e.* full-to-total ratio) to estimate the ratio of SHBLE-encoding over EGFP-encoding transcripts. This ratio was about 69% shble#4, while for shble#6 it

was close to 21% (Sup. Table 2). For the rest of the constructs, there was virtually no read corresponding to spliced transcripts and the ratio was in all cases above 99.96% (Sup. Table 2).

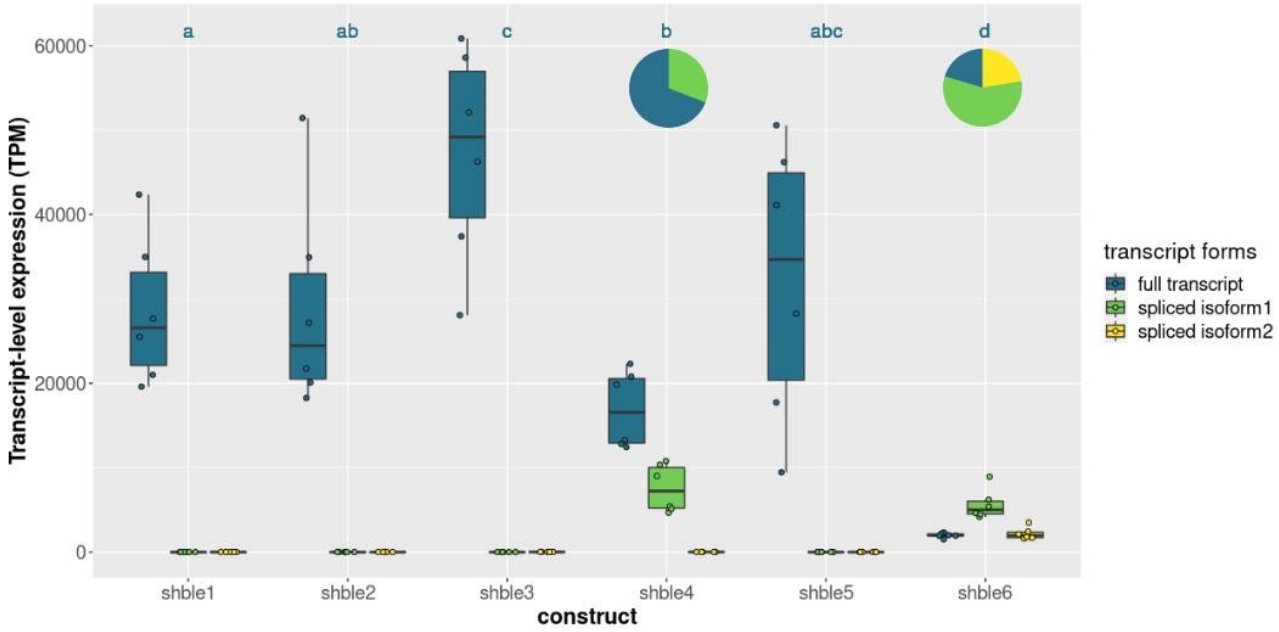

Figure 1. Transcript abundance after transfection with the different shble gene versions. *mRNA-levels are expressed as transcripts per million values (TPM) for the full form (in dark blue) as well as for the two spliced forms (in green and yellow). Median values are given in Sup. Table 2. Pie charts illustrate the proportions of the spliced forms detected in shble#4 and shble#6 conditions. The experiment was performed on six biological replicates. Dark blue letters above the different bars refer to the results of a Wilcoxon rank sum test. Conditions associated with a same letter do not display different median TPM values for the full mRNA (p<0.05 after Benjamini-Hochberg correction).*

2. Alternative CUB of the *shble* gene impacted SHBLE and EGFP protein levels.

Label-free proteomic analysis allowed to detect EGFP proteins for all constructs, with EGFP abundance in shble#3 and shble#6 being significantly lower than in other conditions (respectively 2.05 and 1.35 normalized iBAQ values, compared to an overall median of 10.08 for the other constructs) (Figure 2C, Sup. Table 3). The SHBLE protein was detected in all conditions but, for shble#6, it displayed extremely low abundance in five replicates and was not detected in one replicate (overall normalized iBAQ value of 0.03) (Figure 2B, Sup. Table 3). Further, the shble#3 condition displayed lower SHBLE protein levels than the remaining four other constructs (normalized iBAQ value of 0.93, compared to an overall median of 3.83) (Figure 2B, Sup. Table 3). Within a given condition, values for SHBLE and EGFP protein levels displayed a

strong, positive correlation (Pearson R coefficients ranging from 0.82 to 0.95 depending on the condition; all p-values < 0.05; Figure 2A). The overall SHBLE-to-EGFP ratio was 0.46±0.1 for all constructs (ranging between 0.36 and 0.56 for the individual constructs), the exception being shble#6, which displayed a ratio close to zero, linked to the very low SHBLE levels (Figure 2D). Label-free proteomic quantification results were validated by semi-quantitative western blot experiments (Sup. Fig. 6, 7 and 8).

