

Codon clusters with biased synonymous codon usage represent hidden functional domains in protein-coding DNA sequences

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Outline

[1. Abstract](#)

[2. Introduction](#)

[3. Results](#)

[3.1. Identifying putatively functional codon clusters \(PFCCs\)](#)

[3.2. Codon usage patterns of PFCCs are diverse](#)

[3.3. PFCC distribution is not restricted to specific regions of protein-coding sequences](#)

[3.4. Specific protein functional classes are overrepresented in genes carrying PFCCs while most PFCCs are not associated with known protein domains](#)

[3.5. Voltage-gated sodium channels include a conserved rare-codon cluster associated with the inactivation gate](#)

[4. Discussion](#)

[5. Materials and Methods](#)

[5.1. Reference genome and data pre-processing](#)

25	<u>5.2. Identifying PFCCs</u>
26	<u>5.3. Calculating TCAI</u>
27	<u>5.4. K-mean clustering of PFCCs</u>
28	<u>5.5. Calculating RLI</u>
29	<u>5.6. Searching for transmembrane helices</u>
30	<u>5.7. Searching for Pfam protein domains</u>
31	<u>5.8. Classifying association between PFCCs and protein domains</u>
32	<u>5.9. Alignment of Nav homologs</u>
33	<u>6. Acknowledgements</u>
34	<u>7. References</u>
35	<u>8. Tables</u>
36	<u>9. Figures</u>
37	<u>10. Supplementary Material List</u>

38 1. Abstract

39 Protein-coding DNA sequences are thought to primarily affect phenotypes via the peptides they encode.
40 Yet, emerging data suggest that, although they do not affect protein sequences, synonymous mutations can
41 cause phenotypic changes. Previously, we have shown that signatures of selection on gene-specific codons
42 usage bias are common in genomes of diverse eukaryotic species. Thus, synonymous codon usage, just as
43 amino acid usage pattern, is likely a regular target of natural selection. Consequently, here we propose the
44 hypothesis that at least for some protein-coding genes, codon clusters with biased synonymous codon usage
45 patterns might represent “hidden” nucleic-acid-level functional domains that affect the action of the
46 corresponding proteins via diverse hypothetical mechanisms. To test our hypothesis, we used computational
47 approaches to identify over 3,000 putatively functional codon clusters (PFCCs) with biased usage patterns
48 in about 1,500 protein-coding genes in the *Drosophila melanogaster* genome. Specifically, our data suggest
49 that these PFCCs are likely associated with specific categories of gene function, including enrichment in
50 genes that encode membrane-bound and secreted proteins. Yet, the majority of the PFCCs that we have
51 identified are not associated with previously annotated functional protein domains. Although the specific
52 functional significance of the majority of the PFCCs we have identified remains unknown, we show that in
53 the highly conserved family of voltage-gated sodium channels, the existence of rare-codon cluster(s) in the
54 nucleic-acid region that encodes the cytoplasmic loop that constitutes inactivation gate is conserved across
55 paralogs as well as orthologs across distant animal species. Together, our findings suggest that codon
56 clusters with biased usage patterns likely represent “hidden” nucleic-acid-level functional domains that
57 cannot be simply predicted from the amino acid sequences they encode. Therefore, it is likely that on the
58 evolutionary timescale, protein-coding DNA sequences are shaped by both amino-acid-dependent and
59 codon-usage-dependent selective forces.

60

61 2. Introduction

62 In general, it is assumed that the primary function of a protein-coding sequence is to encode a specific
63 sequence of amino acids whose biochemical properties determine the structure and functions of the encoded
64 peptide. However, emerging data indicate that synonymous mutations, which do not affect amino acid
65 sequences, can still have dramatic phenotypic impacts. Thus, it has been hypothesized that some important
66 factors affecting protein structures and functions are not simply encoded by amino acid residues but by
67 nucleic-acid-level information, such as codon usage bias [1,2]. Therefore, just as a sequence of amino acids
68 with a specific order and/or specific biochemical properties can form a protein domain that performs

69 specific functions, it is also possible that a sequence of codons with a specific codon usage pattern could
70 serve as a nucleic-acid-level domain that affects the functions of the mature protein.

71 Based on the hypothesis that codon-usage-encoded domains can affect protein functions, researchers have
72 identified rare-codon clusters, defined by whole-genome codon usage frequencies, that possibly decelerate
73 translation and thus modify protein functions by affecting co-translational folding and/or modifications of
74 nascent peptide chains [2–7]. Nevertheless, if functional codon clusters do exist, local deceleration of
75 translation may not be the only mechanism through which they affect protein functions. It is also possible
76 that functional codon clusters could correspond to locally accelerated translation, a specific combination of
77 translationally decelerated and accelerated regions, specific RNA secondary structures [8,9], and binding
78 sites for miRNAs [10]. Thus, for generally investigating codon clusters as functional domains that may be
79 “hidden” from the amino acid sequences, exclusive focus on rare-codon clusters may lead to biased results.
80 Therefore, it is necessary to develop statistical methods that generally detect putatively functional codon
81 clusters (PFCCs), no matter what specific codons they prefer or through what mechanisms they may affect
82 protein functions.

83 Consequently, to identify PFCCs, we developed a conservative statistical approach and applied it to the
84 *Drosophila melanogaster* genome with approximately 14,000 protein-coding genes, which yielded over
85 3,000 PFCCs in about 1,500 genes. Interestingly, some of these PFCCs strongly prefer common codons
86 while some others adopt complex codon usage patterns that cannot be simply described as preference for
87 common or rare codons, which has not been reported before. Furthermore, we found that genes encoding
88 transmembrane proteins are more likely to bear PFCCs. However, only a small proportion of the identified
89 PFCCs are associated with the coding sequences of transmembrane helices, which suggests that PFCCs are
90 either associated with other types of protein domains that are overrepresented in transmembrane proteins or
91 not necessarily associated with amino-acid-encoded domains. We further found that the majority of the
92 identified PFCCs are not associated with established protein domains in the Pfam database [11]. These data
93 suggest that most PFCCs likely encode “hidden” nucleic-acid-level functional domains that cannot be
94 predicted solely from amino acid sequences. The rationale for this inference is as follows: first, Pfam is a
95 well-established database of conserved protein domains that have undergone strong natural selection;
96 second, the PFCCs can be identified only when natural selection on local codon usage patterns is strong
97 enough to generate statistically detectable signals; third, if the major impacts of PFCCs on gene functions
98 are mediated by amino-acid-encoded protein domains, most PFCCs are expected to be associated with
99 amino-acid-encoded domains that have undergone strong natural selection; fourth, the actual observation
100 contradicts the expectation, and thus the functions of PFCCs should not be strongly associated with amino-

101 acid-encoded domains. Finally, by implementing comparative analysis between homologs, we showed that
102 the family of voltage-gated sodium channels likely evolved conserved preference for rare codons in a region
103 responsible for the channel inactivation. Together, our data suggest that similar to amino acid sequences,
104 codon clusters can also encode diverse functional domains, which provides an additional level of regulation
105 over the structures, modifications, and functions of proteins.

