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## Potential role of the *X* circular code in the regulation of gene expression

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Keywords: gene expression; codon optimization; genetic code; circular code

## Abstract

It is proposed that the *X* circular code, a set of 20 trinucleotides (codons) that has been identified in the protein-coding genes of most organisms from bacteria to eukaryotes, represents a new genetic signal that contributes to the regulation of gene expression. Circular codes have the important mathematical property of being error-correcting codes, and it has been hypothesized that the *X* circular code represents an ancestor of the standard genetic code, used in primordial systems to simultaneously decode amino acids and synchronize the reading frame. Here, using previously published experimental data, we demonstrate important correlations between the presence of *X* motifs in genes and more efficient gene expression. Our contributions suggest that *X* motifs continue to play a functional role in extant genomes and represent a new genetic signal, contributing to the maintenance of the correct reading frame and the optimization and regulation of gene expression.

## 1 Introduction

Codes are ubiquitous in genomes: for example, the genetic code, the nucleosome positioning code, the histone code, the splicing code, mRNA degradation code, or the 'codon usage' code [1-6], to name but a few. The standard genetic code [1] is probably the most well-known genome code, and represents one of the greatest discoveries of the 20th century. All known life on Earth uses the (quasi-)same triplet genetic code to control the translation of genes into functional proteins. The fact that there are 64 possible nucleotide triplet combinations but only 20 amino acids to encode, means that the genetic code is redundant and most amino acids are encoded by more than one codon. This redundancy allows for the encoding of supplementary information in addition to the amino acid sequence [7-9], and significant efforts have been applied recently to understand the multiple layers of information or 'codes within the code' [10] that can be exploited to increase the versatility of genome decoding.

Here, we focus on an important class of genome codes, called the circular codes, first introduced by Arquès and Michel [11] and reviewed in [12,13]. In coding theory, a circular code is also known as an error-correcting code or a self-synchronizing code, since no external synchronization is required for reading frame identification. In other words, circular codes have the ability to detect and maintain the correct reading frame. For example, comma-free codes are a particularly efficient subclass of circular codes,

1 where the reading frame is detected by a single codon. The genetic code was originally proposed to be  
2 a comma-free code in order to explain how a sequence of codons could code for 20 amino acids, and at  
3 the same time how the correct reading frame could be retrieved and maintained [14]. However, it was  
4 later proved that the modern genetic code could not be a comma-free code [15], when it was discovered  
5 that *TTT*, a codon that cannot belong to a comma-free code, codes for phenylalanine. Other circular codes  
6 are less restrictive than comma-free codes, as a frameshift of 1 or 2 nucleotides in a sequence entirely  
7 consisting of codons from a circular code will not be detected immediately but after the reading of a  
8 certain number of nucleotides.  
9

10 By excluding the four periodic codons {*AAA,CCC,GGG,TTT*} and by assigning each codon to a preferential  
11 frame (i.e. each codon is assigned to the frame in which it occurs most frequently), the so-called *X*  
12 circular code (Fig. 1a,b) was identified in the reading frame of protein coding genes from eukaryotes  
13 and prokaryotes [11,16]. Other circular codes, and notably variations of the common *X* circular code, are  
14 hypothesized to exist in different organisms [16-18].

15 The *X* circular code has important mathematical properties, in particular it is self-complementary [11],  
16 meaning that if a codon belongs to *X* then its complementary trinucleotide also belongs to *X*. Like the  
17 comma-free codes, the *X* circular code also has the property of synchronizability. It has been shown that,  
18 in any sequence generated by the *X* circular code, at most 13 consecutive nucleotides are enough to  
19 always retrieve the reading frame [11]. In other words, any sequence 'motif' containing 4 consecutive *X*  
20 codons is sufficient to determine the correct reading frame (Fig. 1c) [20]. More formal definitions of the  
21 mathematical properties of the *X* circular code are available [12-13,21].

22 The hypothesis of the *X* circular code in genes is supported by evidence from several statistical analyses  
23 of modern genomes. We previously showed in a large-scale study of 138 eukaryotic genomes that *X*  
24 motifs (defined as series of at least 4 codons from the *X* circular code) are found preferentially in protein-  
25 coding genes compared to non-coding regions with a ratio of ~8 times more *X* motifs located in genes  
26 [22]. More detailed studies of the complete gene sets of yeast and mammal genomes confirmed the strong  
27 enrichment of *X* motifs in genes and further demonstrated a statistically significant enrichment in the  
28 reading frame compared to frames 1 and 2 [23-24]. In addition, it was shown that most of the mRNA  
29 sequences from these organisms (e.g. 98% of experimentally verified genes in *S. cerevisiae*) contain *X*

1 motifs. Intriguingly, conserved *X* motifs have also been found in many tRNA genes [25], as well as in  
2 important functional regions of the 16S/18S ribosomal RNA (rRNA) from bacteria, archaea and  
3 eukaryotes [26-27], which suggest their involvement in universal gene translation mechanisms. More  
4 recently, a circular code periodicity 0 modulo 3 was identified in the 16S rRNA, covering the region that  
5 corresponds to the primordial proto-ribosome decoding center and containing numerous sites that  
6 interact with the tRNA and messenger RNA (mRNA) during translation [20].  
7

8 Based on these observations, it has been proposed that the *X* circular code represents an ancestor of the  
9 modern genetic code that was used to code for a smaller number of amino acids and simultaneously  
10 identify and maintain the reading frame [27]. Intriguingly, the theoretical minimal RNA rings, short RNAs  
11 designed to code for all coding signals without coding redundancy among frames, are also biased for  
12 codons from the *X* circular code [28]. These RNA rings attempt to mimic primitive tRNAs and potentially  
13 reflect ancient translation machineries [29-30].  
14

15 The question remains of whether the *X* motifs observed in modern genes are simply a vestige of an  
16 ancient code that might have existed in the early stages of cellular life, or whether they still play a role  
17 in the complex translation systems of extant organisms.  
18