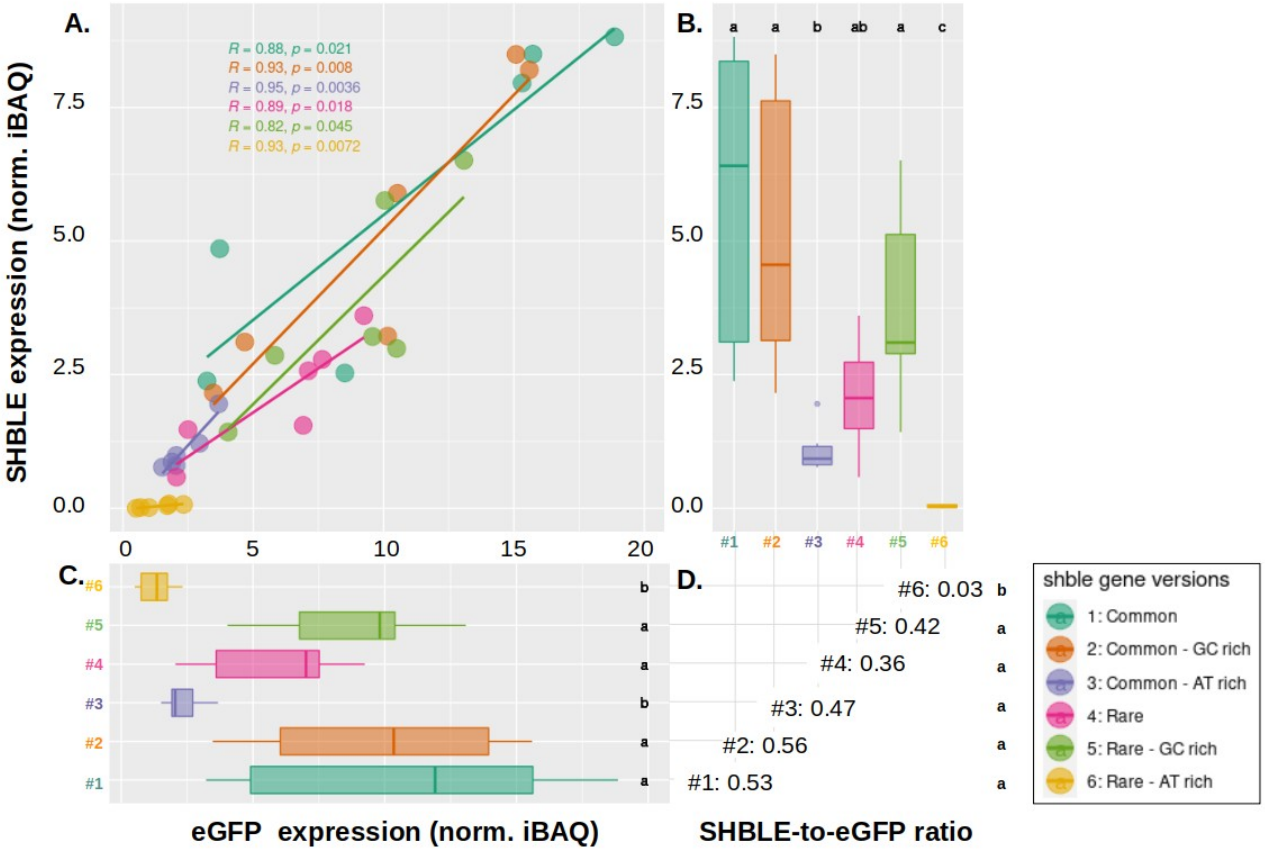

Figure 2. Expression of SHBLE and EGFP at the proteomic level, and relation between them. *Panel A: Pearson's correlation between SHBLE (y axis) and EGFP (x axis) protein levels. Six different conditions are shown: shble#1 (dark green), shble#2 (orange), shble#3 (purple), shble#4 (pink), shble#5 (light green) and shble#6 (yellow). Marginal boxplots (panels B and C) respectively show SHBLE and EGFP protein levels expressed as normalized iBAQ values. Median values are given in Sup. Table 3. The SHBLE-to-EGFP ratio for each of the six conditions (median of the ratios for each replicate) are given in panel D. Six replicates are shown (with three of them corresponding to two pooled biological replicates). Letters in the different panels refer to the results of a pairwise Wilcoxon rank sum test. Within each panel, conditions associated with a same letter do not display different median values of the corresponding variable (p<0.05 after Benjamini-Hochberg correction).*

3. Transcriptomic and proteomic phenotypes matched to different extent, and a combination several composition variables could explain the differences.

After analysing separately mRNA and protein levels in cells transfected with the different *shble* versions, we aimed at establishing a connection between those transcriptomic and proteomic phenotypes. Because SHBLE and EGFP protein analyses led to similar results, we focus here only on SHBLE. Variation in full-length transcripts levels explained 45% of the variation in the protein levels (Figure 3A). Interestingly, the shble#3 condition behaved differently from the rest and rendered similar SHBLE protein values for all replicates, independently of the variation in transcript levels (Figure 3A). When removing this condition from the correlation analysis, variation in full-length transcripts levels explained 83% of the variation in the SHBLE protein levels (Figure 3B).

In order to understand the differential matches between transcriptomic and proteomic phenotypes, we explored the explanatory potential of four sequence composition and mRNA physicochemical parameters. First, an increase of the match between the *shble* sequence and the human average CUB (i.e. COUSIN score) corresponded to an elevation in the protein-to-transcript ratio (Pearson's R=0.67, p=6.6e-6, Figure 3C) - with the exception of the shble#4 condition which displayed the lowest match to the human CUB, but a higher protein-to-transcript ratio than shble#6 and #3 (Figure 3C). In fact, the lower ratio for these two later conditions could be explained at the light of the three other tested parameters. Indeed, an increase of the GC3 content corresponded monotonically to an augmentation in the protein-to-transcript ratio (Pearson's R=0.81, p=1.5e-9, Figure 3D), and shble#6 and #3 had the lowest GC3 content. Then, variations in CpG frequency (Figure 3E), and in mRNA folding energy (Figure 3F), corresponded to a bell-shaped variation in SHBLE protein-to-transcript ratio so that both low and high values resulted in decreased protein-to-transcript ratio (Figure 3E and 3F): shble#6 and #3 had the lowest CpG frequency, and the highest mRNA folding energy. Thus, the shble#3 condition combined suboptimal values for all four studied characteristics and resulted in poorly efficient translation in spite of the high mRNA levels (see part1). In contrast, shble#1 (made of the most used codons), displayed an optimal value for all parameters and resulted in the most efficient translation (highest protein-to-mRNA ratio).

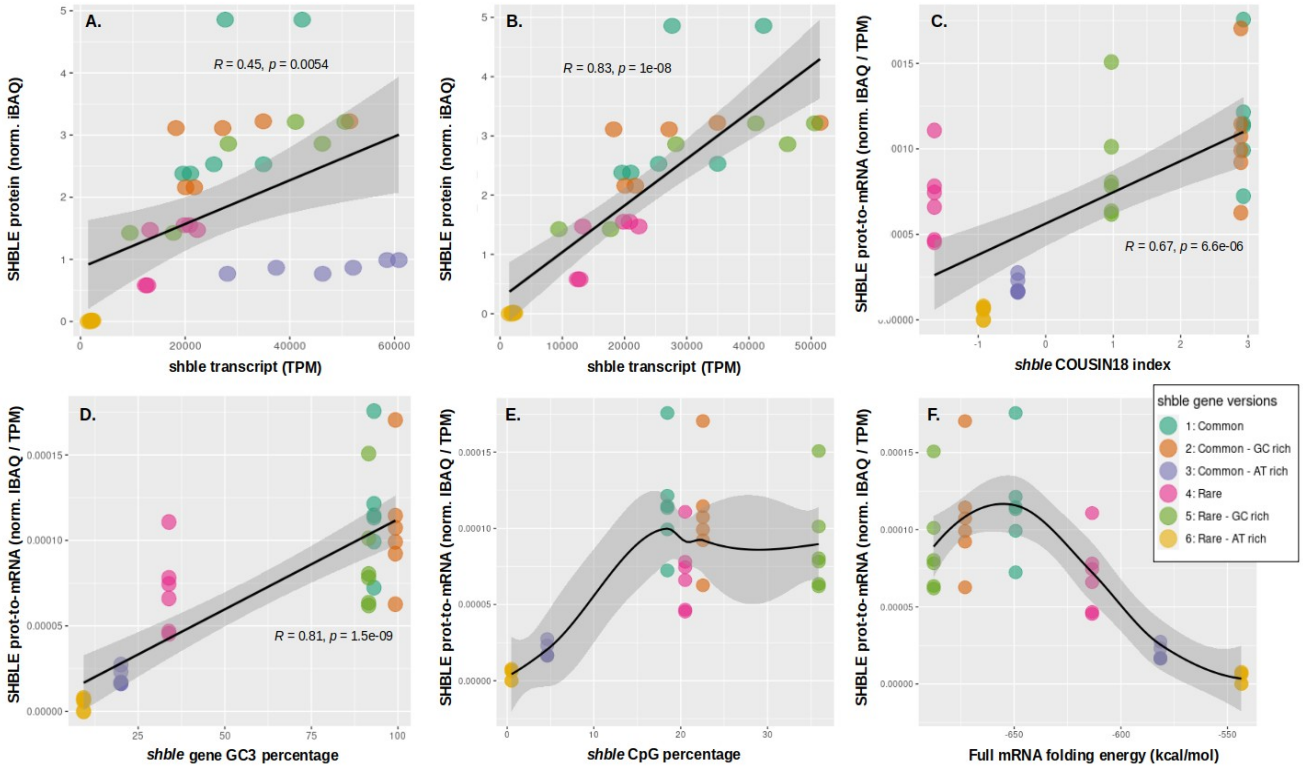

Figure 3. Relation between the transcriptomic and the proteomic phenotypes, and potential explicative parameters for variations in SHBLE protein levels. *Six different conditions are shown, using the colour code: shble#1 (dark green), shble#2 (orange), shble#3 (purple), shble#4 (pink), shble#5 (light green) and shble#6 (yellow).* ***Panel A:*** *Pearson's correlation of SHBLE protein level and* shble *transcript level taking into account all six constructs.* ***Panel B:*** *The construct #3, which displayed a discordant pattern from the others (see panel A), was excluded from the Pearson's correlation.* ***Panel C:*** *Pearson's correlation between SHBLE protein-to-mRNA ratio and COUSIN index of the shble recoded version.* ***Panel D:*** *Pearson's correlation between the SHBLE protein-to-mRNA ratio and the GC3 percentage of the shble recoded version.* ***Panel E:*** *SHBLE protein-to-mRNA ratio variations depending on CpG frequency of the shble recoded version.* ***Panel F:*** *Correspondence between the SHBLE protein-to-mRNA ratio and the folding energy of the corresponding transcript. The results for six biological replicates are shown, each of them with independent RNAseq measurements but pooled by pairs for the label-free proteomic analysis.*

4. Single-cell EGFP protein expression varied within each condition, but CUB variations induced shifts of the whole population.