106

107 [3. Results](#)

108 [3.1. Identifying putatively functional codon clusters \(PFCCs\)](#)

109 If the synonymous codon usage of a codon cluster does not perform specific functions, it should not be
110 affected by natural selection and thus it can be explained by the background codon usage frequencies, which
111 is mainly determined by mutations and genetic drift [12,13]. For example, if a gene locates in a GC-enriched
112 chromosomal region that has resulted from GC-biased mutations, it is expected that the background codon
113 usage are biased towards GC-ended codons; thus, if a sub-genic region is not significantly affected by
114 natural selection on codon usage, its synonymous codon usage should also be biased towards GC-ended
115 codons. Therefore, if the codon usage pattern of a codon cluster cannot be explained by the background
116 codon usage frequencies, it should be significantly affected by natural selection; thus, such a codon cluster
117 is by definition a PFCC. To identify PFCCs, first we needed to choose background codon usage frequencies.
118 Previous studies on synonymous codon usage usually used the whole-genome codon usage frequencies as
119 the background [3,5–7]. Nevertheless, our recent study [14] showed that gene-specific codon usage pattern
120 can be fairly different from whole-genome one. Thus, a codon cluster whose synonymous codon usage
121 cannot be explained by whole-genome codon usage may be adequately explained by gene-specific codon
122 usage, and *vice versa*. Therefore, to filter out the interference from the discrepancies between whole-
123 genome and gene-specific codon usage patterns so that PFCCs are conservatively identified, neither whole-
124 genome nor gene-specific codon usage frequencies should be able to explain the codon usage pattern of a
125 PFCC. Based on the aforementioned logic, we developed a statistical approach to scan protein-coding
126 sequences in order to identify PFCCs (see Materials and Methods: Identifying PFCCs).

127 By applying the approach to 13,821 protein-coding genes from the reference *D. melanogaster* genome, we
128 identified 3,050 PFCCs in 1,445 genes (Table S1). This result indicates that PFCCs do exist, and they
129 impact at least 10% of protein-coding genes in the *D. melanogaster* genome.

130 [3.2. Codon usage patterns of PFCCs are diverse](#)

131 In principle, the codon usage patterns of PFCCs can deviate from the background codon usage frequencies
132 for various non-mutually exclusive biological reasons. First, the enrichment of rare codons in a PFCC might
133 decelerate translation [7]. Second, it is possible that the enrichment of common codons in a PFCC could
134 accelerate translation. Third, PFCCs with more complex codon usage patterns, which cannot be simply
135 described as the preference for common or rare codons, might serve important functions by modifying
136 mRNA secondary structure [8,9] or miRNA accessibility [10]. Thus, classifying the identified PFCCs by
137 their codon usage patterns could be informative for assessing how PFCCs may affect protein functions.

138 Codon adaptation index (CAI) [15] has been widely used to describe a protein-coding sequence's propensity
139 of using common codons. In general, a higher CAI indicates stronger preference for common codons and/or
140 avoidance of rare codons. However, directly using CAI as the index to classify PFCCs could lead to biased
141 results, especially when common codons are not enriched in the PFCCs. This is because the differences
142 between usage frequencies of the synonymous codons for some amino acids are much larger than those of
143 other amino acids. Thus, even if two codon clusters both strictly use rare codons, they could have very
144 different CAIs depending on the amino acid sequences. To circumvent such a weakness of CAI, we propose
145 to use a transformed CAI (TCAI) to describe the general codon usage pattern of a PFCC.

146 TCAI is calculated as below. For a PFCC, the corresponding amino acid sequence and the background
147 codon usage pattern – either the whole-genome or gene-specific codon usage pattern – are used to randomly
148 generate 10,000 “pseudo-clusters” of codons that encode exactly the same amino acid sequences as what is
149 encoded by the PFCC. Thus, on average, the overall codon usage patterns of these pseudo-clusters should
150 follow the background codon usage pattern. Then the CAIs of all pseudo-clusters are calculated, and TCAI
151 is defined as the result of subtracting the proportion of pseudo-clusters whose CAIs are higher than the CAI
152 of the PFCC from the proportion of pseudo-clusters whose CAIs are lower than the CAI of the PFCC. Thus,
153 TCAI varies between -1 and 1. $TCAI = -1$ means that the CAIs of all pseudo-clusters are higher than that of
154 the PFCC, suggesting that the PFCC strongly prefers rare codons; in contrast, $TCAI = 1$ suggests that the
155 PFCC strongly prefers common codons. Thus, TCAI effectively suppresses the interference from different
156 levels of codon usage biases for different amino acids.

157 We calculated TCAIs for all identified PFCCs, either by using whole-genome (Fig. 1A) or gene-specific
158 (Fig. 1B) codon usage pattern as the background. The distribution of TCAI values (Fig. 1) indicates that
159 most of the PFCCs are rare-codon clusters, while common-codon clusters do exist as shown by a small peak
160 in the rightmost part of the histograms. More interestingly, there are also some codon clusters whose TCAI
161 values are intermediate, suggesting that their codon usage patterns are more complex and cannot be simply
162 described by strong preference for common or rare codons. The preponderance of rare-codon clusters may

163 be explained by two reasons that are not mutually exclusive. First, the preponderance may represent the fact
164 that rare-codon clusters are biologically more important than other types of functional codon clusters.
165 Second, the preponderance may also be partly an artifact of technically easier detection of enriched rare
166 codons in a short nucleotide sequence. Nonetheless, it was undoubtedly confirmed that there are different
167 types of codon clusters in terms of synonymous codon usage patterns.

168 We also noted that although the distribution patterns shown in Fig. 1A and Fig. 1B are qualitatively similar,
169 the actual values of corresponding columns in the histograms are quantitatively different. This suggested the
170 possibility that a PFCC could be assigned to different types of codon clusters, depending on which
171 background codon usage pattern was used. Such a possibility may interfere the interpretations of the
172 putative functions of the PFCC. For example, a rare-codon cluster in terms of whole-genome codon usage
173 may be classified as a common-codon cluster in terms of gene-specific codon usage, and thus it could be
174 unclear whether the PFCC may decelerate or accelerate translation. In order to assess the influence of the
175 discrepancy between whole-genome and gene-specific codon usage patterns on the classification of PFCCs,
176 we used a scatter plot to examine the relationship between whole-genome TCAI and gene-specific TCAI
177 (Fig. 2). The data points were then clustered by K-mean clustering to seven types (K=7).

178 We found that most codon clusters have similar whole-genome and gene-specific TCAI (Fig. 2, types I-V).
179 However, some common-codon clusters in terms of whole-genome TCAI were classified as rare-codon
180 clusters in terms of gene-specific TCAI (Fig. 2, type VI), and *vice versa* (Fig. 2, type VII). This result
181 suggests that due to the discrepancy between whole-genome and gene-specific codon usage patterns, it is
182 difficult to predict the exact biological roles of some identified PFCCs. For example, in our previous study,
183 we showed that some whole-genome rare codons can be common and translationally optimal for tissue-
184 specific genes [14]. Thus, a rare-codon cluster in terms of whole-genome codon usage, which would be
185 naïvely considered as a “decelerating codon cluster”, might be a common-codon cluster in terms of gene-
186 specific codon usage, which could actually serve as an “accelerating codon cluster”. Therefore, although
187 PFCCs can be detected by statistical approaches proposed by us and others [6,7], to computationally predict
188 the candidate functional roles of these codon clusters may require extra information such as tRNA
189 expression profile and better tools for predicting the secondary and tertiary structures of RNAs.