19 In this work, we define a simple density parameter representing the coverage of *X* motifs in genes.  
20 Unexpectedly, this parameter identifies several relations between the *X* circular code and translation  
21 efficiency and/or kinetics. Our observations provide evidence supporting the idea that motifs from the  
22 *X* circular code represent a new genetic signal, participating in the maintenance of the correct reading  
23 frame and the optimization and regulation of gene expression.  
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## 28 **2. The *X* circular code and 'optimal' codons/dicodons for translation efficiency**

### 29 **2.1 *X* codons correlate with optimal codons**

30 We compared the 20 codons that belong to the *X* circular code with the 'codon optimality code' resulting  
31 from various statistical and experimental studies in metazoan [31], as well as in *S. cerevisiae* [32]. In these  
32 studies, the codon stabilization coefficient (CSC), defined as the Pearson correlation coefficient between  
33 the occurrence of each codon and the half-life of each mRNA, was used as a robust and conserved  
34 measure of how individual codons contribute to shape mRNA stability and translation efficiency. Thus,  
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2 codons found more frequently in genes with longer mRNA half-lives have higher CSC values. The 61  
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4 coding codons can then be ranked according to their CSC scores in different organisms. Fig. 2 shows the  
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6 mean ranking of optimal codons, according to the CSC score, from four different experiments (in *S.*  
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8 *cerevisiae*, zebrafish, *Xenopus* and *Drosophila*), where the highest ranking codon is the most optimal one.  
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10 The *X* codons are ranked significantly higher than non-*X* codons (i.e. the 41 coding codons which do not  
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12 belong to the circular code *X*), according to a Mann-Whitney signed rank test ( $z$ -score = 4.3,  $p$ -value <  
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14 0.00001). In other words, optimal codons for mRNA stability and elongation rate are significantly  
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16 enriched in *X* codons.  
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## 21 **2.2 *X* codons correlate with the dicodons associated with increased expression**

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23 In recent years, emerging evidence has shown that translational rates may be encoded by dicodons  
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25 rather than single codons [33-35]. For example, a large-scale screen in *S. cerevisiae* [33] assessed the degree  
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27 to which codon context modulates eukaryotic translation elongation rates beyond effects seen at the  
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29 individual codon level. The authors screened yeast cell populations housing libraries containing random  
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31 sets of triplet codons within an ORF encoding superfolder Green Fluorescent Protein (GFP). They found  
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33 that 17 dicodons were strongly associated with reduced GFP expression, i.e. associated with a  
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35 substantial reduction of the translation elongation rate. This set included the known inhibitory dicodon  
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37 *CGA-CGA* and was enriched for codons decoded by wobble interactions. Of these 17 dicodons associated  
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39 with slower translation elongation rates, none are composed of 2 *X* codons (Supplementary Table 1).  
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43 A subsequent statistical analysis of coding sequences of nine organisms [34] identified dicodons with  
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45 significant different frequency usage for coding either lowly or highly abundant proteins. The working  
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47 hypothesis was that sequences encoding abundant proteins should be optimized, in the sense of  
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49 translation efficiency. 16 dicodons were identified with a preference for low abundance proteins, while  
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51 40 dicodons presented a preference for high abundance proteins. None of the 16 dicodons associated  
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53 with low abundance proteins are composed of 2 *X* codons (Supplementary Table S1). In contrast, 27 of  
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55 the 40 dicodons associated with high abundance proteins correspond to 2 *X* codons, and only 3 dicodons  
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57 do not contain any *X* codons (Supplementary Table S1).  
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2 These recent studies support the idea that codons in coding sequences are likely arranged in an  
3 organized way, and that the local sequence context contributes to the effects of codon usage bias on gene  
4 regulation. Strikingly, our observations support the hypothesis that codon context may be linked in  
5 some way to the *X* circular code. In the next section, we describe more detailed analyses that test this  
6 hypothesis further.  
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### 15 **3. *X* motifs are enriched in the minimal gene set**

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17 Based on the increasing evidence of the importance of codon context [35-39], we hypothesized that if the  
18 *X* circular code plays a role in gene regulation, then we might expect to see a non-random use, or  
19 'clusters', of *X* codons along the length of the gene. In previous work [23-24], we defined an *X* motif as a  
20 series of consecutive *X* codons (of length at least 4 codons in order to always retrieve the reading frame,  
21 see Supplementary Materials) in a gene sequence and searched for such *X* motifs in the reading frames  
22 of different genes. This approach allowed us to demonstrate that the reading frames of genes in yeasts  
23 and in mammals are significantly enriched in such *X* motifs.  
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32 To test the hypothesis that the *X* motifs represent a more universal signature, we analyzed a set of 81  
33 genes that were previously defined as a 'minimal gene set' [40]. At that time, the 'minimal gene set' genes  
34 were found to be conserved in all species. We used the *Mycoplasma genitalium* genes provided in the  
35 original study, as well as 15,822 orthologous sequences (5503 eukaryotes, 9205 bacteria and 1114  
36 archaea) from the OrthoInspector 3.0 database [41], and identified all *X* motifs in the reading frame with  
37 a minimum length of 4 codons. Fig. 3 shows the density (defined as the number of *X* motifs per kilobase,  
38 see Supplementary Materials) of the *X* motifs in the mRNA sequences. To evaluate the significance of the  
39 enrichment, as in previous work [23-24], we used a randomization model in which we generated N=100  
40 random codes that preserved most of the properties to the *X* code, except the circularity. We then  
41 identified all random motifs from the 100 random codes and calculated mean values for the 100 codes.  
42 The density of *X* motifs found in the minimal gene set sequences belonging to the three domains of life,  
43 is significantly higher than the density of random motifs according to a one-sided Student's *t*-test ( $p <$   
44  $10^{-100}$ ) for each set of sequences from archaea, bacteria and eukaryota. This study demonstrates that *X*  
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1 motifs are significantly enriched in the minimal gene set, and seem to be a universal feature of gene  
2 sequences in all three domains of life.  
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#### 8 **4. X motifs are enriched in codon-optimized genes**

