Gene- and Genome-Based Analysis of Significant Codon Patterns in Yeast, Rat and Mice Genomes with the CUT Codon Utilization Tool

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Abstract

The translation of mRNA in all forms of life uses a three-nucleotide codon and aminoacyl-tRNAs to synthesize a protein. There are 64 possible codons in the genetic code, with codons for the ~20 amino acids and 3 stop codons having 1- to 6-fold degeneracy. Recent studies have shown that families of stress response transcripts, termed modification tunable transcripts (MoTTs), use distinct codon biases that match specifically modified tRNAs to regulate their translation during a stress. Similarly, translational reprogramming of the UGA stop codon to generate selenoproteins or to perform programmed translational read-through (PTR) that results in a longer protein, requires distinct codon bias (*i.e.*, more than one stop codon) and, in the case of selenoproteins, a specifically modified tRNA. In an effort to identify transcripts that have codon usage patterns that could be subject to translational control mechanisms, we have used existing genome and transcript data to develop the gene-specific Codon UTilization (CUT) tool and database, which details all 1-, 2-, 3-, 4- and 5-codon combinations for all genes or transcripts in yeast (*Saccharomyces cerevisiae*), mice (*Mus musculus*) and rats (*Rattus norvegicus*). Here, we describe the use of the CUT tool and database to characterize significant codon usage patterns in specific genes and groups of genes. In yeast, we demonstrate how the CUT database can be used to identify genes that have runs of specific codons (e.g., AGA, GAA, AAG) linked to translational regulation by tRNA methyltransferase 9 (Trm9). We further demonstrate how groups of genes can be analyzed to find significant dicodon patterns, with the 80 Gcn4-regulated transcripts significantly ($P < 0.00001$) over-represented with the AGA-GAA dicodon. We have also used the CUT database to identify mouse and rat transcripts with internal UGA codons, with the surprising finding of 45 and 120 such transcripts, respectively, which is much larger than expected. The UGA data suggest that there could be many more translationally reprogrammed transcripts than currently reported. CUT thus represents a multi-species codon-counting database that can be used with mRNA-, translation- and proteomics-based results to better understand and model translational control mechanisms.

1. Introduction

Translation is the process by which proteins are synthesized from messenger RNA (mRNA) in the ribosome, using both transfer RNAs (tRNAs) and other trans-acting factors. The codons in mRNA consist of three nucleotides and interact with the tRNA anticodon during translation to specify the insertion of one of ~20 amino acids in a growing peptide chain, or in the case of stop codons, to terminate translation. tRNA molecules consist of 70 to 90 nucleotides folded into a cloverleaf structure containing an anticodon loop and a 3'-CAA that is covalently linked to a specific amino acid. tRNA is initially transcribed to contain adenosine (A), guanosine (G), uridine (U) and cytidine (C), but it is the most heavily modified nucleic acid in the cell and contains an average of 9-11 post-transcriptional modifications at >30 conserved sites [1, 2]. These RNA modifications have been shown play important roles in promoting tRNA stability, optimizing codon-anticodon interactions and preventing translation errors [3, 4].

In mRNA there are 64 possible three-nucleotide combinations that comprise the full set of codons. AUG is generally used as the start codon and the translation initiation site, but UUG, GUG and CUG have also been identified as occasional start sites in some transcripts in several organisms [5]. The ribosome has three sites associated with bound tRNA: one where the aminoacyl-tRNA enters (A site), one that has the tRNA bound to the growing polypeptide chain (P site), and lastly an E site where tRNA exits the ribosome. The polypeptide chain is catalytically transferred from the tRNA at the P site to the tRNA at the A site, at which point the free tRNA exits through the E site and the ribosome moves along to the next codon [6]. Excluding stop codons, there are 61 possible codons that can occupy each site or more than 226,918 three-codon combinations that can associate with A-, P- and E-sites. Translation elongation continues until the ribosome encounters one of three stop codons, UAA, UAG or UGA. There are instances, described below, where a UGA stop codon, in conjunction with trans- and cis-acting factors, can signal for the addition of a unique amino acid and these instances highlight the use of codon-based translational regulatory mechanisms.

The regulation of gene expression by specific patterns of codon usage has been established for both prokaryotic and eukaryotic systems [7-12]. In bacterial systems where transcription is coupled to translation, the expression of tryptophan metabolic enzymes from the Trp operon is linked to the translation of the UGG-UGG dicodon, where UGG codes for tryptophan, in a short leader peptide. The transcription and subsequent

translation of tryptophan metabolic enzymes requires pausing at the UGG-UGG dicodon due to low levels of charged tryptophan tRNA, which promotes specific folding of the upstream transcript (the attenuator) to promote the transcription of the entire Trp operon [7]. Translational regulation has also been demonstrated in budding yeast for some stress response genes over-represented with codons linked to leucine, arginine and glutamic acid, by the regulated levels of modified cytidines and uridines in the wobble position of the tRNA anticodon. tRNA methyltransferase (Trm) 4 and Trm9 complete the formation of 5-methylcytidine (m^5C) and 5-methoxycarbonylmethyluridine (mcm^5U) and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), respectively, in the anticodon of tRNAs specific for Leu, Arg and Glu [8, 9, 13]. Further, the levels of m^5C (H_2O_2 , ROS agents), mcm^5U (HU, MMS, alkylating agents) and mcm^5s^2U (HU, MMS, alkylating agents) modifications have been shown to change in response to stress [8, 9, 14, 15]. Reporter, targeted, and systems-based studies comparing transcripts and proteins have demonstrated that the translation of UUG (Leu), AGA (Arg) and GAA (Glu) codons are dependent on specific wobble base modifications and that some stress response genes that over-use these codons have decreased translation in the absence of the corresponding Trm and wobble base modification [9, 10, 14, 16].