190 To summarize, PFCCs are diverse according to their codon usage patterns. Rare-codon clusters, whose main
191 function is presumably decelerating translation [6,7], seem to be the majority of PFCCs. There are also other
192 types of PFCCs, including common-codon clusters and PFCCs with more complex codon usage patterns,
193 which likely have functions other than decelerating translation. Nonetheless, the discrepancy between

194 whole-genome and gene-specific codon usage patterns makes it hard to predict the possible functions of the
195 PFCCs whose whole-genome TCAI and gene-specific TCAI are dramatically different.

196 **3.3. PFCC distribution is not restricted to specific regions of protein-coding sequences**

197 Except for the codon usage patterns of PFCCs, the locations of PFCCs in protein-coding sequences may
198 also provide hints to the possible functions of PFCCs. Previous studies have shown that a potential
199 important function of codon clusters is that N-terminal rare-codon clusters could affect secretion of proteins
200 [16–18], possibly via interaction with the nascent chains of signal peptides [17,18]. Therefore, we next
201 tested if the PFCCs detected by our approach tend to locate near the N-terminus; if they do, it could suggest
202 that PFCCs are likely associated with secretion of proteins.

203 To measure how close a PFCC-encoded region is to the N-terminus, we defined the relative location index
204 (RLI) of a PFCC as the ratio of the distance between the midpoint of the PFCC-encoded region and the N-
205 terminus to the length of the entire protein. Thus, a small RLI means that the PFCC-encoded region is close
206 to the N-terminus. We then plotted the distribution of PFCCs against their RLIs (Fig. 3A). We found that
207 although the density of PFCCs is apparently higher in the N-terminal region, the distribution of PFCCs is
208 not restricted to this region (Fig. 3A). As we have assigned these codon clusters to seven types (Fig. 2), we
209 also examined if some specific types of PFCCs exhibit skewed distribution towards the N-terminal region
210 (Fig. 3B-H). As expected, type I codon clusters, which can be described as rare-codon clusters, exhibit
211 slight enrichment near the N-terminus (Fig. 3B). To our surprise, type III codon clusters, which can be
212 described as common-codon clusters, also exhibit relatively strong enrichment near the N-terminus (Fig.
213 3D). Other types of codon clusters do not exhibit clear enrichment near the N-terminus. We also performed
214 a gene ontology (GO) analysis [19,20] (<http://geneontology.org/>) on the genes carrying N-terminal codon
215 clusters ($RLI < 0.1$) to see if the genes encoding secreted proteins are enriched. We found that not only
216 some extracellular matrix structural constituents, mostly mucins, are enriched, but also proteins associated
217 with plasma membrane or transcription-level regulation are enriched (Table S2).

218 Together, these data indicate that although N-terminal regions are more likely to harbor PFCCs, many
219 PFCCs actually locate in other regions (Fig. 3). They also suggest that although the function of a subset of
220 the PFCCs may be explained by N-terminal rare-codon clusters' impact on secretion or signal peptides, such
221 a function is unlikely a general role played by other PFCCs. For example, the codon clusters locating in the
222 middle of genes should have little to do with signal peptides. Thus, PFCCs likely perform various biological
223 functions that need further investigation.

3.4. Specific protein functional classes are overrepresented in genes carrying PFCCs while most PFCCs are not associated with known protein domains

To further investigate the biological roles of PFCCs, we next performed GO analyses on the genes carrying PFCCs, in order to test the hypothesis that PFCCs are associated with various functional features of protein-coding genes. We found that in all 1445 genes that carry the PFCCs, genes encoding membrane-binding proteins and transcription-related proteins are overrepresented, while genes encoding ribosomal and mitochondrial proteins are underrepresented (Table S3). This result suggests that functional codon clusters might be associated with transmembrane domains, so we then tested if the amino acid sequences encoded by the PFCCs are near or overlapped with the transmembrane helices predicted by TMHMM, an algorithm predicting transmembrane helices [21]. Unexpectedly, we found that only about 6% of the PFCCs are near or overlapped with some transmembrane helices (Table 1). Thus, there seems to be a discrepancy between the overrepresentation of transmembrane proteins in the genes carrying PFCCs and relatively few PFCCs that are near or overlapped with the sequences encoding transmembrane helices. Nevertheless, such a discrepancy could be explained by that PFCCs may be functionally more important for the non-transmembrane regions in transmembrane proteins. The discrepancy may also be explained by that transmembrane helices are less sensitive to the change in codon usage since the helices are strongly affected by the biochemical properties, such as hydrophobicity, of amino acid residues [21,22].

If most PFCCs are not associated with transmembrane helices, then what other types of protein domains might be associated with the PFCCs? To answer this question, we examined the association between PFCCs and annotated protein domains in the Pfam database [11,23]. We found that about 1/4 of the PFCCs are near or overlapped with some annotated Pfam protein domains, yet it is still unclear how the other 3/4 might influence protein functions (Table 2, Table S4). Among the PFCCs of which each is associated with only one Pfam protein domain, about 1/2 locate within protein domains (Table 2), which was consistent with what was recently reported by Chaney et al. [7]. These data suggest that although some PFCCs likely affect protein functions by modifying the co-translational processes concerning protein domains defined by amino acid sequences, the majority of PFCCs seem to be associated with unknown functional domains.

To summarize, although specific protein functional classes are overrepresented in the genes carrying PFCCs, most of the PFCCs are not associated with known protein domains defined by amino acid sequences. Therefore, PFCCs likely represent “hidden” nucleic-acid-level domains that regulate protein functions.

3.5. Voltage-gated sodium channels include a conserved rare-codon cluster associated with the inactivation gate

To identify possible specific functions of some PFCCs, we next investigated PFCCs identified in the *D. melanogaster* voltage-gated sodium channel (Nav) genes as a proof of principle for the following reasons. First, Nav has multiple transmembrane domains [24–26] and we have shown that transmembrane proteins are associated with PFCCs (Table S3). Second, Nav is a well-characterized protein family in terms of its physiological roles and structure-function relationship. Third, the *D. melanogaster* genome harbors two Nav paralogs whose divergence was dated back to the origin of Bilateria, which allows us to identify the PFCCs with conserved codon usage patterns.