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10 If *X* motifs modify the codon usage in favor of optimal codons for translational efficiency, then we would  
11 expect that increasing the number of *X* motifs in a gene would increase the expression level. In an  
12 indirect way, we have shown that this is indeed the case. We previously showed that synthetic genes,  
13 which were re-designed for optimized protein expression, generally have more *X* motifs [24].  
14 Supplementary Fig. 1A shows an example of the protein L1h from human papillomavirus (HPV-16),  
15 optimized for expression in mammalian cell lines and leading to significantly increased expression [42].  
16 Here, the wild type gene contains only 3 *X* motifs, while the optimized gene construct has a total of 21 *X*  
17 motifs. It is important to note that classical codon optimization strategies do not always increase protein  
18 expression levels. Supplementary Fig. 1B shows another example involving the L1s protein from human  
19 papillomavirus (HPV-11) optimized for expression in the potato *Solanum tuberosum* [43]. In this case,  
20 only a low level of L1 expression was observed for the codon-optimized gene. In this example, we did  
21 not observe a significant difference between the number of *X* motifs in the wild type and optimized  
22 sequences (5 *X* motifs in the wild type gene compared to 4 in the optimized construct).  
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38 Codon replacement strategies have also been applied to the design of attenuated viruses, although in  
39 this case frequent codons are replaced with rare ones. Using quantitative proteomics and RNA  
40 sequencing, the molecular basis of attenuation in a strain of bacteriophage T7 (*Escherichia coli* K-12)  
41 was investigated [44]. The authors engineered the *E. coli* major capsid protein gene (gene 10A) to carry  
42 different proportions of suboptimal, rare codons. Transcriptional effects of the recoding were not  
43 observed, but proteomic observations revealed that translation was halved for the completely recoded  
44 major capsid gene, with subsequent effects on virus fitness (measured as doublings/hour). We obtained  
45 the sequences with 10%, 20%, 30% and 50% recoding from [45] and identified the density (defined in  
46 Supplementary Materials) of *X* motifs in each construct. Fig. 4 clearly shows the correlation between the  
47 fitness obtained for each recoded sequence and the density of *X* motifs observed. The authors suggested  
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1 that recoding of gene 10A reduced capsid protein abundance probably by ribosome stalling rather than  
2 ribosome fall-off.  
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6 In general, codon optimization is a successful strategy for improving protein expression in heterologous  
7 systems. However, simply replacing all rare codons by frequent codons can have negative effects *in vivo*  
8 [46]. Rare codons have the potential to slow down the translation elongation rate, due to the relatively  
9 long dwell time of the ribosome while searching for rare tRNAs. Several studies have suggested that  
10 gene-wide codon bias in favor of slowly translated codons serves as a regulatory means to obtain low  
11 expression levels of protein when desired, for example, in the case of regulatory genes, or where excess  
12 of the protein may be detrimental or lethal to the cell. An example, in *Neurospora crassa*, demonstrated  
13 that codon optimization of the central clock protein FRQ actually abolished circadian rhythms [47].  
14 Different optimized constructs of the wild type gene *frq* were used in the study, where either the N-  
15 terminal end (codons 1-164) or the middle region (codons 185-530) was optimized. All optimized  
16 constructs gave higher levels of FRQ protein, this led to a different structural conformation. The density  
17 of *X* motifs (defined in Supplementary Materials) identified in the different wild type and optimized  
18 constructs is shown in Supplementary Table 22. As in the previous examples, the optimized constructs  
19 contain significantly more *X* motifs (for instance, density of 10.2 in the N-terminal end of the fully  
20 optimized construct compared to 4.1 in the wild type). This example shows how non-optimal codon  
21 usage, and the associated reduction in the number of *X* motifs, can be used *in vivo* to regulate protein  
22 expression and to achieve optimal protein structure and function.  
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42 In nature, the translation efficiency of a gene may vary at different conditions, cell types and tissues [48-  
43 51]. Thus, it has been proposed that the codon optimization should take into account other factors in  
44 addition to replacing rare codons by frequent ones, a process termed 'codon harmonization' [52-54]. Taken  
45 together, the examples described above suggest that it may be important for such harmonization  
46 strategies to consider the effect of codon replacement on the insertion or deletion of *X* motifs.  
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## 55 **5. *X* motifs correlate with translation efficiency and mRNA stability**

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2 In this section, we examine the recent evidence resulting from high-throughput technologies such as  
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4 ribosome profiling, and demonstrate that the presence of *X* motifs in genes can be used as a predictor of  
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6 gene expression level.  
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8 We have shown previously that the reading frames of genes in *S. cerevisiae* are significantly enriched in  
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10 *X* motifs [16]. Since then, ribosomal profiling has enabled a more detailed study of translation efficiency  
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12 for a large set of 5450 genes from this organism [55]. A central assumption of ribosome profiling is that  
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14 indirect measurement of the kinetics of translation *via* ribosome footprint occupancy on transcripts is  
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16 directly reflective of true protein synthesis. The authors thus performed a simulation of translation  
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18 based on the totally asymmetric simple exclusion process (TASEP) model, using experimental  
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20 measurements of the number of ribosomes on each transcript as well as RNA copy numbers to calibrate  
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22 the parameters. They used the simulation to estimate the average translation rate of each gene, using  
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24 experimental measurements of ribosome occupancy. Again, we identified the *X* motifs in the complete  
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26 set of 5450 genes and calculated the density of *X* motifs (defined in Supplementary Materials) in three  
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28 subsets of the genes having different estimated translation rates (Fig. 5). We observed that genes with  
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30 higher translation rates had significantly more *X* motifs than those with lower translation rates. The  
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32 density of *X* motifs is higher for the sequences with medium translation rates than for those with low  
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34 translation rates (one-sided Student's t-test  $p < 10^{-10}$ ) and for the sequences with high translation rates  
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36 than for those with medium translation rates (one-sided Student's t-test  $p < 10^{-14}$ ). This result  
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38 demonstrates the link between the total time needed for ribosome transition on a mRNA and density of  
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43 *X* motifs along the length of the sequence.