Links between the regulation of translation and distinct codon usage patterns has also been demonstrated for mammalian transcripts that use UGA stop codon reprogramming or programmed translational read-through (PTR). Stop codon recoding is used to incorporate selenocysteine into selenoproteins, with the corresponding activities involved in ROS detoxification, selenium utilization and thyroid function [12, 17]. Selenocysteine has been referred to as the 21st amino acid, with no dedicated triplet codon [18, 19]. As such, decoding for selenocysteine is unconventional and requires a "recoding" of the UGA stop codon for incorporation in a process termed stop codon reprogramming [17, 19, 20]. Similar to the yeast Trm9 example noted earlier, stop codon recoding utilizes specifically modified wobble uridine bases, mcm^5U and 5-methoxycarbonylmethyl-2'-O-methyluridine (mcm^5Um), to promote optimal anticodon-codon interactions [17, 21, 22], as well as 3'-UTR regulatory sequence and trans-acting factors. The 3'-UTR found in transcripts encoding selenoproteins contains a selenocysteine insertion sequence (SECIS) that helps identify the internal UGA codons being re-coded for selenocysteine [23, 24]. PTR has also been shown to occur at UGA stop codons, as in the recent notable example of human vascular endothelial growth factor-A (VEGFA) isoform VEGF-Ax, in which Ax denotes an extended form [25]. VEGFA encodes a growth factor that promotes

endothelial cell migration and growth, with roles in angiogenesis and tumorigenesis, among others [26].

VEGFA contains another stop codon that is 22 codons (66 nts) down from the first UGA stop codon, with the nucleotide sequence well conserved in humans, rats and mice. PTR of the first UGA stop codon inserts serine and promotes the formation of a 22 amino acid extended version (VEGF-Ax), with PTR requiring a cis-acting element found in the Ax region that is 63 nts downstream of the canonical stop codon and corresponds to an hnRNP1 A2/B1 element [25]. With similarities to stop codon recoding, PTR uses cis-acting sequences, but the precise tRNA involved in PTR has not been clearly identified. PTR has also been shown for Ago1 and Mtch2 mRNA, and is predicted for Nr1d1, Adamts4 and Tox [25]. Other examples of translational read-through (Mdh1 and LdhB) have been reported to include stop codons outside of UGA, with targeted and genome-wide approaches identifying real and potential targets in humans, fruit flies, yeast and viruses [27].

Codon usage is one of the many parameters involved in the translational regulation of specific transcripts. Monocodon usage data is readily available to describe genome-based trends [28] and, in the case of yeast, to describe individual genes [13]. Bacterial studies on the Trp and other operons for other metabolic processes have demonstrated that dicodons in individual genes can have regulatory effects [29]. To investigate the potential regulatory effects of gene-specific dicodons and other codon combinations, we have developed a Codon UTilization (CUT) tool and database to analyze all genes or transcripts banked for a species, and we applied this tool to the analysis of yeast, mouse and rat genome sequence data (<http://pare.sunycnse.com/cut/index.jsp>). We demonstrate in yeast that specific dicodon and quadruple codon runs can be linked to optimal translation by Trm9-catalyzed tRNA. Further, we show that the AGA-GAA dicodon is over-represented in 80 Gcn4-regulated transcripts, which provides a novel link between the regulation of translation initiation in the Gcn2-eIF2 α -Gcn4 pathway and regulation of translational elongation by Trm9. Finally, we demonstrate how CUT can be used as an exploratory tool in identifying 45 mouse and 120 rat transcripts that contain an internal UGA codon, which greatly expands the potential mammalian targets that could be undergoing stop codon recoding or some form of PTR.

2. Materials and Methods

2.1 CUT tool algorithms and database design

The CUT tool was implemented using a standard “Model View Controller” (MVC) software design pattern as a Java 2 Enterprise Edition (J2EE) package deployed on a JBoss application server. The “Model” is comprised of a MySQL relational database and Enterprise Java Bean (EJB), the “View” is provided by Java Server Pages (JSPs), and the “Controller” is formed by Java Servlets. Offline Java code was written to populate the database by parsing GTF annotation files (obtained from SGD and the UCSC table browser) and using this data to assemble coding sequences for each protein coding gene or transcript from the relevant genome sequences. The coding sequences then undergo a brief quality “control” check (e.g., ensure complete reading frame and accepted start codons). Accepted coding sequences were then analyzed for specific codon and codon combination usage. Codon sequence bias (for every contiguous combination of length 1 to 5) in each transcript is calculated as a Z-score (number of standard deviations above/below the population mean) by comparison to the full population of transcripts within a particular annotation set. The procedure for calculating the expected codon frequency for each gene/transcript was based on the observed global frequency of each mono-, di-, tri-, quadra- and quint-codon sequence in the set of all gene/transcripts in a given species. In addition, we treated all alternative isoforms of a given gene as a separate transcript, which resulted in the exon sequences found in each isoform being counted. We reasoned that multiple isoforms could be present in the cell at the same time, to support our use of codon sequences specific to each isoform as separate variables that compose the expected frequency value. Z-scores are a simple measure of whether a gene/transcript is using a codon sequence more or less than the global frequency. Specifically Z-scores detail how many standard deviations above or below the global frequency the specific codon sequence is used in the gene/transcript, which can be misleading when dealing with expected frequencies that approach zero. For example the Z-score measures for quintuplet codon combinations should be used with caution as the expected frequency of all quintuplet codons approaches zero and a single use in a gene/transcript results in a Z-score > 100. We note that the number of times each quintuplet codon is used in a gene is detailed in the CUT database and these can be more informative measures than Z-scores. The online tool does not support right clicking due its use of dynamically generated gene/transcript pages.