We have demonstrated above that the EGFP reporter was a relevant proxy for SHBLE abundance, as their iBAQ values were highly correlated (Figure 2A). On this basis, and in order to further assess the

phenotypic variation at the single-cell level, we performed an extensive analyse on the cell-based fluorescence values of 16 transfection replicates. We verified first that the total fluorescence signal (i.e. adding all cells) was strongly correlated to the EGFP level estimated by the label-free proteomics (Pearson's R=0.86, p=4.8e-15, Sup. Fig. 9). We observed then that the distribution of this fluorescence signal was 1. for all conditions, different from that obtained with cells expressing EGFP alone (i.e. "empty" control; individual Anderson-Darling test results shown in Table 2); and 2. multimodal for all the conditions expressing EGFP (Figure 4A, Sup. Fig. 10). We described these multimodal populations by means of curve deconvolution, and showed that an approximation based on two underlying Gaussian populations fitted well the observed distributions (Sup. Fig. 11). Thus, 1. the construct expression changed the fluorescence phenotype, and synonymous variations of the upstream *shble* sequence modulated it; 2. for a given version of the *shble* sequence, cells were differentially impacted by the construct expression, overall defining two subpopulations of low or high EGFP expression.

Table 2. Quantitative parameters of green fluorescence signal distribution per condition.

Condition	Distribution similarity to #empty (AD score and associated p-value)*		Percentage of fluorescent cells	Total fluorescence value for the whole population$		Mean fluorescence value for the underlying first Gaussian subpopulation (log10)	Mean fluorescence value for the underlying second Gaussian subpopulation (log10)
#shble1	1580	0	89.56%	105.269 e9	bc	4.84	6.61
#shble2	1480	0	90.17%	98.311 e9	b	4.78	6.59
#shble3	497	4.637 e-272	79.37%	39.384 e9	d	4.31	5.86
#shble4	463	7.325 e-254	88.00%	63.395 e9	ac	4.58	6.28
#shble5	108	4.244 e-59	83.85%	70.719 e9	abc	4.63	6.32
#shble6	11600	0	51.78%	13.990 e9	e	3.97	5.05
#empty	0	1	82.26%	57.692 e9	a	4.44	6.18
#superempty	64100	0	0.45%	135.449 e6	*n.a.*	*n.a.*	*n.a.*
mock	62500	0	1.00%	141.163 e6	*n.a.*	*n.a.*	*n.a.*

**"AD", results of an Anderson-Darling test for distribution similarity, comparing each curve distribution in Figure 4A against that obtained for the "empty" condition (the null hypothesis being that the samples compared could have been drawn from a common population). $The statistical test is a pairwise Wilcoxon rank sum test. Conditions associated with a same letter do not display different median values for the corresponding variable (p<0.05 after Benjamini-Hochberg correction).*

For each condition, we summarized the fluorescence behaviour of the whole cell population by describing the following statistics (Table2): (i) the fraction of cells displaying fluorescence over 99th

percentile of the "mock" fluorescence distribution (*i.e.* 14,453 'FITC-A' fluorescence units, which corresponded to the cell autofluorescence, as the mock did not carry any plasmid); (ii) the total fluorescence value of the whole population; (iii) the median fluorescence value of the population; (iv) the mean fluorescence value for each underlying Gaussian populations. We observed that the central fluorescence value (i.e. the median) of the population correlated very well with the overall fluorescence (R=0.85, p-value<2.2e-16, Sup. Fig. 12), but that the later allowed a better discrimination between conditions. Shble#1 and #2 thereby appeared more fluorescent than the #empty control, and shble#6 displayed a lower fluorescence than all the other conditions, as did shble#3 in a lower magnitude (Table 2, Sup. Fig. 12). Those differences in the total signal reflected in fact an reproductible impact of the synonymous construct expression on all cells, independently of their affiliation to the low or high fluorescent populations: indeed, between each condition, both underlying Gaussian curves shifted following the same pattern, as illustrated by the variations of their mean values (Figure 4B, Table 2). When combining all our summary statistic variables for describing the population cellular fluorescence we observed that indeed shble#6, and to a lesser extent shble#3, were the most divergent conditions, characterized by the highest proportion of negative or low-fluorescent cells, while shble#1 and shble#2 displayed very similar behaviour characterized by high fluorescence values in all scores (Sup. Fig. 13). Those results strengthened the observations obtained by the label-free proteomic experiments, and underlied the cell-to-cell reproductibilty of the impact of synonymous substitutions.