Each Nav α -subunit consists of four transmembrane domains (Domains I-IV) linked by cytoplasmic chains, plus an N-terminal and a C-terminal cytoplasmic chains. The inactivation gate, which is responsible for stopping the sodium influx during action potential, is formed by the cytoplasmic chain between Domain III (DIII) and Domain IV (DIV) that will be refer to as DIII-IV linker below [26]. In general, most invertebrates have two types of Nav, namely type 1 Nav (Nav1) and type 2 Nav (Nav2), while vertebrates have lost the Nav2 gene but have gained multiple Nav1 paralogs [26]. As aforementioned, *D. melanogaster* has two paralogs of Nav, namely *para*, the Dmel/Nav1, and *NaCP60E*, the Dmel/Nav2 [27,28].

Multiple PFCCs were identified in Dmel/Nav1 and Dmel/Nav2, but the PFCCs in Dmel/Nav1 and those in Dmel/Nav2 are not always homologous. Nonetheless, we found that both genes have PFCCs in the DIII-IV linkers (Fig. 4). To assess the potential functions of these PFCCs, we then scanned the DIII-IV linkers with a 15-amino-acid sliding window and calculated TCAI for each window. We found that these PFCCs exhibit strong preference for rare codons (Fig. 5A, Dmel; Fig. 5B, Dmel), suggesting that decelerating translation during synthesizing the inactivation gate may be the key function of these PFCCs. We further scanned the DIII-IV linkers of Nav homologs in several other representative eukaryotic species, and found that the majority of them also have sub-regions preferring rare codons (Fig. 5, TCAI < -0.8).

Considering that the divergence between Nav1 and Nav2 was dated back to the origin of Bilateria [26], the conserved preference for rare codons in the DIII-IV linkers further support the hypothesis that the normal function of inactivation gate requires decelerated translation of this region. Decelerated translation is possibly critical for the correct folding pattern or phosphorylation of the DIII-IV linker [29–32]. In this regard, we hypothesize that synonymous mutations from rare codons to common codons in the DIII-IV linker could induce changes in the action potential through prolonged or shortened depolarization. Also, as some nonsynonymous mutations in the DIII-IV linker could cause cold-induced paralysis [33], it is possible

285 that the synonymous mutations from rare codons to common codons in this region can cause similar
286 phenotypes.

287 Furthermore, we noticed that not all DIII-IV linkers bear obvious rare-codon clusters (Fig. 5A, Bmor, Dpul,
288 Lgig, Hsap5, Hsap8, Hsap10). Therefore, it is possible that for some species, synonymous codon usage in
289 the DIII-IV linker is less sensitive to natural selection, perhaps due to other mechanisms that compensate the
290 effects of rare codons on protein folding. More interestingly, we found that among the Nav1 paralogs in
291 human, some have rare-codon clusters in the DIII-IV linkers while others do not. We also found that among
292 the paralogs with rare-codon clusters, the specific locations of rare-codon clusters can be different. These
293 findings perhaps suggest that rare-codon clusters are associated with the division of labor between Nav1
294 paralogs. As Nav1 paralogs have differentiated tissue-specific expression profiles [34], one mechanism
295 underlying the possible codon-usage-mediated division of labor may be that these paralogs adapt their DIII-
296 IV linkers' codon usage patterns to tissue-specific tRNA pools [14,35,36], so that the corresponding protein-
297 coding sequences are able to more finely regulate the function of inactivation gate.

298

299 4. Discussion

300 Here we show that clusters of codons with biased codon usage patterns may serve as nucleic-acid-level
301 domains that affect gene functions, just as a sequence of amino acids with a specific order and/or specific
302 biochemical properties can form a protein domain. We accomplished this by developing a conservative
303 statistical approach to identify PFCCs in the *D. melanogaster* genome. We have identified over 3000
304 PFCCs, and most of them strongly prefer rare codons. Nevertheless, we also found that a small proportion
305 of the PFCCs exhibit other patterns of codon usage, such as preference for common codons, which was not
306 reported before. We showed that although the PFCCs are associated with specific protein functional classes
307 including transmembrane proteins and transcription factors, most of them are not associated with known
308 protein domains defined by amino acid sequences. As a proof-of-principle, we used the example of a rare-
309 codon cluster associated with the inactivation gate of Nav to propose a hypothesis concerning how a PFCC
310 could affect specific biochemical and physiological properties of a protein. Together, our results suggest that
311 it is likely a general phenomenon that codon clusters with biased codon usage patterns serve as diverse
312 “hidden domains” involved in regulating protein functions.

313 In this paper, based on a widely used codon usage index CAI [15], we proposed an alternative codon usage
314 index TCAI (see Materials and Methods: Calculating TCAI) that was used for classifying PFCCs.

315 Compared to CAI, TCAI is better at describing the preference for rare codons. This is because when CAI is

316 calculated, codon usage frequencies are all normalized to the frequencies of the most common synonymous
317 codons. Thus, the CAI value of any codon cluster that strictly uses common codons will always be 1, while
318 if two codon clusters that strictly use rare codons but have different amino acid sequences, they may have
319 fairly different CAI values. However, by using the newly proposed TCAI, rare-codon clusters will have
320 similar TCAI values that are -1 or very close to -1, while common-codon clusters keep TCAI values at 1 or
321 near 1. Thus, TCAI is a good choice when researchers intend to identify rare-codon clusters.

322 In comparison to previous methods for detecting functional codon clusters [7], the method presented here is
323 more conservative in terms of detecting rare-codon clusters due to the usage of both whole-genome and
324 gene-specific codon usage patterns as the background codon usage. Yet, it is more powerful in terms of
325 detecting other types of codon clusters due to a more relaxed assumption about the possible functional roles
326 of codon clusters. The diverse codon usage patterns and locations of the PFCCs suggest that codon clusters
327 may affect protein functions through various mechanisms. The major mechanism through which codon
328 clusters regulate protein functions is possibly the deceleration of translation, as shown by the preponderance
329 of rare-codon clusters in the identified PFCCs. However, we must admit that the preponderance of rare-
330 codon clusters may be partly an artifact of technically easier detection of the preference for rare codons by
331 our approach. To increase the power of codon-cluster-detection algorithms and more accurately assess the
332 prevalence and importance of different types of codon clusters, researchers may need to incorporate
333 phylogenetic analyses of homologous protein-coding genes in order to identify codon clusters with
334 conserved codon usage patterns.

335 Consistent with previous reports [7], we found that some of the PFCCs are associated with known protein
336 domains defined by amino acid sequences, which suggests that some codon clusters do have the potential to
337 assist correct folding and modifications of protein domains. However, we also found that the majority of
338 PFCCs are not associated with known protein domains [11,23], indicating that these PFCCs may carry
339 necessary information for regulating protein functions and such information cannot be predicted from amino
340 acid sequences. Thus, codon clusters could serve as “hidden domains” in protein-coding sequences. For
341 example, some “free coiled regions” of proteins may not be actually “free”: their folding and modifications
342 could be restricted by the codon usage patterns of the corresponding genomic regions. Further investigation
343 into the codon clusters that may encode “hidden domains” could be important for biologists to better
344 understand how genetic information directs the functions of proteins.