2.2 Gene specific codon and dicodon analysis

Species-containing genes or transcript data stored in the CUT database can be accessed using the search tab found at <http://pare.sunycnse.com/cut/controller?action=search>. The organism, genome, annotation and gene of interest must be specified in the pull down menu, with the organism designation leading to single genome and annotation choices. The gene/tx pull-down can be searched with partial or full gene names or transcript (NM_numbers) identifiers using the search gene button. Results on each search are displayed below the search gene button and their CUT data can be accessed by clicking on the gene/tx hyperlink, which will bring users to a page that displays the gene sequence and provides access to monocodon to quintupletcodon data and graphs for each gene. In addition there is a download spreadsheet tab for each gene that allows for the export of all monocodon and dicodon data that is linked to the gene-specific codon sequence, Z-score, expected frequency and actual frequency. The resulting download is produced in tab-delimited form. Resulting data can be imported into a graphing program (*i.e.*, Excel) and data can be organized to identify the most and least used moncodons and dicodons in the gene sequence. **On each gene/transcript webpage the hyperlinks detailing the monocodon to quintupletcodons used in each gene can be clicked to bring the user to bar graphs detailing the sequence, number of times used and Z-score.**

2.3 Gene codon painting and immunoblot analysis

Codon *painting* can be performed using the codon search input box found on each gene/transcript page tied to the database. Once the specific codon pattern is searched (using the input field to specify sequence, search button to search and then highlight selected tab to paint), resulting output is highlighted to paint sequence occurrences in each target sequence. Protein expression analysis can be performed in the organism of choice. In the past in mouse and yeast models reported here we have analyzed specific proteins in the presence or absence of specific anticodon wobble base modifications [8-11, 13, 30]. For example, in yeast specific protein levels in wild-type (By4741) and *trm9Δ* cells were determined as previously described

using corresponding C-terminal TAP tagged strains [31] and immunoblots. Briefly, each TAP-tag strain was made *trm9Δ* using a PCR amplified cassette derived from YML014W, with selection occurring on G418. Protein extracts from each strain were then analyzed by immunoblots using an anti-TAP antibody, with equal loading probed using an anti- β -tubulin antibody, as previously described [13].

2.4 CUT downloads, gene parsing and dicodon statistical analysis

Using the Genomes tab, users can access a page that allows for the download of all gene-specific monocodon and dicodon data for yeast, mouse and rat genes/transcripts. Data on genome wide monocodon and dicodon frequencies for all species-containing genes/transcripts can be downloaded from the CUT database (<http://pare.sunycnse.com/cut/controller?action=listAnnot>) by clicking on the Download Stats button. The Heatmap Files icon that is shown on each gene/transcript page can be used to download all monocodon and dicodon Z-scores for all genes/transcripts in a specific organism. The resulting download is a compressed folder that when extracted will provide a tab-delimited file detailing Z-scores for all monocodons and dicodons used in each gene. Outside of CUT these lists can be used to compile sets of genes (*i.e.*, visual basic programmed search of gene list in Excel) and, by using random sampling approaches, determine if summed Z-scores for sets of genes are higher than average values. For example, visual basic based scripts can be programmed in Excel to randomly order all yeast genes, pick 80 random genes to obtain and then sum dicodon Z-score values, with this process performed for N = 300 occurrences. The average value and standard deviation for the summed Z-score can then be compared to an actual value to determine a measure of significance (Z-score), similar to our previously described studies [13, 32].

2.5 Multi-codon searches to identify species-containing genes with specific in frame sequences

Multi-codon searches to identify species-containing genes with specific sequence can also be performed in the CUT database using the search tab (<http://pare.sunycnse.com/cut/controller?action=search>). The organism, genome and annotation information must be specified in the pull down menu, with the organism designation

only providing a single genome and annotation choice. The “containing codon sequence” input can be specified with a codon string (*i.e.*, UGANNN, with N being either U, G, A or C), followed by a click on the search gene button. Results of each search are displayed below the search gene button and their CUT data can be accessed by clicking on the gene/tx hyperlink, which will bring users to a page that displays gene sequence and other information, as described above, for each gene.

3. Results and Discussion

The CUT algorithm was developed to methodically count the codon content in gene or transcript sequences beginning with the start codon. To facilitate bulk gene analysis, data was imported (**Fig. 1**) as either gene annotations (SGD, 6664 yeast genes) or as RefSeq annotated transcripts (30,392 mouse and 16,711 rat transcripts). A key difference between the yeast and mammalian data imports is that genes are represented as single entities, while multiple transcripts are present for specific genes, respectively, with the latter including splice variants. Each entry was methodically analyzed to catalog the number of each possible monocodon as well as each possible dicodon (64x64), tricodon (64x64x64), quadruplecodon (64x64x64x64) and quintuplecodon (64x64x64x64x64) combinations (<http://pare.sunycnse.com/cut/>). For each gene/transcript, there are 64 to over 1 billion possible features to catalog and store, but no gene had codon sequences that represent the entire combination space. The CUT database was designed using a table based format (**Fig. 2**) and allows for individual gene queries and the retrieval of all of the codon combination data noted earlier, and describes whether a codon or codon combination is over-represented in a gene sequence using a Z-score metric. Search functions that are associated with CUT include queries based on Z-score cutoffs, codon combinations, and gene identity. Bulk download of codon usage frequencies and Z-scores for all monocodon and dicodon combinations is enabled for individual genes as well as for all genes or transcripts specific to each species.

We have previously reported that the yeast translation elongation factor 3 (*YEF3*) transcript is translationally regulated by Trm9-catalyzed uridine modifications on tRNA and that *YEF3* overuses GAA (91 total count, 3.4 Z-score) and AGA (41 total count, 3.6 Z-score) codons [13, 33]. In *trm9Δ* cells, we have shown

that Yef3 protein levels are dramatically reduced, with little difference observed in transcript levels [10, 13]. *YEF3* was used to query the database (**Fig. 3A**) to demonstrate results that report monocodon usage patterns in bar graph format (**Fig. 3B**). In addition, *YEF3* was searched to detail dicodon usage pattern numbers (**Fig. 3C**), such as GAA-GAA, which is represented 8 times and has a Z-score of 3.2. Z-scores that detail pattern usage can be exported for all represented dicodons in a gene. For example, data specific to *YEF3* have been exported, sorted from low to high, and plotted in **Fig. 3D**. Dicodons overused in *YEF3* are found on the far right side of **Fig. 3D** and have Z-scores >8. It is interesting to note that 22 of the 25 most overused dicodons in *YEF3* contain either GAA or AGA, with both codons decoded by tRNAs modified by Trm9. Notably, the dicodon combinations AGA-AGA, GAA-AGA, AGA-GAA and GAA-GAA were in the top 25 most over-represented list for *YEF3*, having Z-scores of 4.8, 4.1, 3.3 and 3.2, respectively. We propose that dicodon patterns are important because they report on the specific tRNAs that will need to be sequentially accessed for translation. In the case of AGA-AGA, GAA-AGA, AGA-GAA, GAA-GAA, they are all decoded by tRNAs modified by Trm9, such that these dicodons should be difficult to translate if there is a deficiency in Trm9-dependent uridine modifications.