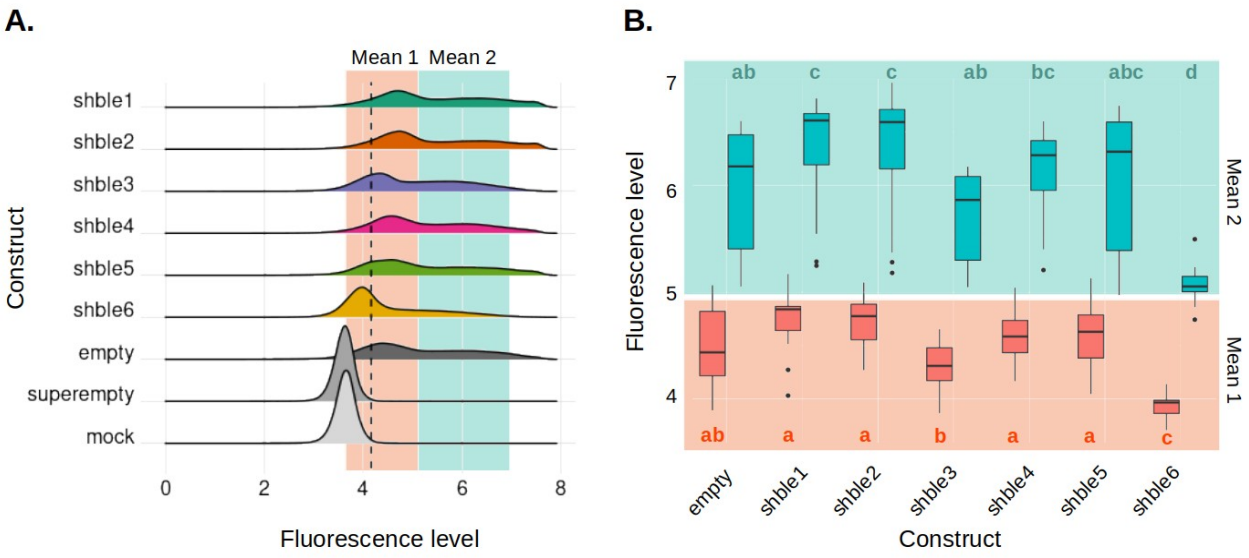

Figure 4. Distribution of the fluorescence signal for the different constructs, and mean values of the two gaussian curves modeling the fluorescence distribution. *Panel A depicts the density of the green fluorescence signal (log10(FITC-A)) considering 480,000 individual cells for each condition: shble#1 (most common codons, dark green), shble#2 (common and GC-rich codons, orange), shble#3 (common and AT-rich codons, purple), shble#4 (rarest codons, pink), shble#5 (rare and GC-rich codons, orange light green), shble#6 (rare and AT-rich codons, yellow). The positive control is "empty" (i.e. transfected cells, expressing EGFP without expressing SHBLE, in dark grey); and the negative controls are "superempty" (i.e. transfected cells, not expressing EGFP nor SHBLE, in medium grey) and "mock" (i.e. untransfected cells, in light grey). The dashed black line shows the threshold for positivity (14,453 green fluorescence units, corresponding to 4.16 in a log10 scale). Panel B represents the first gaussian mean1 (population of lower intensity, in red), and the mean2 (population of higher fluorescence intensity, in blue). For each category of mean, the statistical test is a pairwise wilcoxon rank sum test, with Benjamini-Hochberg adjusted p-values on sixteen biological replicates: for each color, conditions associated to a same letter do not display different median values of the corresponding variable.*

5. Alternative CUB of the *shble* gene resulted in different cell growth dynamics.

To assess the functional impact of the molecular phenotypes described above, we performed a real-time cell growth analysis, both in presence and in absence of antibiotics. We anticipated a trade-off between a potential benefit confered by the antibiotic resistance, and a potential cost through protein overexpression and its associated burden. Thus, to disentangle the effects linked to the total expression of heterologous proteins (SHBLE + EGFP), and the effect of the antibiotic resistance gene alone, we tested two additional constructs solely containing versions shble#1 and shble#4 of the *shble* gene, not linked to the *EGFP* reporter

(labelled shble#1* and shble#4* in Figure 5 and Sup. Fig. 14). For all conditions, we monitored over time a dimensionless parameter named "Cell Index", that integrates cell density, adhesion, morphology and viability; and we evaluated the total area below the curve as a proxy for cell growth (Sup. Method 2.8, Sup. Fig. 14). We fitted to a Hill's equation these values of cell growth as a function of the antibiotic concentration to recover, for each condition: 1. the maximum growth in the absence of antibiotic (Figure 5, y axis); and 2. the estimation for the antibiotic concentration value that inhibited cell growth to half the maximum (IC50; Figure 5, x axis).

First, we observed that transfection with an empty vector (i.e. the "superempty" control, not expressing EGFP nor SHBLE) resulted in a drop of about 50% in max. growth in the absence of antibiotic (Figure 5, y axis), and in a drop of about 85% in IC50 value (Figure 5, x axis) compared to the mock. This meant that, independently of heterologous gene expression, the transfection alone had an impact on cell fitness.

Second, all cell populations transfected with any of the constructs displayed a lower max. growth in the absence of antibiotics than the "superempty" control (Figure 5, y axis). Only shble#4 and #6, expressing SHBLE and EGFP at low level, and shble#1* and shble#4* lacking EGFP, had a higher IC50 value (Figure 5, x axis). Further, all transfected cells resisted less the presence of antibiotics than the mock independently of the construct used (Figure 5, x axis), at the exception of shble#1* (high level of SHBLE and no EGFP). This meant that the burden induced by the expression of heterologous proteins was stronger than the potential benefit of the antibiotic resistance.

Focusing on the comparison of the *shble* versions #1 and #4 with or without EGFP, both shble#1* and shble#4* versions displayed a similar increase in max. growth in the absence of antibiotic with respect to their EGFP+ relative counterparts (respectively 38% and 24%, shown as coloured arrows on Figure 5, y axis). However, while the IC50 of shble#4* remained similar to shble#4, the antibiotic resistance for version shble#1* dramatically increased with respect to that of shble#1 (green arrow on Figure 5, x axis). As mentioned before, shble#1* was the only condition in which resistance to the antibiotic was actually better than for the untransfected cells, in spite of a remaining substantial negative impact on maximum growth on the absence of antibiotics (Figure 5, y axis). This meant that 1. in absence of antibiotics, higher amount of heterologous protein (whatever if it correspond to SHBLE or EGFP) had a more pronounced negative impact

on cell fitness; 2. in presence of antibiotic, the optimum between the confered resistance and the cost of protein burden was determined by both, the total amount of the two heterologous proteins (including EGFP, which was unecessary to the cell fitness), and the abundance of the protein confering the resistance itself (SHBLE).

Overall, even if no significant correlation could be established because of the limited number of experimental conditions, a trend appeared: variation in cell fitness in absence of antibiotics seemed to be inversely related to variation in total amount of heterologous proteins (using fluorescence as a proxy, showed as dot size in Figure 5), so that conditions displaying strong cellular fluorescence (*e.g.* shble#1) grew less in the absence of antibiotics, and resisted worse the presence of antibiotics, than conditions displaying lower fluorescence (*e.g.* shble#6) (Figure 5 , Sup. Fig. 14). Our results suggested thus: first, the existence of an important stress related to plasmid transfection; and second, the establishment of a trade-off between the benefit of heterologous protein expression conferring resistance and the additional burden of fluorescent protein expression coupled to the resistance.