345 As we have shown by the example of rare-codon clusters in the DIII-IV linkers of Nav proteins, functional
346 codon clusters may be important for some key functions of proteins. This could have important implications
347 for molecular evolutionary studies and biological engineering practice. For molecular evolutionary studies,

348 codon clusters with critical functions suggest that synonymous sites in such functional codon clusters may
349 bias the estimation of the rate of neutral evolution if researchers consider synonymous mutations as neutral
350 mutations. Moreover, it is possible that the selective pressure on synonymous codon usage may be even
351 stronger than that on nonsynonymous mutations, which could greatly interfere the results and inferences of
352 the evolutionary analyses based on the comparison between synonymous and nonsynonymous sites. For
353 biological engineering practice, functional codon clusters suggest that when transgenes are designed, simple
354 codon optimization [37], which generally uses common codons to encode amino acid residues, may not be
355 the best choice to achieve desired structure and functions of the engineered proteins. Instead, the codon
356 usage of different regions within a transgene may need to be more delicately controlled.

357 Together, our data support the broad existence of diverse and functional codon clusters that may affect
358 protein functions and associated phenotypes through various mechanisms. In this regard, we suggest that
359 functional codon clusters should be seriously considered if researchers are to thoroughly understand how
360 genetic information is interpreted into functional, phenotypic, and evolutionary outputs *in vivo*.

361

362 [5. Materials and Methods](#)

363 [5.1. Reference protein-coding sequences](#)

364 Reference protein-coding sequences of *D. melanogaster* were downloaded from Ensembl 89 [38]. Protein-
365 coding sequences fulfilling the following criteria were chosen. 1) The sequence length is a multiple of three.
366 2) The sequence uses standard genetic code. 3) For each gene, only the longest mRNA isoform was used; if
367 there were multiple isoforms of the same length, then the first record shown in the FASTA file was used.

368 The protein-coding sequences of Nav1 and Nav2 in analyzed species can be found in Table S5.

369 [5.2. Identifying PFCCs](#)

370 Fig. S1 depicts how to identify PFCCs in a protein-coding sequence. For a window W_i starting with the i th
371 codon in a protein-coding sequence, the window size S is set to vary between 5 to 50 codons. For each
372 window size, two χ^2 tests are performed by comparing the codon usage of the window respectively to
373 whole-genome codon usage and gene-specific codon usage, and the higher p -value is selected as the
374 representative p -value. Then the representative p -values are plotted against window sizes, which generates a
375 p - S curve representing a function $p(S)$ that describes the relationship between p -value and window size (Fig.
376 S1A-D). If $p(S)$ is monotonic, the lowest p -value together with its corresponding S are selected as the
377 representative p and S for W_i , namely p_i and S_i ; otherwise the p -value and the S that correspond to the lowest

378 stationary point of $p(S)$ are selected as p_i and S_i . For the focal protein-coding sequence, all p_i 's are corrected
379 by setting the false discovery rate (FDR) [39] to 0.05 so as to get the corrected p -values $p_{i,corrected}$'s; then
380 windows with $p_{i,corrected}$ values lower than the threshold 0.05 are detected as positive segments with
381 unexpected codon usage patterns (Fig. S1E). Finally, isolated positive segments, together with the codon
382 clusters generated by merging overlapped positive segments, are detected as PFCCs.

383 5.3. Calculating TCAI

384 To calculate the TCAI of a given sequence of codons, the background relative codon usage frequencies need
385 to be calculated first. For example, if a gene uses 10 AAA and 30 AAG to encode Lys, the gene-specific
386 background relative codon usage frequencies of AAA and AAG will respectively be $10/(10+30)=0.25$ and
387 $10/(10+30)=0.75$. Then the focal sequence of codons is translated to an amino acid sequence. The next step
388 is to generate a pseudo-sequence of codons according to the amino acid sequence and the background
389 relative codon usage frequencies. For example, assuming that the amino acid sequence is Lys-Lys and the
390 background relative codon usage frequencies are 0.25 for AAA and 0.75 for AAG, the first Lys will have a
391 25% chance to be encoded by AAA and 75% chance to be encoded by AAG, and so will the second Lys.
392 This step of pseudo-sequence generation is repeated for 10,000 times so that there will be 10,000 pseudo-
393 sequence of codons, which represent the expected results if codons are used randomly to encode the amino
394 acids. Then the CAIs [15] of all pseudo-sequences and the CAI of the actual codon sequence are calculated.
395 Finally, TCAI is calculated by subtracting the proportion of pseudo-sequence whose CAIs are higher than
396 the CAI of the actual sequence from the proportion of pseudo-sequences whose CAIs are lower than the
397 CAI of the actual sequence.

398 When TCAI is -1, it means that none of the pseudo-sequences has a CAI lower than the actual sequence;
399 thus, the actual sequence strongly prefers rare codons. In contrast, when TCAI is 1, the actual sequence
400 strongly prefers common codons.

401 5.4. K-mean clustering of PFCCs

402 K-mean clustering is done by using the online tool at <http://scistatcalc.blogspot.com/2014/01/k-means-clustering-calculator.html>. The number of clusters (i.e., K) is determined by the elbow method, according to
403 <https://pythonprogramminglanguage.com/kmeans-elbow-method/>. Each input data point of K-mean
404 clustering is specified by its gene-specific and whole-genome TCAIs.

406 5.5. Calculating RLI

407 For a protein-coding sequence with L codons, the RLI of a PFCC which starts at the i th codon and has a size
408 of S_i codons is calculated as $(i + S_i / 2) / L$.

409 **5.6. Searching for transmembrane helices**

410 For a focal PFCC, the protein sequence from the first residue or the 150th residue upstream to the PFCC-
411 encoded region, whichever is closer to the PFCC-encoded region, to the last sense codon or the 150th codon
412 downstream to the PFCC-encoded region, whichever is closer to the PFCC-encoded region, is input to
413 TMHMM [21] in order to search for transmembrane helices near or overlapped with the PFCC-encoded
414 region. The coordinates of identified transmembrane helices are recorded.

415 **5.7. Searching for Pfam protein domains**

416 For a focal PFCC, the protein sequence from the first residue or the 150th residue upstream to the PFCC-
417 encoded region, whichever is closer to the PFCC-encoded region, to the last sense codon or the 150th codon
418 downstream to the PFCC-encoded region, whichever is closer to the PFCC-encoded region, is input to the
419 hmmscan program of HMMER [23] on <https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan> in order to
420 search for Pfam protein domains [11] near or overlapped with the PFCC-encoded region. The coordinates
421 and names of identified Pfam domains are recorded.

422 **5.8. Classifying association between PFCCs and protein domains**

423 The association between a PFCC and a protein domain is classified to one of the following categories.

- 424 1) No association: The closest distance between the PFCC-encoded region and the protein domain is longer
425 than 20 residues.
- 426 2) 1-to-multiple association: Multiple protein domains are overlapped with the region that starts from the
427 20th residue upstream to the PFCC-encoded region and ends at the 20th residue downstream to the PFCC-
428 encoded region.
- 429 3) Cluster in domain: Only one protein domain is associated with the PFCC. The PFCC-encoded region
430 locates within the protein domain.
- 431 4) Domain in cluster: Only one protein domain is associated with the PFCC. The protein domain locates
432 within the PFCC-encoded region.
- 433 5) Domain overlap left of cluster: Only one protein domain is associated with the PFCC. The start of the
434 protein domain is upstream to the PFCC-encoded region and the end of the protein domain locates within
435 the PFCC-encoded region.