We have also used CUT to explore codon usage patterns in yeast genes (**Fig. 4**) other than *YEF3*. Our goal was to determine if we could highlight significant dicodon and quintupletcodon patterns that correlate with decreased protein levels in *trm9Δ* cells, relative to wild-type cells. Specifically, we have used the codon painting function of CUT to highlight the AGA codon in the gene *ENT2* (**Fig. 4A**), with the finding that this gene contains two AGA-AGA doublets (Z-score = 2.0). The Ent2 protein is required for actin patch assembly and endocytosis. Based on over-use of AGA-AGA dicodons, we predicted that Ent3 protein levels would decrease in *trm9Δ* cells, a prediction that was confirmed by immunoblot analysis (**Fig. 4B**). As a negative control we have included immunoblot data on Met6 protein levels from wild-type and *trm9Δ* cells (**Supplemental Figure S1**). The Met6 gene is over-represented with AGA codons (31 total, Z-score = 2.5) but contains 0 AGA-AGA dicodons. Met6 protein levels are not affected by a Trm9 deficiency, further supporting the idea that complex codon patterns are an important determinant of MoTTs.

The GAA codon has also been shown to have its translation linked to Trm9. We searched the CUT database to identify genes that use five GAA codons in a row and identified *NAB3*. In **Fig. 4C**, we painted

NAB3 to identify GAA codons, with the finding that there are nine instances of five GAA codons in a row (Z-score = 28). These nine instances were found in runs of 7 and 10 GAA codons. Nab3 is an RNA-binding protein that is part of the Nrd1 complex involved in some 3'-end processing. The GAA codon runs found in Nab3 are extremely rare and, based on previously published reporter data, the Nab3 protein should be decreased in *trm9Δ* cells compared to wild-type, which we confirmed by immunoblot analysis (**Fig. 4D**).

Translation of the AAG codon has been linked to Trm9 [9]. We have previously used an AAG-AAG-AAG-AAG reporter system to observe a modest but statistically insignificant decrease in reporter activity in *trm9Δ* cells, relative to wild-type [9]. The reporter system is an artificial construct and we wanted to determine if any endogenous yeast genes had AAG codon combinations greater than four and, if so, was the corresponding protein expression linked to Trm9. We used the CUT database to find the *HMO1* gene, which was significantly over-represented with AAG-AAG-AAG-AAG-AAG. This quintuplet codon occurred four times, with a Z-score of 55 (**Fig. 4E**). In fact, all four AAG-AAG-AAG-AAG-AAG quintuple codons are found in runs of 8 AAG codons. We predicted that, due to the presence of 8 AAG codons in a row, the levels of the Hmo1 protein should be strongly dependent on Trm9, which was again supported by immunoblot data (**Fig. 4F**). The *HMO1* gene is an interesting case because it contains 23 AAG codons in total, which is an average amount. The significance of the AAG codon in *NAB3* becomes apparent when dicodons are analyzed, with the AAG-AAG dicodon occurring 10 times and has a Z-score of 8.0. *NAB3* represents a good example of how complex codon patterns can be more informative for modeling translation than simple monocodon based descriptors.

In some cases, regulation of gene expression requires the coordination of many genes and, based on monocodon patterns, we have previously reported that groups of genes have the potential to be regulated by changes in tRNA wobble base modifications. The CUT database can be used to download all dicodon data for all genes in a species and determine if groups of genes over-use some dicodon combinations. In response to nutrient starvation, the Gcn2-eIF2 α -Gcn4 pathway is activated by translational regulation [34-39], with subsequent Gcn4-based transcriptional regulation of many genes. Computational studies have identified Gcn4-binding sites in 80 transcripts [40]. We identified the 80 Gcn4-regulated genes in the CUT database download detailing dicodon usage, and then summed the Z-scores in each dicodon category, to get a group measure of dicodon over-usage (**Fig. 5**). The five dicodons with the highest summed Z-scores (>65) for the

group of 80 Gcn4-regulated transcripts were GGU-GCC (Gly-Gly), GCA-GGU (Ala-Gly), GCU-GUG (Ala-Val), GGU-GUU (Gly-Val) and AGA-GAA (Arg-Glu). We were intrigued that the Trm9-regulated codons AGA and GAA were found in the list of top-scoring dicodons for the 80 Gcn4 transcripts, so we used a random sampling of 80 transcripts (300 times from all yeast genes) to determine significance (**Fig. 5, inset**). We note that significance of a group score was performed outside of CUT and this functionality is planned for later versions of the tool. The average summed AGA-GAA dicodon Z-score for 80 randomly-sampled genes was 30 with a standard deviation of 7.2, making a highly significant ($P < 10^{-5}$) Z-score of 68 for Gcn4-regulated transcripts. While we can directly link the AGA-GAA dicodon to regulation by Trm9, the other top scoring Gly-, Ala- and Val-based codons are dependent on other tRNAs. It is interesting that the Gcn2-eIF2 α -Gcn4 pathway, which begins with regulation of translation initiation, can now be linked to regulation of translation elongation through the AGA-GAA dicodon.