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45 To investigate whether *X* motifs might play a role in modulating ribosome speed in specific regions in  
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47 mRNA, we considered single protein studies, where local translation elongation rate has been studied  
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49 in detail. The first example concerns the study of a gene in *S. cerevisiae*, to investigate the link between  
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51 translational elongation and mRNA decay [56]. In this study, various HIS3 protein constructs (length of  
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53 699 nucleotides) were designed with increasing codon optimality (measured by the CSC index) from  
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55 0% to 100%. We identified *X* motifs in the different constructs as before and compared them to the  
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57 experimentally measured mRNA half-life. As the authors point out, the mRNA half-life is largely  
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59 determined by the codon-dependent rate of translational elongation, since mRNAs whose translation  
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1 elongation rate is slowed by inclusion of non-optimal codons are specifically degraded. The density of *X*  
2 motifs ranges from 0 in the 0% optimized construct to more than 7 in the 100% optimized sequence  
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4 (Fig. 6a). The results suggest that the introduction of individual *X* motifs in specific regions can be used  
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6 to increase the mRNA half-life.  
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10 The second example concerns a *Drosophila* cell-free translation system that was used to directly  
11 compare the rate of mRNA translation elongation for different luciferase constructs with synonymous  
12 substitutions [57]. The OPT construct was designed with the most preferred codons in all positions except  
13 for the first 10 codons, while the dOPT construct had the least preferred codons in all positions. The N-  
14 OPT, M-OPT and C-OPT constructs were created by replacing the N-terminal part (codons 11–223),  
15 middle part (codons 224–423) and C-terminal part (codons 424–550) of the dOPT sequence with the  
16 corresponding optimized sequence, respectively. For each construct, the authors measured the time  
17 when the luminescence signal was first detected after start of translation. The time of first appearance  
18 (TFA) should thus reflect the speed of translation process. Higher TFA values were observed for each  
19 construct in the order dOPT < C-OPT < M-OPT < N-OPT < OPT, correlating well with an increasing  
20 density of *X* motifs (Fig. 6b). These results suggest that the introduction of *X* motifs in different regions  
21 of the gene significantly increased the rate of translation elongation, probably by speeding up ribosome  
22 movement on the mRNA.  
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26 We have highlighted the potential effects of *X* motifs on translation elongation speed, protein folding  
27 and function. The examples selected include studies in very different organisms, including viruses, fungi  
28 and insects with different codon usage bias (codon usage tables for these organisms are provided in  
29 Supplementary Table S3), but in all the examples a strong correlation is observed between ‘optimal’  
30 codons and *X* codons. Taken together, the results support the idea that the use of *X* motifs is a conserved  
31 mechanism from viruses to animals that may participate in the modulation or regulation of the  
32 translation elongation rate along the mRNA.  
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## 38 6. Conclusions and Prospects

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40 In this work, we have combined two very distinct research domains: gene translation through the  
41 genetic code and the theory of circular codes which allows two processes simultaneously: reading frame  
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1 retrieval and amino acid coding. Our hypothesis is that at least two codes operate in genes: the standard  
2 genetic code, experimentally proved to be functional, and the *X* circular code that has been shown to be  
3 statistically enriched in genes. For the first time here, we shed light on a number of biological  
4 experimental results by using the definition of a very simple parameter to analyze the density of *X* motifs  
5 in genes, i.e. motifs from the circular code *X*.  
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12 We would first like to make some comments about the mathematical structure of these two codes. The  
13 standard genetic code consists of 60 codons coding for 19 amino acids, the start codon *ATG* that codes  
14 for the amino acid *Met* and establishes the reading frame, and three non-coding stop codons  
15 {*TAA, TAG, TGA*}. The genetic code has a weak mathematical structure: a surjective coding map for the 60  
16 codons and an incomplete self-complementary property for the 60 codons (e.g. the complementary  
17 codon of *TTA* coding the amino acid *Leu* is the stop codon *TAA*) implying a non self-complementary  
18 property for the four start/stop codons. The circular code *X* consists of 20 codons coding for 12 amino  
19 acids and has a strong mathematical structure: circularity for retrieving the reading frame, a surjective  
20 coding map, a complete self-complementary property for the 20 codons, a  $C^3$  property, etc. (reviewed in  
21 [12-13]).  
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34 We propose that the theory of circular codes can be used to shed light on many of the observed  
35 phenomena related to optimal codons/dicodons and the effects of codon optimization on different  
36 factors of gene expression, from transcriptional regulation to translation initiation, retrieval of the open  
37 reading frame, translation elongation velocities, and protein folding. We showed that optimal codons at  
38 the species and gene levels correlate well with the 20 codons that define the *X* circular code. Importantly,  
39 the optimal codons identified in diverse species [31] that increase translation elongation rates and mRNA  
40 stability are significantly enriched in *X* codons. We then studied a number of published experiments that  
41 used recent technologies to perform more detailed investigations of codon usage along the length of a  
42 gene, which suggest that codon context and local clusters of optimal or non-optimal codons may  
43 represent important regulatory signals for translation bursts and pauses [6,58]. In all these experiments,  
44 increased translation efficiency correlates with the number of *X* motifs present in the gene sequences.  
45 These observations raise the question: do *X* motifs somehow orchestrate elongation rate? Since it is  
46 known that translational elongation rate is intimately connected to mRNA stability, it is also tempting  
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2 to suggest that *X* motifs are linked to the universal code of codon-mediated mRNA decay proposed by  
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4 Chen and Coller [59].

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6 The theory of the *X* circular code will have practical implications for improving the prediction of gene  
7  
8 expression levels based on the gene sequence. Most of the current codon usage measures are dependent  
9  
10 on the studied organism and the chosen expression system. In contrast, the presence of *X* motifs  
11  
12 represents a universal signature that is significantly correlated with increased expression. Our previous  
13  
14 work has already shown that *X* motifs can predict functional versus dubious genes in yeast [23] and can  
15  
16 be used for rational gene design [24].