436 6) Domain overlap right of cluster: Only one protein domain is associated with the PFCC. The start of the
437 protein domain locates within the PFCC-encoded region and the end of the protein domain is downstream to
438 the PFCC-encoded region.

439 7) Domain upstream to cluster: Only one protein domain is associated with the PFCC. The end of the
440 protein domain is upstream to the PFCC-encoded region.

441 8) Domain downstream to cluster: Only one protein domain is associated with the PFCC. The start of the
442 protein domain is downstream to the PFCC-encoded region.

443 [5.9. Alignment of Nav orthologs and identification of DIII-IV linkers](#)

444 Nav orthologs were aligned by using MAFFT algorithm [40,41]. The annotated DIII-IV linkers of
445 Dmel/Nav1 (<https://www.uniprot.org/uniprot/P35500>) and Dmel/Nav2
446 (<https://www.uniprot.org/uniprot/Q9W0Y8>) were used to locate the DIII-IV linkers of the Nav1 and Nav2
447 in other analyzed species.

448

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457 raw data are available from Zhen Peng (peng.z@wustl.edu) upon reasonable request.

458

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557 **8. Tables**

558 **Table 1. Biased codon clusters overlap with transmembrane helices.**

		Association type	Number of clusters		
Clusters associated with transmembrane helices	1-to-1 association	cluster in helix	14	115	195
		helix in cluster	16		
		helix overlap left of cluster	21		
		helix overlap right of cluster	24		
		helix upstream to cluster	20		
		helix downstream to cluster	20		
	1-to-multiple association		80		
All clusters			3050		

559 A codon cluster is defined to be associated with a transmembrane helix if the distance between at least one
 560 amino acid residue of the helix and the closest residue encoded by the codon cluster does not exceed 20
 561 amino acids.

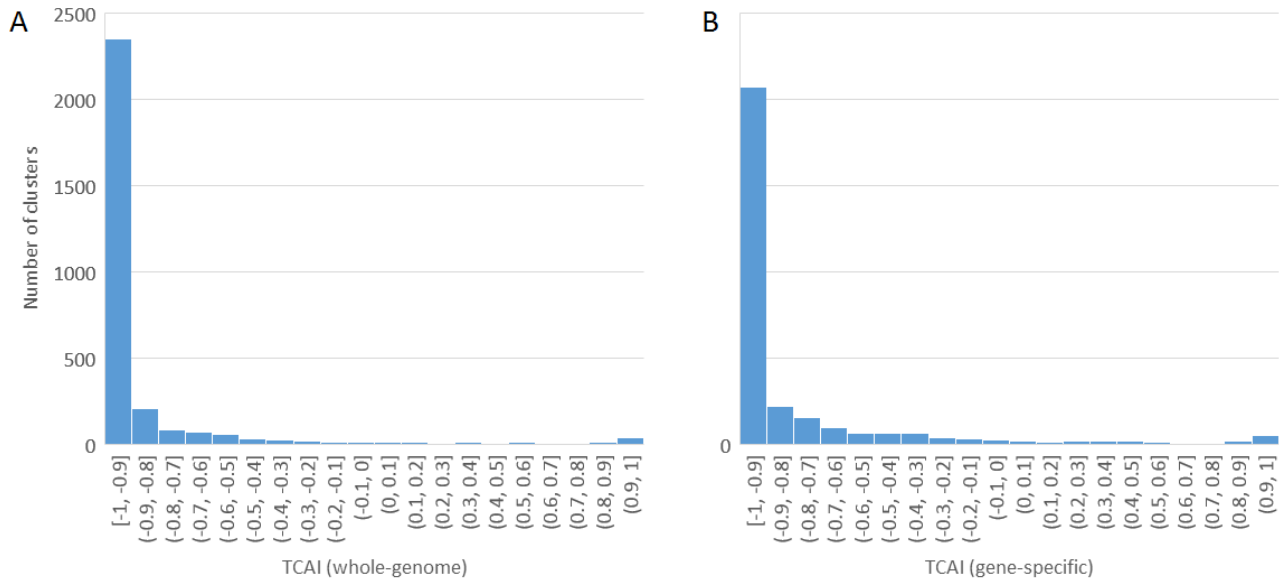
562 **Table 2. Biased codon clusters overlap with Pfam domains.**

		Association type	Number of clusters		
Clusters associated with Pfam domains	1-to-1 association	cluster in domain	299	584	746
		domain in cluster	3		
		domain overlap left of cluster	63		
		domain overlap right of cluster	75		
		domain upstream to cluster	58		
		domain downstream to cluster	86		
	1-to-multiple association		162		
All clusters			3050		

563 A codon cluster is defined to be associated with a Pfam domain if the distance between at least one amino
 564 acid residue of the Pfam domain and the closest residue encoded by the codon cluster does not exceed 20
 565 amino acids.