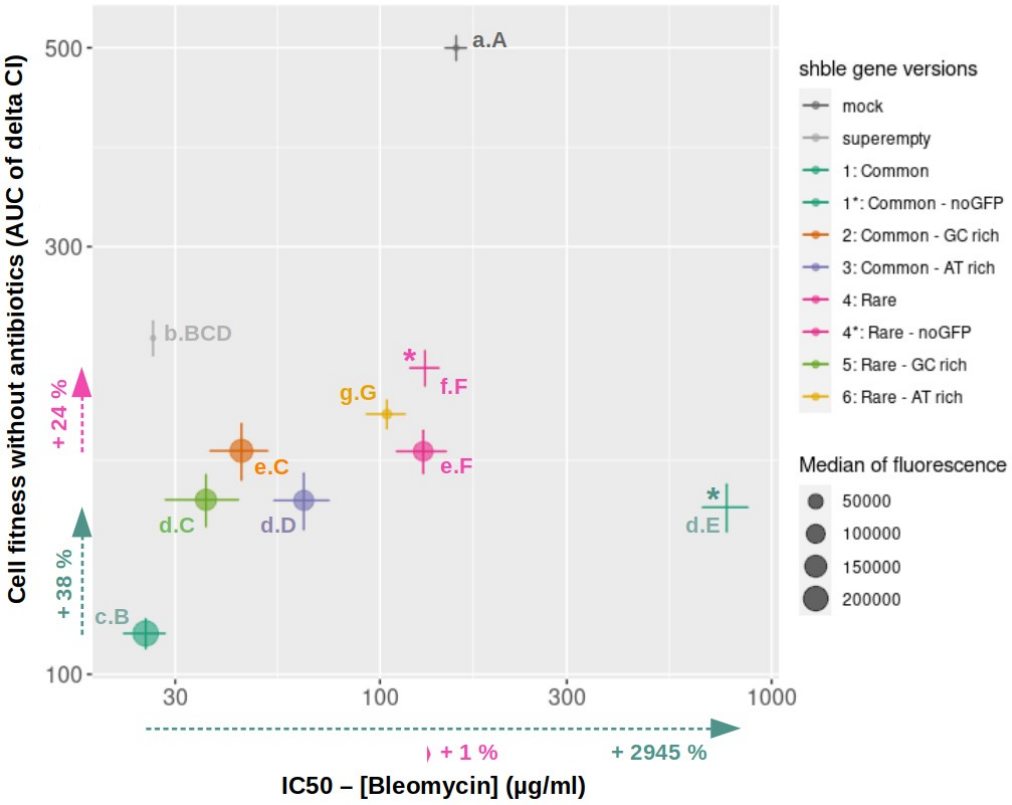

Figure 5. Variation of cell growth in presence or in absence of antibiotics. *The y axis represents maximum cell growth in absence of antibiotics, proxied as the area under the curve of the delta Cell Index (AUC, log scale). The x axis represents the bleomycin concentration reducing to 50% the corresponding maximum growth (e.g. IC50; log10 scale). Represented central values were estimated fitting Cell Index data to Hill's equation (pooled data, 3 to 6 biological replicates), and bars correspond to the standard error (left standard error for superempty IC50 was out of the graph limit and is not plotted – but see Sup. Fig. 15 for representation on linear axes). Statistical tests are Welch modified two-sample t-tests, performed for the AUC (small letters, y axis) or the IC50 (big letters, x axis): for each size of letters, conditions associated with a same letter do not display different median values of the corresponding variable (p<0.05 after Benjamini-Hochberg correction). The size of the dots is proportional to the corresponding median of fluorescence, which is used as a proxy for the level of heterologous proteins. Nine different conditions are shown: mock control (dark grey), superempty control (light grey), shble#1 (dark green), shble#2 (orange), shble#3 (purple), shble#4 (pink), shble#5 (light green), shble#6 (yellow) and versions shble#1* (dark green) and shble#4* (pink) lacking the EGFP reporter gene. Arrows on the margins represent the shift of values (expressed as percentage of the initial value) for shble#1* and shble#4* against shble#1 and shble#4 respectively.*

DISCUSSION

In the present manuscript we have analysed the multilevel molecular *cis*-effects of codon usage preferences on gene expression, and have further explored higher-level integration consequences at the cellular level. The global *trans*-effects of codon usage preferences of our focal gene on the expression levels of other cellular genes have been analysed and described in an accompanying paper (62). We summarize our observations of these *cis*-effects in Figure 6, which displays variation in the each of the composition and phenotypic variables monitored for the different genotypes analysed. This representation highlights that a combination of synonymous changes results in important multilevel variation in gene expression levels and leads to dramatic differences in the cellular phenotype.

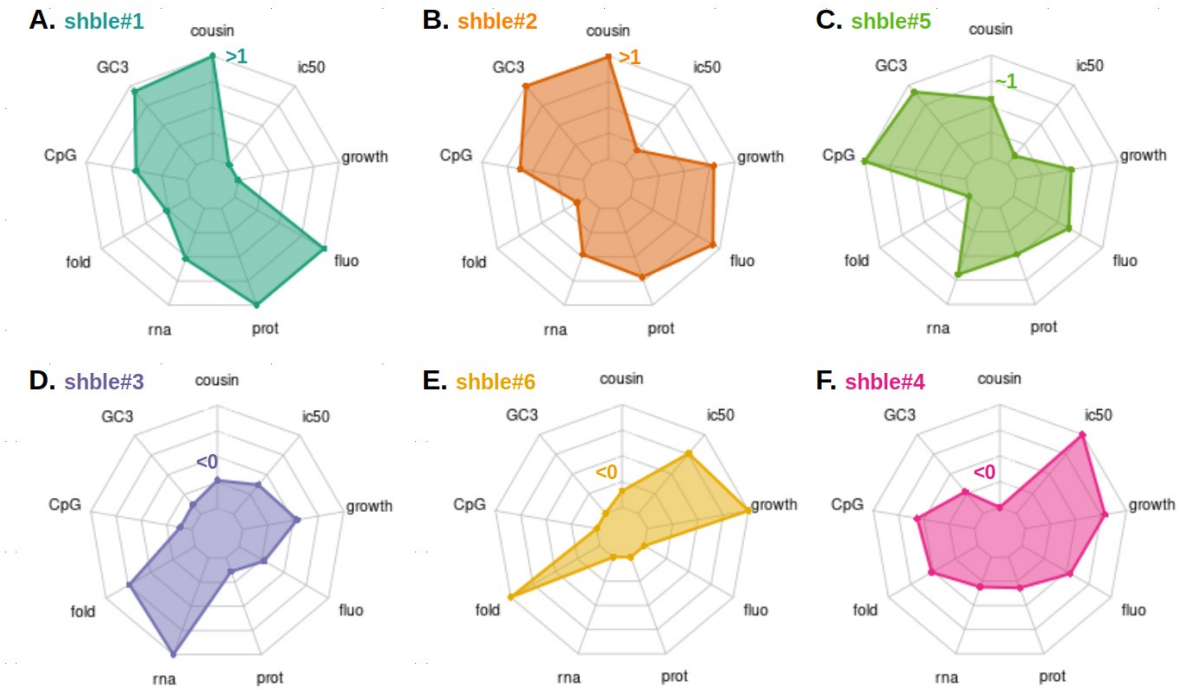

Figure 6. Summarizing combination of sequence composition parameters and multi-level phenotypes for each customized version of the* shble *antibiotic resistance gene. *The six versions, designed with the one amino acid – one codon strategy, are shown by decreasing similarity to the human CUB (i.e. cousin score). They are defined as follow:* ***A****. shble#1 (most common codons, cousin > 1, in dark green),* ***B****. shble#2 (common and GC-rich codons, cousin > 1, in orange),* ***C****. shble#5 (rare and GC-rich codons, cousin ~ 1, in light green),* ***D****. shble#3 (common and AT-rich codons, cousin < 0, in purple),* ***E****. shble#6 (rare and AT-rich codons, cousin < 0, yellow) and* ***F****. shble#4 (rarest codons, cousin < 0, in pink). The sequence characteristics are from the top to the left: "cousin" (expressing the similarity to the average genome human CUB: a score < 0 is opposite; ~1 is similar; >1 is similar human CUB, but of larger magnitude (59)), "CpG" (the CG dinucleotide frequency), "GC3" (the GC content at the third base of the codons), and "fold" (the mRNA folding energy). The different phenotypes, from the bottom to the right: "rna" (the SHBLE coding full mRNA amount), "prot" (the SHBLE protein amount), "fluo" (the total fluorescence signal), "growth" (proxy of the cellular fitness in absence of antibiotics) and "ic50" (proxy of the cellular fitness in presence of antibiotics).*

Variation in codon usage preferences modifies alternative splice patterns and leads to differences in mRNA levels. The two versions having the most dissimilar CUB with respect to the human average (shble#4 and shble#6) were characterized by splicing events, which were not detected by leading splice site predicting algorithms (60,61), and which reduced the coding potential of the resulting mRNA by 30 to 80%. CUB variations across intron-exon boundaries have been described in several eukaryotes (*e.g.*

human, fishes, fruit flies, nematodes, plants (11,63,64)); and splicing regulatory motifs, that can be disrupted by synonymous mutations, have been described in mammals (9,64–67). As signature for selective pressure, a reduced SNP density and decreased rate of synonymous substitutions have further been reported in these regulatory regions (68,69). Thus, selection against mRNA mis-processing can constitute an important selective force that results in concomitant selection for a precise local CUB (70), and this selective force has even been proposed to outperform translational selection in *D. melanogaster* (71).