Next we determined if the CUT tool and database could be used to identify interesting codon trends in mammalian transcripts. Specifically, we have applied the same algorithms and developed the same database query forms used to study yeast genes and analyzed all transcripts available in the RefSeq datasets for mice and rat genes (**Fig. 6A**). We highlight mouse *Brca1* and *Gpx1* genes, which encode key stress response proteins whose activities play important roles in DNA repair and ROS-detoxification, respectively [41, 42]. We downloaded dicodon Z-score data for all *Brca1* (**Fig. 6B**) and *Gpx1* entries (**Fig. 6A**) and then visualized the gene specific data in a scatter plot. Note that the maximum value for the Y-axis is 25 for *Brca1* and 65 for *Gpx1*. As can be observed in both *Brca1* and *Gpx1* plots (**Fig. 6B-C**), most of the data stays on a similar horizontal plane near the Y-axis (Z-score) values of 0 to 10 (*Brca1*) and 0 to 5 (*Gpx1*). There are a number of dicodon outliers for *Brca1* that have Z-scores (~22), including AGU-AAA and UUA-CCG (**Fig. 6B**). A similar analysis of *Gpx1* was performed (**Fig. 6C**), with the most dramatic outlier found at a Z-score of 55 for UGA-GGC, which was twice the score of any dicodon found in *Brca1*. The high Z-score for the UGA-GGC dicodon in *Gpx1* was expected as the corresponding protein contains selenocysteine, which is incorporated by stop codon recoding and utilizes a non standard internal UGA. *Gpx1* and the UGA-GGC dicodon are examples of extreme codon usage, as there are only 24 reported selenoproteins in mice [43], and UGA is not usually followed by another codon in most transcripts, because UGA usually signals for the end of translation.

In addition to using CUT to identify significant codon patterns in individual or groups of genes, the program is also designed to be used as an exploration tool. We decided to use CUT to identify all mouse and rat transcripts that contain an internal UGA codon (**Fig. 7**), as this should identify transcripts that have the potential to undergo stop codon recoding (*i.e.*, selenoproteins) or to participate in some form of translational read-through to generate an extended protein with a different function (e.g., VegF-Ax) [25] or targeting to a specific organelle (Mdh1) [27]. We searched both the mouse and rat data loaded in CUT using the sequence UGA-NNN, where NNN represents any of the 64 codons. Our search identified 45 mouse (**Table I**) and 120 rat (**Table II**) transcripts with internal UGA codons, which was more than the 25 - 30 expected hits for each organism. We had expected 23-24 selenoproteins and a small number of other reported transcripts that undergo translational read-through in mice and rats. The number of internal UGA transcripts noted for each of our identified targets ranged from 1 to 20 for mouse transcripts and 1 to 36 for rat transcripts. While the transcripts that contain more than 1 UGA codon could be RefSeq annotation errors, we note that transcripts for selenoproteins can include 1 to 10 internal UGA codons [12, 43], with Sepp1 corresponding to the latter in both our mouse and rat datasets (**Tables I and II**).

Specific to the mouse UGA-NNN search using the CUT tool, we identified all 24 known mouse selenoproteins as well as an additional 21 transcripts with internal UGA codons. **We speculate that the 21 mouse transcripts have their UGA codon translated, with this speculation supported for 3 below detailed proteins.** Specific to the rat UGA-NNN search using the CUT tool, we identified 23 of 24 known rodent selenoproteins, with the exception of RGD1560938/Seli/Ept1, which does not have an internal UGA codon. Remarkably, we identified an additional 97 transcripts with internal UGA codons. All identified mouse and rat transcripts were compared to the NCBI database using a nucleotide blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure correct gene identification, with criteria of >99% identical gene BLAST being employed. The biochemical functions of selenoproteins include reduction of ROS, selenium transport and selenocysteine synthesis. Additionally, some selenoproteins play major roles in cancer prevention, thyroid metabolism, male fertility, and immune, muscle, and central nervous system functions [4]. The roles of some selenoproteins such as selenoprotein O, T and H (identified by CUT as 2700094K13Rik), are still unknown.

Mouse- and rat-specific transcripts that possess internal UGA codons but do not contain selenocysteine were also most likely identified in our UGA-NNN search. For example, VegFA was identified in our mouse and rat searches. The human version of VegF, known as *VegF-Ax*, was reported by Fox and co-workers to use translational read-through of the UGA codon to form a longer protein with anti-angiogenic activity [25]. In addition, studies have provided evidence for UGA read-through targets identified in our search, and they included *Ago1* and *Mtch2* [25], as well as *LdhB* and *Mdh1* (mouse and rat) [27]. In some cases, specific *cis*-acting sequence elements are believed to facilitate UGA read-through, with the resulting C-terminally extended proteins taking on physiologically distinct roles compared to those produced using the upstream (or canonical) UGA. For VegF-Ax (a VegF-A isoform with 22 additional C-terminal amino acids), the ribonuclear protein A2/B1 was identified as a *trans*-acting factor critical for VegF-Ax production. In parallel, for selenocysteine-decoding transcripts, recognition of the SECIS element by the RNA binding protein Sbp2 is critical for UGA translation as selenocysteine [44]. 3'-UTR and other bioinformatics parameters that can be used to further parse the identified UGA-containing transcripts are discussed below.

LdhB and Mdh1 can both be considered stress-response enzymes critical for intracellular redox homeostasis and they have CU bases immediately following the UGA stop codon. It has been shown that changing the CU dinucleotide results in decreased read-through efficiency, as does changing the stop codon [27]. Lactate dehydrogenase B (LdhB) is one subunit of the lactate dehydrogenase enzyme, which converts lactate to pyruvate and vice versa via the interconversion of NADH and NAD⁺. The C-terminally extended LdhB, termed LdhBx, has 6 extra amino acids and contains a peroxisomal targeting signal type 1 (PTS1) sequence in the 3' extension. LdhBx has also been shown to co-import LdhA, another lactate dehydrogenase subunit, into peroxisomes [45]. Similarly, two protein isoforms of malate dehydrogenase 1 (Mdh1) exist, one of which is created via translational read-through to create a 20 amino acid C-terminally extended protein. Mdh1 catalyzes the reversible oxidation of malate to oxaloacetate using NADH/NAD⁺, with the extended protein also containing a PTS1 [2]. It is therefore likely that peroxisomal localization of both LdhB and Mdh1 is dependent on stop codon read-through.