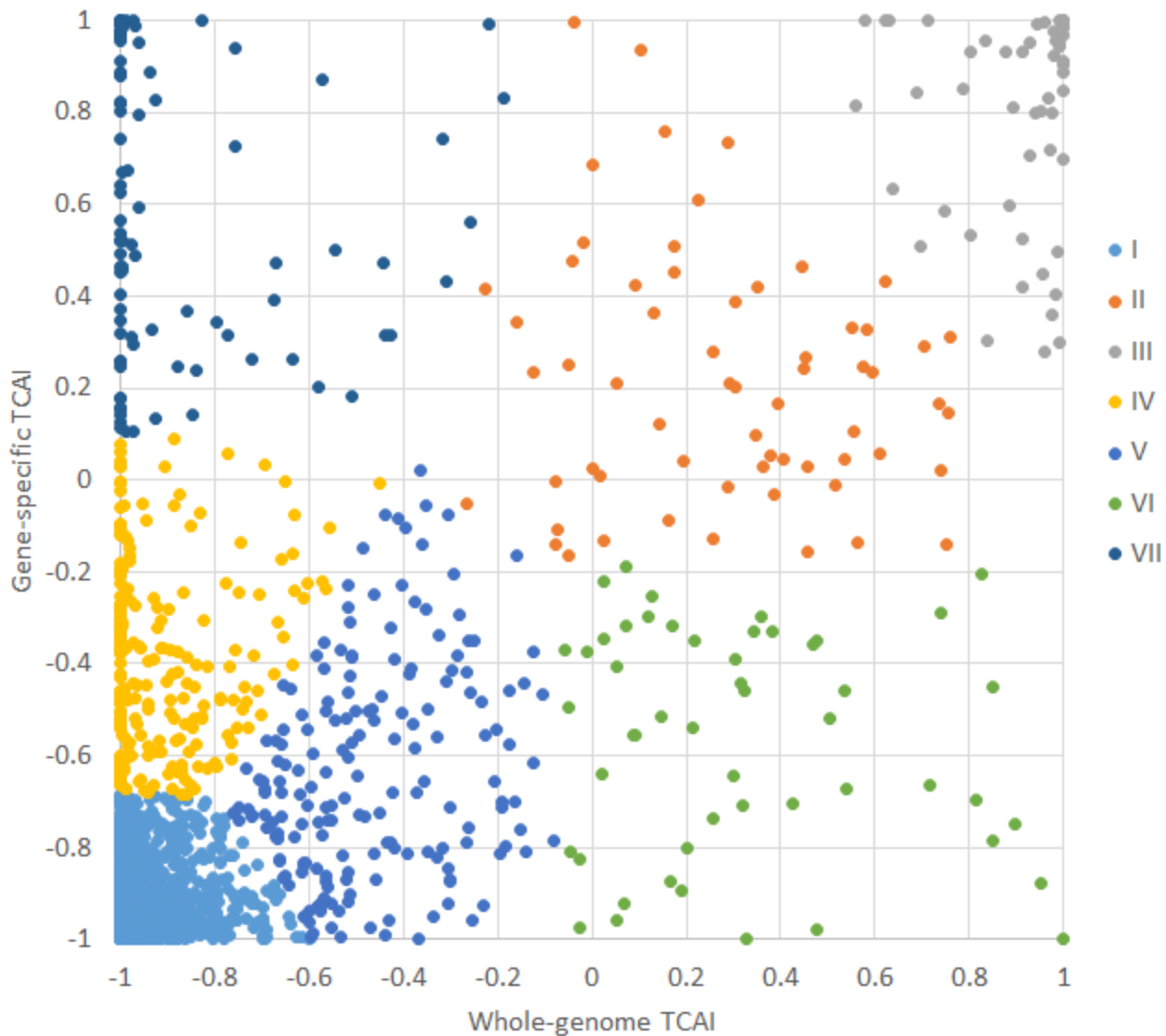
566

9. Figures



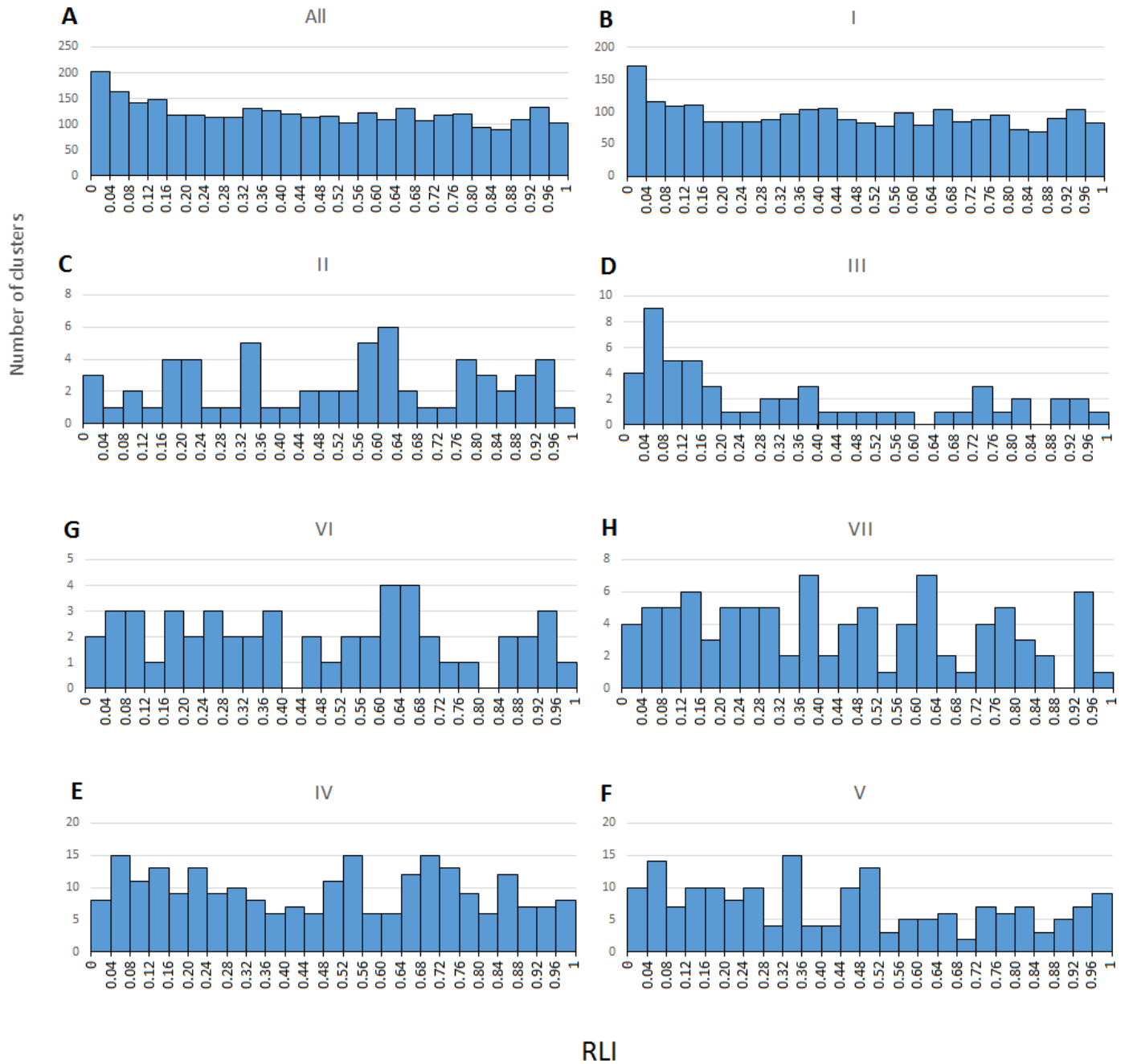
567

568 **Fig. 1. Distribution of TCAI values.** TCAI values were calculated by using the whole-genome (A) or
569 gene-specific (B) codon usage patterns as the background codon usage. The TCAI of a rare-codon cluster is
570 near -1, while that of a common-codon cluster is near 1.