17  
18 Translation of mRNA by the ribosome is a universal mechanism, and the most parsimonious explanation  
19  
20 for the observed correlation between the presence of *X* motifs and increased translation elongation rates  
21  
22 is that *X* motifs are somehow recognized by the ribosome. It is known that codon usage has effects on  
23  
24 the major steps of translation elongation, including codon-anticodon decoding and peptide bond  
25  
26 formation [58], as well as translocation which can be slowed down by mRNA secondary structure  
27  
28 elements, such as pseudoknots and stem-loops [60]. Our hypothesis that *X* motifs in mRNA are  
29  
30 recognized by the ribosome is further supported by recent ribosome profiling experiments in  
31  
32 *Neurospora crassa*, which suggest that codon optimization increases the rate of ribosome movement on  
33  
34 mRNA [61], and by the observation that translation elongation and mRNA stability are coupled through  
35  
36 the ribosomal A-site [62]. Interestingly, our previous work has identified *X* motifs in the anticodon region  
37  
38 of multiple tRNA genes, as well as in important functional regions of the ribosomal rRNA including the  
39  
40 decoding centre [25-27].

41  
42 How could motifs from the *X* circular code work? If the decoding unit at the ribosome is the anticodon  
43  
44 then the comma-free codes would immediately return to the reading phase while the general circular  
45  
46 codes would have a delay associated with reading at most four codons (exactly 13 nucleotides). If the  
47  
48 decoding unit at the ribosome is the anticodon with adjacent nucleotides then the general circular codes  
49  
50 could also immediately return to the reading phase. Does the self-complementary property of the *X*  
51  
52 circular code contribute to coordination between *X* motifs in mRNA and *X* motifs in tRNA and/or rRNA?  
53  
54 So far we have mainly discussed the effects of codon choices on the throughput of translation, but  
55  
56 changes in the translation elongation process can clearly affect translation fidelity and accuracy,  
57  
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1 reviewed in <sup>[63]</sup>. For example, clustering of rare codons could deplete cognate tRNAs, increasing the  
2 probability of a near- or non-cognate tRNA occupying the decoding site, and this probability could be  
3  
4 reflected in the frequency of miss-incorporation. In this case, it has been shown that the standard genetic  
5  
6 code minimizes the impact of the mutations on the translated protein <sup>[56]</sup>. Clustering of identical rare  
7  
8 codons also increases the probability of a frameshift during translation. Ribosome stalling at *Lys* codons  
9  
10 triggers ribosome sliding on successive *AAA* codons. When ribosomes resume translation, they may shift  
11  
12 in an incorrect reading frame. The ribosomes translating in the -1 or +1 frame usually quickly encounter  
13  
14 out-of-frame stop codons that result in termination. Again, it has been suggested that the genetic code  
15  
16 might be in some way optimized for frameshift mutations <sup>[64]</sup>. Given the inherent error correcting  
17  
18 properties of circular codes, it is possible that the *X* circular code may play a role in the synchronization  
19  
20 of the correct reading frame.  
21  
22

23  
24 In the future, we hope to show that the simple parameter defined in this work to estimate the coverage  
25  
26 of *X* motifs in genes is a useful factor that should be taken into account in codon optimization strategies  
27  
28 or other experimental approaches involving gene expression. We also plan to investigate more complex  
29  
30 parameters linked to *X* motifs, such as localized density patterns within specific regions of the genes.  
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35

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45  
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48 interest.  
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## Figure Legends