Genome-wide analysis and published reports have identified two additional targets of translational read-through, Ago1 and Mtch2, which contain 37 and 10 amino acids, respectively. Argonaute 1 (Ago1) is

involved in RNA interference and RNA silencing by binding to micro-RNAs or siRNAs and repressing translation of complementary mRNA [46]. Mitochondrial carrier 2 (Mtch2) is a key player in the mitochondrial death pathway via the recruitment of proapoptotic truncated BID (tBID) [47]. It is still unclear how translational read-through alters the function of these two proteins as they have only recently been identified as targets for PTR. Ago1 and Mtch2 do not share the same CU dicodon following UGA that is necessary for translational read-through of LdhB and Mdh1. Instead, both Ago1 and Mtch2 are followed by an “AG” dicodon. Interestingly, the read-through efficiency of these two transcripts is highest among the five known PTR targets, at 13% for Mtch2 and 24% for Ago1, compared to 11% for VegF-Ax [25] and 1-2% for LdhB and Mdh1 [45].

We identified 97 rat transcripts with internal UGA codons that are not currently classified as selenoproteins. **Similar to the mouse results we speculate that these UGA codons are translated.** Considering the links between toxicant-induced tRNA modifications and the translational decoding of stress response transcripts,[48] several of these gene transcripts stand out. For example, *Bag4* belongs to a family of *Bag*-related chaperone proteins that have been shown to regulate cell death and growth decisions by interacting with both anti-apoptotic factors, as well as growth stimulatory kinases such as PI3K [49-52]. Other internal UGA-containing transcripts can be linked to cellular stress responses through their involvement in DNA damage-induced cell cycle checkpoint controls: the *Cdc25b* phosphatase targets cyclin dependent kinase 2 for dephosphorylation allowing entry into mitosis, and is itself targeted by *Chk1* in response to DNA damage [53, 54]; the *Taok1* kinase mediates chromosome-microtubule interactions during M phase of the cell cycle, guarding against chromosomal instability [55]. Last, *Fam120a* (also *Ossa/C9orf10*) is an RNA binding protein implicated in tumor cell resistance to oxidative stress by mechanisms thought to involve the activation of anti-apoptotic signals [56]. It will be interesting to determine the extent of translational recoding during stress responses, as it could provide new activities to optimize the response.

A total of 32 rat olfactory receptors were identified as having at least one internal UGA codon, and several had greater than five. Rat olfactory proteins comprise seven-transmembrane receptors and sense smell via direct interaction with odorant molecules and they belong to the largest multi-gene family in rats [57]. Perhaps differential UGA recoding represents a further means of diversification for olfactory receptors in rats, contributing to their advanced ability to differentiate between a myriad of odorants. It is further remarkable to

find such a high number of internal UGA-containing transcripts in rat compared to mouse. This finding raises the possibility that rat uses UGA recoding to diversify protein expression from an individual transcript to an extent greater than that observed in other rodents. It remains to be seen whether the internal UGA of these transcripts is decoded as selenocysteine, or serine, as is the case for *VegF-Ax* [25, 58]. The first step has been taken in our methodical identification of transcripts with internal UGA codons as candidates for recoding; the next step will be to employ proteomic analysis of candidate transcripts in order to determine which amino acid is used. Additionally it will be interesting to investigate whether the UGA containing mouse and rat transcript genes are encoded by transcripts that display a further codon bias and whether their regulation is influenced by tRNA modifications, according to the MoTT hypothesis [48, 59].

4. Conclusions

We have demonstrated the utility of the CUT tool and database by applying and analyzing gene specific dicodon patterns in yeast, mice and rat genes and transcripts. Development of the CUT database has permitted the first genome-wide exhaustive analysis of internal UGA stop codons. Our results reveal a diversity of potential new transcripts that undergo translational recoding, which can provide a means to expand the genetic code and increase the functional diversity of gene products without requiring the addition of new genetic material. While our UGA-NNN search has identified many transcripts with the potential to be translationally decoded, bioinformatics analyses of functional classification, 3'-UTR sequence space, and conservation between species will need to be performed to add further proof. **One approach that could be employed to validate that the UGA-NNN transcripts are translated could be mass spectrometry based proteomics to identify the corresponding peptide.** In addition, experimental evidence that provides direct support for translational decoding in the form of new peptides that contain selenocysteine or that correspond to amino acid sequences downstream of standard stop codons will be needed to confirm that the UGA codon was translationally decoded. Nevertheless the CUT tool thus provides a powerful new approach to gain insight into the complexity of mono- and multi-codon usage across yeast and mammalian genomes which can be used to elucidate the biological function of codon usage patterns in the translational control of gene expression. **The**

version 1 CUT tool and database provides simple and complex codon usage data for all yeast, mouse and rat genes/transcripts in their respective genomes. The future evolution of CUT will include the addition of specific modules that include human, bacterial and other model organism data sets. Complex codon usage patterns have the potential to be regulatory in many species, and the CUT tool and database approach has the potential to be applied to all sequenced and gene-defined genomes. Future capabilities that include advanced search functions based on gene function and codon count criteria, statistical analysis to identify significant codon patterns in groups of genes and genome-genome comparisons are also planned.