In our experimental setup, variation in mRNA levels between conditions was independent of variation in DNA abundance, ruling out a possible effect of differential transfection efficacy. The two versions with the largest deviation in mRNA levels were the AT-richest ones (shble#3 and #6, respectively the highest and the lowest level of mRNA). Transcript abundance at a given time point is the result of integrating mRNA synthesis and degradation kinetics. As all versions share the same CMV promoter, and 5' untranslated region, we hypothesize that the observed differences in mRNA levels may most probably result from differential mRNA stability and decay, rather than primary transcription regulation. Such an effect has been described for bacteria (*E. coli* (72)), unicellular eukaryotes (*S. cerevisae, S. pombe* (35), *N. crassa, T. brucei (73,74)),* and metazoa (fruit fly (75) or zebrafish (76)). The effects on version shble#6 are difficult to address as only 20% of the total transcript contain the customized *shble* sequence; it is thus impossible to disantangle the effect of sequence composition on the full mRNA level from the consequences of the splicing defect. The very high transcript levels of version shble#3 are interesting in the light of recent findings on CUB linked mRNA degradation and/or storage: indeed, AU-rich mRNAs have been found to accumulate in P-bodies, which can lead to an accumulation of those transcript in the cell (77). In addition, the P-body retention of those transcripts reduce their availability to translation and could further explain the reduced protein level for this condition (see below).

Variation in mRNA nucleotide composition, codon usage preference and structure lead to differences in translation efficiency. Considering all conditions together, our experimental setup allowed us to determine that the mRNA levels explain only around 40% of the variation in protein levels, which fits well previous descriptions in the literature for a wide diversity of experimental systems (78–80). Such weak explanatory power would not be expected if mRNAs were translated at a constant rate, and has thereby

motivated studies to elucidate which factors are involved in the regulation of translation (81). Here, we evidenced that this discrepancy between mRNA and protein level was unequal between CUB. Particularly, the version using the AT-richer codons among the two most common ones in the average human genome (shble#3) displayed the highest mRNA levels but contrasting low amount of the corresponding protein. A possible explanation for this phenomenon could be the selective translation impairment of AT-rich transcripts. As proposed before, this can result from P-body retention (77), but other mechanisms may alternatively, but not exclusively, be involved. For instance, in human cells, the protein Schlafen11 has been shown to prevent translation of viral transcript (known to be AU-enriched), in a codon usage dependant manner (82,83). Noticing that the AT-rich condition #4 displays a moderated impairment of translation, we interpret that condition #3 dramatic phenotype arises in fact from the combination of suboptimal variables for which a role in optimizing the expression of heterologous genes had already been evidenced (11) : (i) similarity to human average CUB, (ii) the CpG frequency, and (iii) the mRNA folding energy.

(i) Regarding similarity in codon bias between the focal gene and the expression system (i.e. average human CUB), gene versions with a better match resulted in higher protein-to-mRNA ratios. This result is in disagreement with previous reports, as well as with descriptions showing the very limited impact of CUB on gene expression in mammals, compared to other features (30,84). Nevertheless, it is complicated to disentangle the effect of CUB from other composition characteristics, such as GC and GC3 content. It is even more difficult to interpret them in terms of neutralist or selectionist origin, as both evolutionary hypotheses could account for variation in either parameter (10).

(ii) Regarding intragenic CpG frequency, we report a negative impact of extreme values (either too high or too low) on translation. Such direct effect on translation efficiency had never been reported before, and CpG frequency had been shown to impact heterologous protein amount through its impact on *de novo* transcription instead (85,86). More precisely, high CpG depletion was previously associated to low mRNA level, that weren't evidenced as a result of changes in nuclear export, alternative splicing or mRNA stability (85,86). Indeed, a signature for selection towards decreased values of CpG has been consistently reported (87,88), experimentally verified by the detrimental effects of increased CpG levels on protein synthesis (89,90), and further corroborated through experimental evolution (91).

(iii) Regarding the total mRNA folding energy, we also report a negative impact of extreme values (either too high or too low) on the translation. Molecular modeling, along with experimental studies, suggest a prominent role of the initiation, rather than elongation, on the efficacy of translation (41,92,93). And indeed, several studies adressing the impact of mRNA folding on translation, established the importance of the 5' mRNA secondary structure in translation initiation. A shared trend was identified in bacteria, yeast, protists, and mammals (31,55,93–96): a reduced mRNA stability near the site of translation initiation is correlated to a higher protein production. In bacteria and yeast, strong folding around the start codon prevents ribosome recruitment (31,93); and a "ramp" of rare codon along the 50 to 100 first coding nucleotides has been reported, with the effect of reducing mRNA folding energy and with the proposed function of avoiding ribosome traffic jam (96,97). A systematic exploration using 244,000 synthetic DNA sequences on *E. coli* shows that the strength of the secondary structures predicted 60 nucleotides around the start codon is capturing around 36% of the total variance in protein synthesis, while variation in downstream mRNA folding energy accounts only for *ca.* 4-5% (98). Nonetheless, an important role of translation elongation cannot be ruled out. Particularly, in human transcripts, de Sousa Abreu *et al.* describe no effect of the initiation rate on translation efficiency (78). A recent study, in human cell lines, highlight the consequences of the secondary structures along the CDS in the functional half life of mRNA (95). In our experimental setup all constructs share by design the nucleotide sequence around the start codon: the 5’UTR corresponds to the plasmid backbone and the first 24 coding nucleotides are identical (AU1 tag). Thus, there are actually no differences in folding energy when considering only the immediate sequence stretch around the start codon, but there are instead differences when considering the full mRNA length. We interpret thus that our observation of a non-monotonic effect of the full-length mRNA folding energy on the protein-to-mRNA ratio is related to translation elongation impairment rather than to an effect on translation initiation.

Transfected human cells differentially express heterologous genes, independently of their sequence; but sequence recoding impact the individual cell expression in a reproductible way. We report phenotypic variability of transfected human cells, observable as multimodal distribution of cellular fluorescence. The multimodal distribution of cellular fluorescence intensity on the transfected cells could be captured in all cases by fitting to a combination of two Gaussian curves. This pattern was similar for all

constructs expressing *egfp*, including the empty control, which contains only the *egfp* sequence, and was thus not influenced by codon usage preferences of the recoded *shble* sequence. We interpret that phenotypic variability in cell-based fluorescence levels reflects phenotypic plasticity and may be related to transient cellular states, such as cell division status and/or differential kinetics of recovery from transfection-induced cellular stress. Cell cycle-dependent differences in gene expression have been actually reported when using cytomegalovirus-based expression vectors (99). Beyond the shared bimodal distribution of fluorescence levels, we observe significant and concerted shifts of both cellular subpopulations towards higher (*e.g.* for the constructs enriched in the most used codons) or lower (*e.g.* for constructs using AT-rich codons) values of fluorescence intensity. Thus, differences in overall EGFP-based fluorescence between recoded constructs do not arise from differences in the number of positive cells expressing a given quantity of EGFP, but rather from differences in EGFP synthesis at the individual cell level. Our experimental model using human cells shows that CUB exerts an important effect on the overall levels but also in the cell-based levels of the heterologous protein produced.

We showcase a trade-off between the cellular burden imposed by extra protein synthesis and the benefit conferred through antibiotic resistance. In the absence of antibiotic all transfected cells grow less than the parental cells: *ca.* 2 to 4.8 times less, even for cells transfected with a control plasmid containing an empty expression cassette (i.e. not expressing neither *shble* nor *egfp).* In addition to this basal cost, related to transfection alone, strong heterologous expression imposes an enormous basal burden on the cellular economy. A similar effect has been described in bacteria (100) and the interpretation is consistent with the broad literature about the direct (-cis) and indirect (-trans) costs of translation (70): first, translation is the per-unit most expensive step during biological information flow (101), consuming ca. 45% of the whole energy supply in human cells in culture (102); second, and virtually all ribosomes are bound to mRNA molecules and potentially engaged in translation (102), so that highly-transcribed heterologous mRNA increase overall ribosome demand and cause loss of opportunity for cellular gene translation; and third, heterologous protein synthesis can lead to additional downstream costs by protein folding, protein degradation and off-target effects of mistranslated proteins (45,103,104). Additionally, mismatch between the CUB of the heterologous gene can display strong trans-effects on the cellular homeostasis, by sequestering

ribosomes onto mRNAs that hardly progress over translation but also by creating a competition for the tRNA pools (31,105). Scarcity of the less common tRNAs is actually a severe limiting factor for protein synthesis in bacteria (106), and this pressure over rare tRNAs can become extreme in conditions of stress, or changes in nutritional status (10,107,108).