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47 Fig 1. a) Definition of the *X* circular code. b) Circular representation of the genetic code, adapted from  
48 [19], with the 20 codons of the *X* circular code shown on the circumference. The numbers after the  
49 nucleotides indicate their position in the codon. *X* codons that are complementary to each other are  
50 highlighted in the same color. c) Retrieval of the reading frame in a *X* motif constructed with the *X*  
51 circular code. Codons belonging to the *X* circular code are indicated in blue, while non-*X* codons are  
52 shown in red. Among the three possible frames, only the reading frame 0 contains codons of the *X*  
53 circular code exclusively.  
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4 Fig 2. Optimal codons for translation elongation rate and mRNA stability in different eukaryotic species  
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6 (*S. cerevisiae*, zebrafish, *Xenopus* and *Drosophila*). Codons are ordered according to their mean ranking  
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8 obtained in four different experiments. Codons belonging to the X code are identified by a blue star.  
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12 Fig 3. Density of X motifs in the mRNA sequences of the 'minimal gene set'. The distributions of the  
13  
14 number of X motifs identified in the sequences from the three domains of life are indicated by boxplots  
15  
16 representing the mean number with a  $\pm 0.99$  confidence interval. The distributions of the number of R  
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18 random motifs (see Supplementary Materials) identified in the same sequences are shown for statistical  
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20 evaluation. There is a very strong statistical significance as confirmed by a one-sided Student's *t*-test  
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22 with a *p*-value  $p < 10^{-100}$  for each set of sequences from archaea, bacteria and eukaryota.  
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26  
27 Fig 4. Histogram of the density of X motifs in the recoded version of the gene 10A from *Escherichia coli*  
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29 K-12, compared to the wild type sequence. The orange plot indicates the viral fitness values  
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31 corresponding to each construct.  
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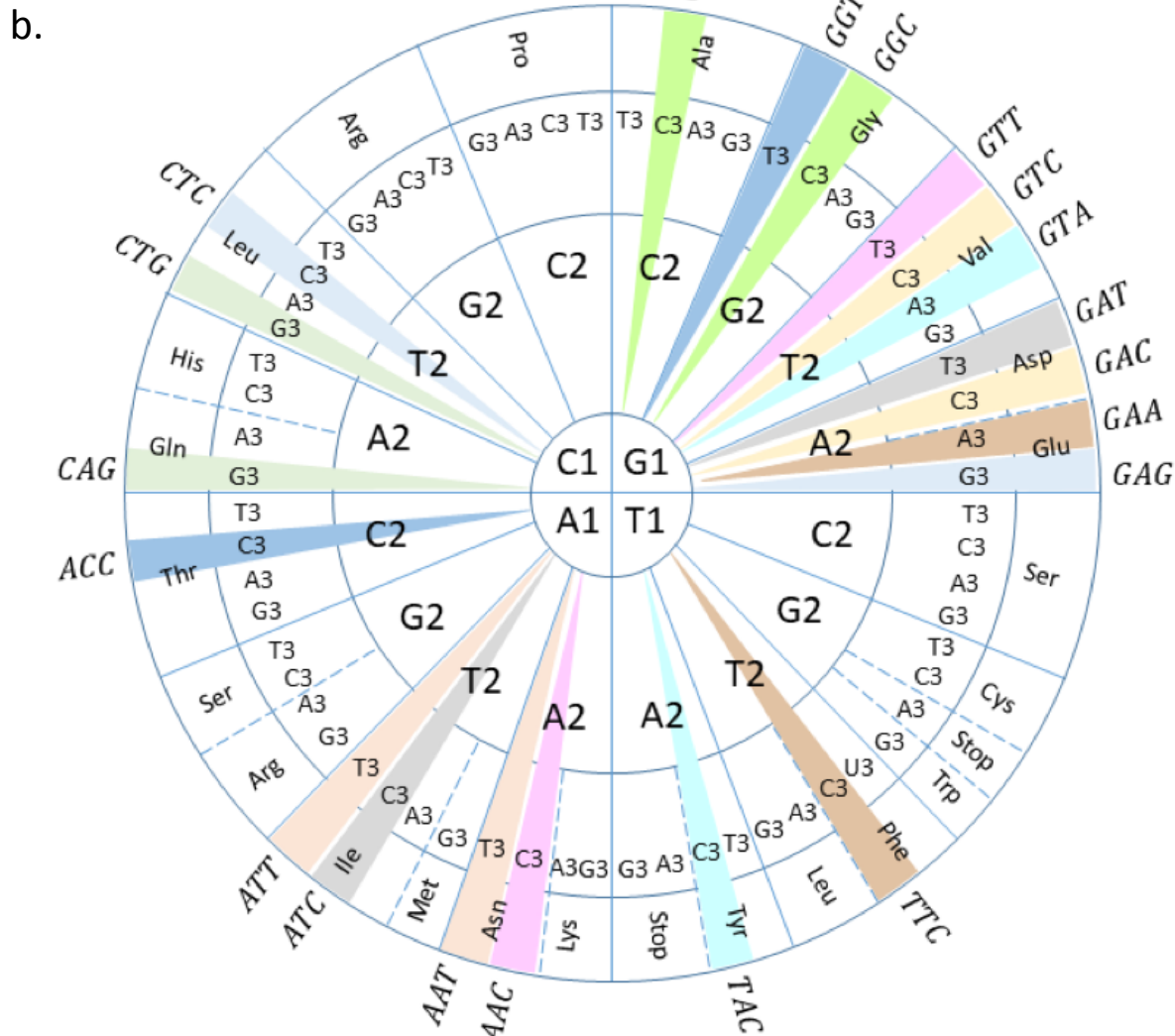
34  
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36 Fig 5. Density of X motifs for *S. cerevisiae* genes: 1323 genes with low translation rates (estimated  
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38 translation rate  $< 0.03$ ), 1378 genes with medium translation rates (estimated translation rate 0.05-  
39  
40 0.09) and 1324 genes with high translation rates (estimated translation rate  $> 1.1$ ). The distributions of  
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42 the number of X motifs identified in the genes are indicated by boxplots representing the mean number  
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44 with a  $\pm 0.99$  confidence interval. The statistical significance is confirmed by two one-sided Student's *t*-  
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46 tests with:  $p < 10^{-10}$  between the sequences with medium translation rates and those with low  
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48 translation rates; and  $p < 10^{-14}$  between the sequences with high translation rates and those with medium  
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50 translation rates.  
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55 Fig 6. a) Histogram of the density of X motifs for different constructs corresponding to the *S. cerevisiae*  
56  
57 HIS3 gene with 0-100% optimized codons. The orange plot indicates the mRNA half-life values  
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59 corresponding to each construct. b) Density of X motifs in the different constructs corresponding to the  
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1  
2 *Drosophila* luciferase gene. Sequence regions shown in blue are codon optimized, and in red are the wild  
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4 type sequence. The numbers above the sequences indicate the codon positions of the optimized regions.  
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- a. X circular code contains 20 codons:  
 {AAC, AAT, ACC, ATC, ATT, CAG, CTC, CTG, GAA, GAC,  
 GAG, GAT, GCC, GGC, GGT, GTA, GTC, GTT, TAC, TTC}
- X codes for 12 amino acids:  
 {Ala, Asn, Asp, Gln, Glu, Gly, Ile, Leu, Phe, Thr, Tyr, Val}



- c.
- $f_0$  ATG ... **G G T A A T T A C G A G** ... TAA
- $f_1$  ATG ... G **G T A A T T A C G** A G ... TAA
- $f_2$  ATG ... G G **T A A T T A C G A** G ... TAA