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Table I. CUT-identified Mouse Transcripts with Internal UGA Codons

Mouse Gene Name	# of <i>internal</i> UGA codons	Transcript ID		Mouse Gene Name	# of <i>internal</i> UGA codons	Transcript ID
4933416I08Rik	9	NM_027700		Msrb1	1	NM_013759
2700094K13Rik	1	NM_001033166		Mtch2	1	NM_001317241
Ago1	1	NM_001317173		Oas1b	1	NM_001083925
Art2a-ps	1	NM_007490		Olfr421-ps1	1	NM_146720
BC089491	1	NM_175033		Olfr915	1	NM_146785
Clec7a	1	NM_020008		Pira6	6	NM_011093
Dio1	1	NM_007860		Ptpv	1	NM_007955
Dio2	2	NM_010050		Selk	1	NM_019979
Dio3	1	NM_172119		Selm	1	NM_053267
Ept1	1	NM_027652		Selo	1	NM_027905
Gm10058	1	NM_001109969		Selt	1	NM_001040396
Gm10096	1	NM_001102678		Sep15	1	NM_053102
Gm10147	1	NM_001099919		Sephs2	1	NM_009266
Gm10230	1	NM_001099347		Sepn1	1	NM_029100
Gm10486	1	NM_001109970		Sepp1	10	NM_009155
Gm14819	1	NM_001110250		Sepw1	1	NM_009156
Gpx1	1	NM_008160		Smcp	3	NM_008574
Gpx2	1	NM_030677		Txnrd1	1	NM_015762
Gpx3	1	NM_008161		Txnrd2	1	NM_013711
Gpx4	1	NM_008162		Txnrd3	1	NM_153162
Ldhb	1	NM_001316322		Vegfa	1	NM_001317041
Mdh1	1	NM_001316675		Vimp	1	NM_024439
Mia3	20	NM_177389				

Table II. CUT-identified Rat Transcripts with Internal UGA Codons

Rat Gene Name	# of internal UGA codons	Transcript ID	Rat Gene Name	# of internal UGA codons	Transcript ID
Abcb11	6	NM_031760	Hdx	26	NM_001134568
Aass	12	NM_001100963	Impg1	5	NM_023958
Abca1	8	NM_178095	Itpr3	12	NM_013138
Abi2	12	NM_173143	Kb23	1	NM_001008813
Acat2	1	NM_001006995	Kbtbd7	1	NM_001302944
Acp1	4	NM_001313735	Kif1c	14	NM_145877
Aldh1a7	6	NM_017272	Kng2	4	NM_001102418
Atn1	10	NM_017228	Krt75	1	NM_001008828
Bag4	9	NM_001025130	Krt77	1	NM_001008807
Batf3	1	NM_021865	Ldhb	1	NM_001316333
Cacna1a	9	NM_012918	LOC100911576	4	NM_001271241
Ccd25b	10	NM_133572	LOC498592	6	NM_001166307
Ces1e	9	NM_031565	LOC500684	12	NM_001047959
Ces2a	3	NM_144743	Lrp1b	2	NM_001107843
Cntnap5a	5	NM_001047865	Ly6c	3	NM_020103
Cstf3	7	NM_001077672	Mdh1	1	NM_001316877
Cyp3a9	1	NM_147206	Mfsd14a	17	NM_001106467
Dio1	1	NM_021653	Msrbl	1	NM_001044285
Dio2	2	NM_031720	Mybpc1	14	NM_001100758
Dio3	1	NM_017210	Ndr3	3	NM_001013923
Doc2b	8	NM_031142	Nkr-plc	1	NM_001040189
Dync2h1	2	NM_023024	Olr1000	1	NM_001000077
Eml1	8	NM_001025741	Olr1052	4	NM_001001363
Fam120a	2	NM_001191816	Olr1064	6	NM_001001076
Fdsp	1	NM_031840	Olr1065	5	NM_001000498
Gnb5	1	NM_031770	Olr1065	5	NM_001000498
Gpx1	1	NM_030826	Olr1226	8	NM_001000442
Gpx2	1	NM_183403	Olr1227	6	NM_001000443
Gpx3	1	NM_022525	Olr1228	5	NM_001000964
Gpx4	1	NM_001039849	Olr1229	10	NM_001000444
Grcc10	3	NM_001198725	Olr1237	8	NM_001000811

Table II (continued). CUT-identified Rat Transcripts with Internal UGA Codons (continued)

Rat\$Gene\$Name	#\$of\$Internal\$ UGA\$codons	Transcript\$D	Rat\$Gene\$Name	#\$of\$Internal\$ UGA\$codons	Transcript\$D
Olr1238	7	NM_001001013	RGD1307621	1	NM_001108025
Olr1239	8	NM_001000811	RGD1563348	1	NM_001114939
Olr1240	6	NM_001000448	Sell	1	NM_001134754
Olr1241	6	NM_001000449	Selk	1	NM_207589
Olr1247	7	NM_001000807	SelM	1	NM_001115013
Olr1254	7	NM_001001085	SelO	1	NM_001085485
Olr1256	6	NM_001001086	Selt	1	NM_001014253
Olr1257	10	NM_001000596	Selv	1	NM_001166396
Olr1273	6	NM_001000458	Sephs2	1	NM_001079889
Olr1273	4	NM_001000458	Sepp1	10	NM_0191921
Olr1274	2	NM_001000801	Sep15	1	NM_001166396
Olr1275	4	NM_001000800	Sepw1	1	NM_013027
Olr1475	2	NM_001000027	Slc21a4	7	NM_030837
Olr19	9	NM_001000117	Snap25	1	NM_001270576
Olr20	7	NM_001000118	Snx9	1	NM_001127637
Olr440	6	NM_001000282	Stau2	2	NM_001007149
Olr440	6	NM_001000282	Stxbp5l	26	NM_001271250
Olr5	2	NM_001000112	Taok1	9	NM_173327
Olr520	1	NM_001000930	Trpm7	36	NM_053705
Olr703	1	NM_001000359	Txnrd1	1	NM_031641
Olr917	5	NM_001001354	Txnrd2	1	NM_022584
Olr943	1	NM_001001368	Txnrd3	1	NM_001184712
Plaur	5	NM_017350	Usp3	3	NM_001025424
Plb1	17	NM_138898	Vegfa	1	NM_001317043
Psmc10	4	NM_053925	Vimp	1	NM_173120
Ptpn18	4	NM_001013111	Vom2r73	1	NM_001099486
Rabif	12	NM_001007678	Zfp14	21	NM_001100991
Rasgrf1	1	NM_001105753			
Rbm25	4	NM_001108984			
Reln	1	NM_080394			
RGD1305537	1	NM_001108822			

Figure Legends

Figure 1. Algorithm outputs and database design for CUT. Species specific inputs for yeast, rat and mouse genes are listed in the upper panel, with these gene (yeast) and transcript (rat and mouse) entries analyzed (middle panel) to catalog and provide searchable fields. Data provided in the CUT database includes species- and gene specific information on codon usage patterns and measures of the significance of said codon patterns in a specific gene (Z-score), relative to species specific genome measures.