The *shble* gene that we have used as a base for synonymous recoding encodes for a small protein that confers resistance to bleomycin through antibiotic sequestering (109). This protein-antibiotic binding is equimolecular and reversible: a SHBLE protein dimer binds two bleomycin molecules (110). It can be thus hypothesised that the strength of the antibiotic resistance conferred is a direct, probably monotonic, function of the SHBLE amount produced. Our results, however, show that the benefit conferred by SHBLE synthesis in the presence of antibiotic is largely exceeded by the cost and burden of heterologous protein synthesis. Thus, as described above for the fitness in the absence of antibiotic, we state an important trade-off between the intensity of heterologous SHBLE+EGFP protein synthesis and the actual bleomycin resistance levels; and an important rescue of the antibiotic resistance for cells expressing SHBLE but not EGFP. Altogether, we conclude that: (i) transfection alone introduces an important cellular stress that impairs cellular growth; (ii) heterologous gene overexpression imposes a strong burden on the cell economy, sufficient to severely affect cell growth; (iii) CUB of heterologous gene differentially impact cellular fitness as a function of the differences in heterologous protein synthesis.

CONCLUSION

The main conundrum for scientists approaching codon usage bias remains the contrast between, on the one hand, the large and sound body of knowledge showing the strong molecular and cellular impact of gene expression differences arising from codon usage preferences and, on the other hand, the thin evidence for codon usage selection at the organismal level. Under the neutral hypothesis, differences in average genome CUB can be explained by biochemical biases during DNA synthesis or repair (e.g. polymerase bias) (111); and, in vertebrates, CUB at the gene level may be shaped by their relative position to isochores (e.g. alternation between GC-rich and AT-rich stretches along the chromosomes) (112). Further, GC-biased gene conversion mechanisms can enhance those local variations (111,113,114). The selective explanation, often referred to as "translational selection", proposes that different codons may led to differences in gene

expression, by changes in alternative splicing patterns, mRNA localisation or stability, translation efficiency, or protein folding (115). If such codon-bias induced variation in gene expression were associated with phenotypic variation that results in fitness differences, it may, by definition, be subject to natural selection. Nevertheless, differences in fitness associated with individual synonymous changes seem to be mostly of low magnitude, so that selection may only act effectively in organisms with large population sizes (116) such as bacteria (7), yeast (117), nematodes (118), but also in fruit flies (19,20,119,120), branchiopods (121) and amphibians (122). In organisms with small population sizes, such as mammals, and particularly humans, evidences of selection for (or against) certain codons remain nevertheless controversial (22). In the present manuscript, we have intended to contribute to this debate by exploring the phenotypic consequences of codon usage differences of heterologous genes in human cells. We claim that the potential evolutionary forces at play in shaping human CUB, select for a strict control of mRNA processing: splicing, and secondary structure (potentially affecting stability and decay); and that the resulting mRNA properties *in fine* impact translation elongation. Notwithstanding, the disparity between predictions and findings encountered in powerful, codon-usage related experimental evolution approaches highlights the gap in our understanding at connecting phenotype and fitness over different integration levels: molecules, cells, tissues and organisms. Despite, or thanks to, the immense body of knowledge accumulated over the last fifty years, the quest for interpreting and integrating the riddle of codon usage preferences over broad scales of time and biological complexity remains tempting and unsolved.

MATERIAL AND METHODS

Design of the *shble* synonymous versions and plasmid constructs. Six synonymous versions of the *shble* gene were designed applying the "one amino acid - one codon" approach, *i.e.*, all instances of one amino acid in the *shble* sequence were recoded with the same codon, depending on their frequency in the human genome (Table 1): shble#1 used the most frequent codons in the human genome; shble#2 used the GC-richest among the two most frequent codons; shble#3 used the AT-richest among the two most frequent codons; shble#4 used the least frequent codons; shble#5 used the GC-richest among the two less frequent codons; and shble#6 used the AT-richest among the two less frequent codons. An invariable *AU1* sequence

was added as N-terminal tag (amino acid sequence MDTYRI) to all six versions. Nucleotide contents between versions are compared in Sup. Table 1. The normalized COUSIN 18 score (COdon Usage Similarity Index), which compares the CUB of a query against a reference, was calculated on the online tool (http://cousin.ird.fr) (59). A score value below 0 informs that the CUB of the query sequence is opposite to the reference CUB; a value close to 1 informs that the query CUB is similar to the reference CUB , and a value above 1 informs that the query CUB is similar the reference CUB , but of larger magnitude (59). All *shble* synonymous sequences were chemically synthesised and cloned on the *XhoI* restriction site in the pcDNA3.1+P2A-EGFP plasmid (InvitroGen), in-frame with the *P2A-EGFP* reporter cassette. In this plasmid, the expression of the reporter gene is located under the control of the strong human cytomegalovirus (CMV) promoter and terminated by the bovine growth hormone polyadenylation signal. All constructs encode for a 1,602 bp transcript, encompassing a 1,182 bp *au1-shble-P2A-EGFP* coding sequence (Sup. Fig. 1). The folding energy of the 1,602 bp transcripts was calculated on the RNAfold Webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi), with default parameters (Table 1). During translation, the P2A peptide (sequence NPGP) induces ribosome skipping (123), meaning that the ribosome does not perform the transpeptidation bond and releases instead the AU1-SHBLE moiety and continues translation of the EGFP moiety. The HEK293 human cell line used here is proficient at performing ribosome skipping on the P2A peptide (124) The transcript encodes thus for one single coding sequence but translation results in the production of two proteins: SHBLE (theoretical molecular mass 17.2 kDa) and EGFP (27.0 kDa). As controls we used two plasmids: (i) pcDNA3.1+P2A-EGFP (named here "empty"), which encodes for the EGFP protein; (ii) pcDNA3.1+ (named here "superempty") which does not express any transcript from the CMV promoter (Table 1). In order to explore the burden of EGFP expression we generated two additional constructs by subcloning the AU1-tagged shble#1 and shble#4 coding sequences in the XhoI restriction site of the pcDNA3.1+ backbone, resulting in the constructs shble#1* and shble#4*, lacking the *P2A-EGFP* sequence.

Transfection and differential cell sampling. As mentioned above, all experiments were carried out on HEK293 cells. Cell culture conditions, transfection methods and related reagents are detailed in Sup. Methods 2.2. Cells were harvested two days after transfection and submitted to analyses at four levels

(Figure 6): (i) nucleic acid analyses (qPCR and RNAseq); (ii) proteomics (label-free quantitative mass spectrometry analysis and western blot immuno-assays); (iii) flow cytometry; and (iv) real-time cell growth analysis (RTCA). Overall, the different experiments were performed on 33 biological replicates, corresponding to a variable number of repetitions depending on the considered analysis (Sup. Method 1). Transfection efficiency was evaluated by means of qPCR targeting two invariable regions of the plasmid and revealed no significant differences between the constructs (Sup. Methods 2.3).