Figure 1



Figure 2

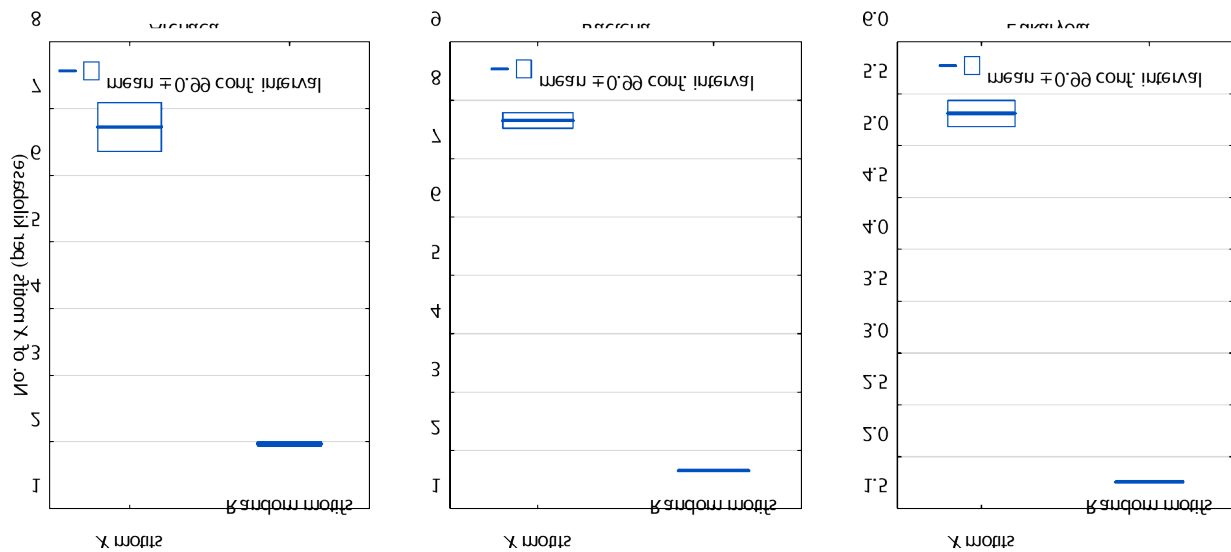


Figure 3



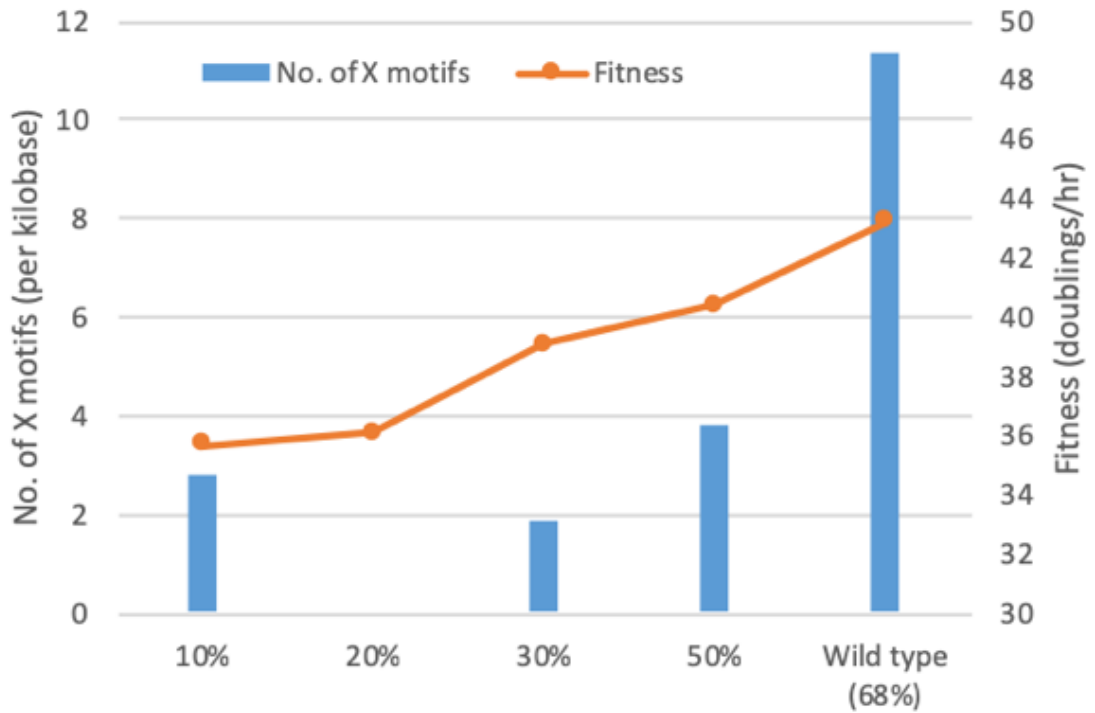