Figure 2. CUT database overview. The design of the database utilized tables that track the codon sequence usage for each transcript and for each annotation set (genome).

Figure 3. Design features and data outputs using CUT. (A) Species specific data can be accessed and searched to provide data on specific genes, or identify genes with specific codon patterns (1 to 5 codons) and Z-scores, with a data search of the Budding Yeast gene *YEF3* demonstrated. (B) Single codon patterns for the *YEF3* highlight the number of highly used codons and their gene specific over usage (Z-score). For example in *YEF3* the GAA codon is used 91 times and based on actual and expected frequencies a significant Z-score is observed. (C) As exemplified using the *YEF3* gene and the GAA-GAA dicodon that is found 8 times, CUT can be used to determine the number of times a dicodon is represented in a gene, with (D) all dicodons from a gene being able to be exported into a tab delimited file and analyzed to determine the most significant dicodon combinations. The top five most overused dicodons in *YEF3* are represented on the graph, with the inset table detailing the next 25.

Figure 4. Codon painting of specific genes. CUT can be used to identify codon patterns on a specific gene to identify the general position of specific codon runs in (A) *ENT2*, (C) *NAB3* and (E) *HMO1*. AGA, GAA and AAG codons have all been linked to Trm9 regulated translation. Immunoblot analysis of the levels of specific

proteins with **(B)** AGA-AGA for Ent2, **(D)** GAA-GAA-GAA-GAA-GAA for Nab3 and **(F)** AAG-AAG-AAG-AAG-AAG for wild-type and *trm9Δ* cells.

Figure 5. Dicodon trends in similarly regulated group of genes. All 64x64 dicodon patterns have been cataloged for each organism's genes/transcripts in CUT. Dicodon data on all species-containing genes can be downloaded and specific groups of genes can be analyzed for dicodon trends. Specifically, 80 transcripts that have been linked to regulation by Gcn4 were analyzed by summing each dicodon Z-score, with these values plotted for all 4,096 possible combinations. The AGA-GAA summed dicodon Z-score was determined to be significantly increased ($p < 10^{-5}$) for the Gcn4-regulated genes (inset panel). Significance was determined by random sampling of 80 transcripts 300-times from the 6,664 yeast genes to determine the average summed Z-score and standard deviation for the AGA-GAA dicodon in a group. The summed Z-score for the 80 Gcn4-regulated transcripts (68) was compared to the average score range of 80 random transcripts (28 to 33).

Figure 6. CUT tool organism downloads and dicodon data specific to mammalian genes. **(A)** Species specific information and gene specific monocodon and dicodon data for yeast, mouse and rat annotated genes and transcripts can be downloaded by clicking on the Download Stats Icon. Unique dicodon Z-scores indicating whether they are significantly over-used in mouse **(B)** *Brca2* and **(C)** *Gpx1*. Note that the Y-scale is different when comparing *Brca2* and *Gpx1* data, as the latter translationally recodes UGA.

Figure 7. Translational recoding in mammals and CUT-based search for targets. In black, two potential mechanisms that can translate a UGA codon are detailed. In red, we describe the search space used to identify the 45 and 120 transcripts that contain internal UGA codons in mice and rats, respectively.

Figure1

Figure 2 (new)

Figure3

A Codon Utilization Tool

Home Search View Genomes Analytics Tutorial Contact Info

Search genes:

Annotation Filters:

Organism: Yeast Genome: sacCer3 Annotation: SGD_sacCer3 Gene/Tx Name: yef3

CDS Filters:

Having any codon sequence with Z-Score greater than +/-: 0.0
Having any codon sequence of length 1 with Z-Score greater than +/-: 0.0
Having any codon sequence of length 2 with Z-Score greater than +/-: 0.0
Having any codon sequence of length 3 with Z-Score greater than +/-: 0.0
Having any codon sequence of length 4 with Z-Score greater than +/-: 0.0
Having any codon sequence of length 5 with Z-Score greater than +/-: 0.0
Containing codon sequence: with Z-Score greater than +/-: 0.0

Search Genes

B

Codon Utilization Tool

Using guest access Login

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Gene CDS Viewer

Annotation Set Info:

Species (common): Yeast
Species (scientific): Saccharomyces cerevisiae
Annotation Name: SGD_sacCer3
Description: SGD_sacCer3
Total Transcript Count: 6,664
Total Codons In Annotation Set: 3,008,007

Transcript Info:

Gene: YEF3
Codon count: 1,045
Transcript: YLR249W
Location: chrXII(+): 636780-639914

Codon sequences present by length:

1: 44 unique singlets
2: 539 unique doublets
3: 988 unique triplets
4: 1035 unique quadruplets
5: 1040 unique quintuplets

Highlight Selected

Download Spreadsheet

D

E

Figure5

Figure6

A

Genome Annotations in CUT

Species	Annotation Name	Transcript Count	Total Codon Count	Genomic Codon Usage Statistics
Yeast(Saccharomyces cerevisiae)	SGD_sacCer3	6,664	3,008,007	Download Stats
Mouse(Mus Musculus)	RefSeq_mm10	30,392	16,534,768	Download Stats
Rat(Rattus Norvegicus)	RefSeq_rn5	16,711	8,261,454	Download Stats

Figure 7 (new)