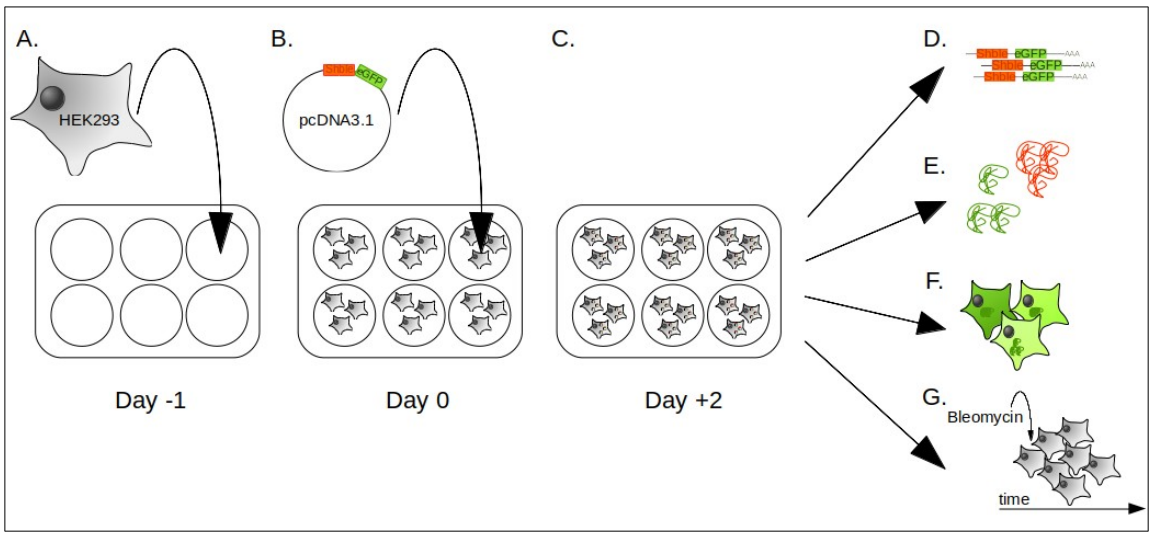

Figure 7. Overview of the sampling protocol and the measured phenotypes. HEK293 cells were seeded on 6-well plates (A) one day before transfection with the customized pcDNA3.1 plasmids (B). Transfected cells were harvested two days later (C). mRNA levels were assessed by RNAseq (D), protein levels were measured by label-free proteomics (E), EGFP fluorescence was assessed at the single cell level by flow cytometry (F) and cell growth was assessed by xCELLigence RTCA (Real Time Cell growth Analysis) in presence of different concentrations of the bleomycin antibiotic (G).

RNA sequencing and data analysis. The transcriptomic analysis was performed on six biological replicates and eight conditions: shble#1 to shble#6, #empty, and mock (for which the sample is submitted to the exact same procedures, including the transfection agent, but in absence of plasmid). Paired 150bp Illumina reads were trimmed (Trimmomatic v0.38) (125) and mapped on eight different genomic references (HISAT2 v2.1.0) (126), corresponding to the concatenation of the human reference genome

585 (GCF_000001405.38_GRCh38.p12_genomic.fna, NCBI database, 7th of February 2019) and the
586 corresponding full sequence of the plasmid. For the mock condition, we considered the human genome and
587 all possible versions of the plasmid. Virtually no read of those negative controls mapped to the plasmid
588 sequences. For all other conditions, read distribution patterns along the plasmid sequence were evaluated
589 with IGVtool (127). In all cases the *au1-shble-p2a-EGFP* coding sequence displayed highly similar coverage
590 shape for all constructs, except for shble#4 and shble#6 for which respectively one and two alternative
591 splicing events were observed (Sup. Fig. 3 and 4). None of these splice sites were predicted when the
592 theoretical transcripts were evaluated using *Human Splicing Finder* (HSF, accessed via
593 https://www.genomnis.com/access-hsf) (60), or with *SPLM - Search for human potential splice sites using*
594 *weight matrices* (accessed via http://www.softberry.com/) (61). When relevant, the three alternative transcript
595 isoforms identified were further used as reference for read pseudomapping and quantification with Kallisto
596 (v0.43.1) (128). Details on RNA preparation and bioinformatic pipeline are provided in Sup. Methods 2.4
597 and Sup. Methods 3.

598 **Label-free proteomic analysis.** The label-free proteomic was performed on nine biological
599 replicates (three of them measured independently, and six pooled by two), and eight different conditions:
600 shble#1 to shble#6, #empty, and mock. 20 to 30 µg of proteins were in-gel digested and resulting peptides
601 were analysed online using a Q Exactive HF mass spectrometer coupled with an Ultimate 3000 RSLC
602 system (Thermo Fisher Scientific). MS/MS analyses were performed using the Maxquant software (v1.5.5.1)
603 (129). All MS/MS spectra were searched by the Andromeda search engine (130) against a decoy database
604 consisting in a combination of *Homo sapiens* entries from Reference Proteome (UP000005640, release
605 2019_02, https://www.uniprot.org/), a database with classical contaminants, and the sequences of interest
606 (SHBLE and EGFP). After excluding the usual contaminants, we obtained a final set of 4,302 proteins
607 detected at least once in one of the samples. Intensity based absolute quantification (iBAQ) was used to
608 compare protein levels between samples (131).

609 **Western blot immunoassays and semi-quantitative analysis.** Western blot immunoassays were
610 performed on nine replicates and nine conditions: shble#1 to shble#6, #empty, #superempty, and mock.
611 Three different proteins were targetted: β-TUBULIN, EGFP, and SHBLE (*via* the invariable AU1 epitope

tag). Semi-quantitative analysis from enzyme chemoluminiscence data was performed with ImageJ (132) by «plotting lanes» to obtain relative density plots (Sup. Fig. 7).

Flow cytometry analysis. Flow cytometry experiments were performed on a NovoCyte flow cytometer system (ACEA biosciences). 50,000 ungated events were acquired with the NovoExpress software, and further filtering of debris and doublets was performed in R with an in-house script (filtering strategy is detailed in Sup. Method 2.7). For subsequent analysis, 30,000 events were randomly picked up from each sample. Seven samples had less than 30,000 events and, in order to ensure the same sample size for all conditions, the four corresponding replicates were excluded. After a first visualization of the data, two replicates were ruled out because they displayed a typical pattern of failed transfection for the condition shble#1 (Sup. Method 2.7), resulting in 16 final replicates being fully examined.

Real time cell growth analysis (RTCA). RTCA was carried out on an xCELLigence system for the mock and the superempty controls, and further eight constructs: the previously analysed shble#1 to shble#6, plus the shble#1* and shble#4* lacking the *EGFP* reporter gene. Cells were grown under different concentrations of the Bleomycin antibiotic ranging from 0 to 5000 µg/mL (Sup. Method 2.8). Three to six biological replicates were performed, including technical duplicates for each replicate. Cells were grown on microtiter plates with interdigitated gold electrodes that allow to estimate cell density by means of impedance measurement. Measures were acquired every 15 minutes, over 70 hours (280 time points). Impedance measurements are reported as "Cell Index" values, which are compared to the initial baseline values to estimate changes in cellular performance linked to the expression of the different constructs. For each construct we estimated first cellular fitness by calculating the area below the curve for the delta-Cell index *vs* time for the cells grown in the absence of antibiotics. We estimated then the ability to resist the antibiotic conferred by each construct through calculation of IC50 as the bleomycin concentration that reduces the area below the curve to half of the one estimated in the absence of antibiotics (detailled methods in Sup. Method 2.8).

Data availability. RNAseq raw reads were deposited on the NCBI-SRA database under the BioProject number PRJNA753061. R scripts and input files are available at https://github.com/malpicard/synonymous-but-not-neutral.git.

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