Figure 4

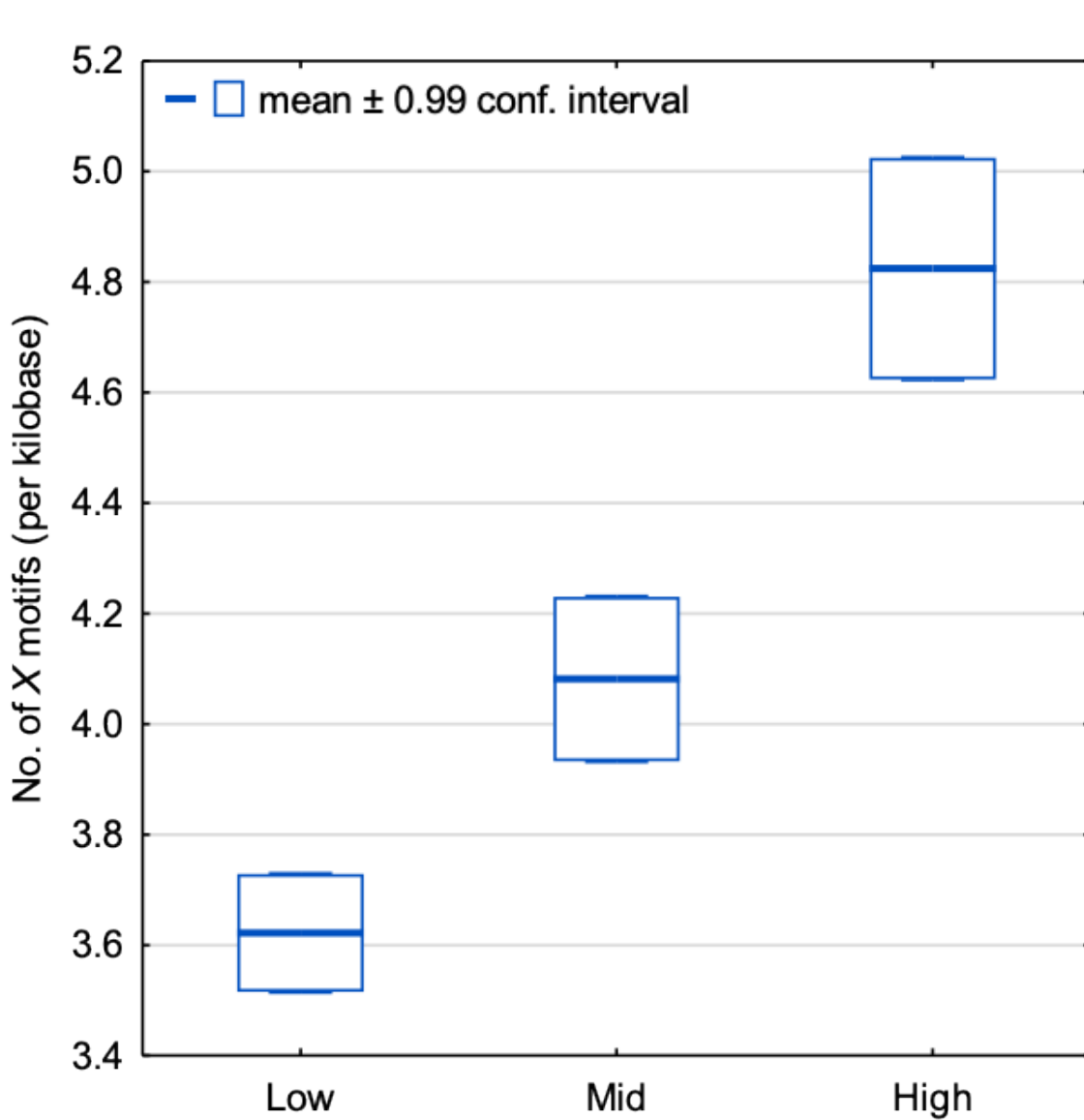


Figure 5

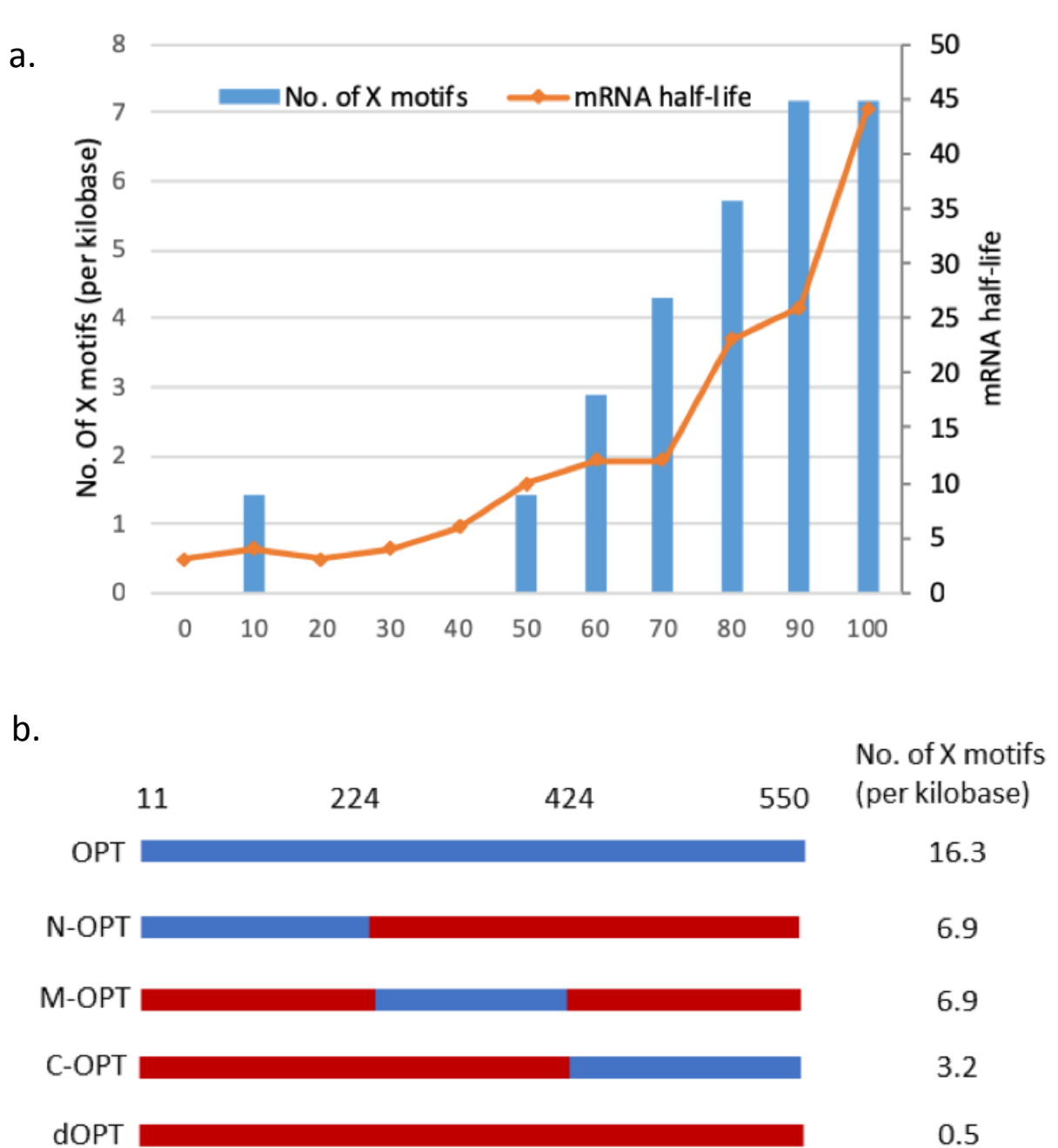


Figure 6