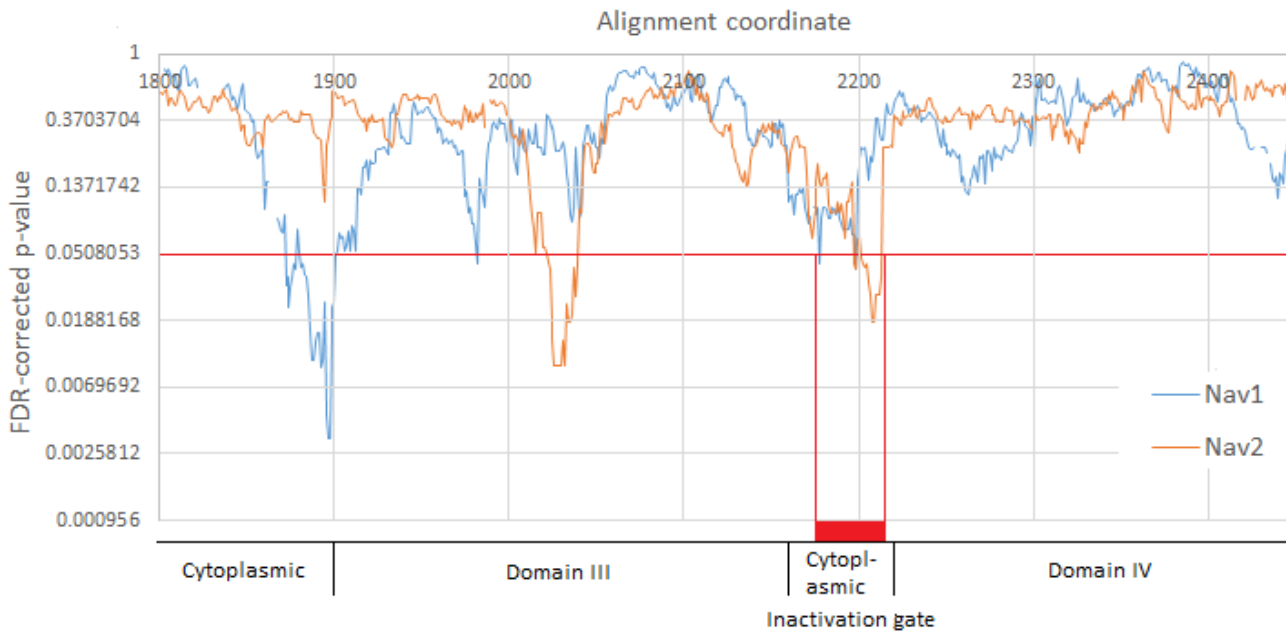
571

572 **Fig. 2. Influence of the discrepancy between whole-genome and gene-specific codon usage patterns on**
573 **classifying PFCCs.** Codon usage patterns of identified PFCCs were described by TCAI. Since gene-
574 specific and whole-genome-level TCAI values for the same codon cluster could be different, we plotted the
575 gene-specific TCAI against whole-genome TCAI for all codon clusters and then classified codon clusters by
576 K-mean clustering (K=7).



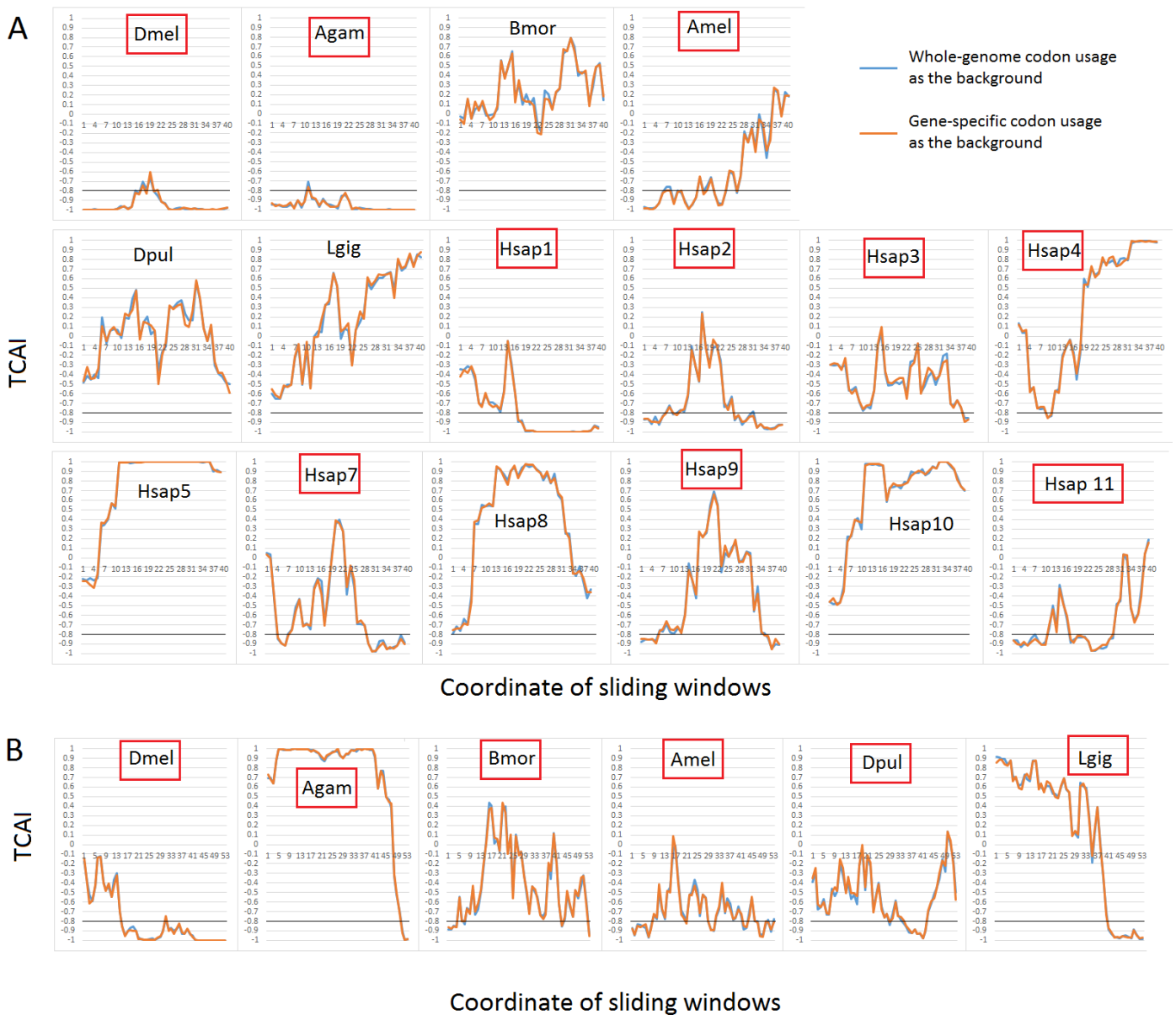
577

578 **Fig. 3. Spatial distribution of putatively functional codon clusters.** For all identified PFCCs and each
579 type of PFCCs shown in Fig. 2, the distribution of PFCCs is plotted against the location coordinates,
580 measured by RLI (RLI=0 means N-terminus; RLI=1 means C-terminus).



581

582 **Fig. 4. Identifying PFCCs in *D. melanogaster* Nav paralogs.** Dmel/Nav1 and Dmel/Nav2 are aligned by
583 amino acid sequences. p -values were corrected by FDR (FDR = 0.05), and those lower than the threshold
584 indicate codon clusters whose codon usage patterns are significantly different from both whole-genome and
585 gene-specific codon usage patterns. Both Dmel/Nav1 and Dmel/Nav2 have PFCCs in the DIII-IV linkers,
586 shown by the red bar.



587

588 **Fig. 5. Nav paralogs generally bear rare-codon clusters in DIII-DIV linkers. (A) Nav1. (B) Nav2.**

589 Dmel: *Drosophila melanogaster*, fruit fly; Agam: *Anopheles gambiae*, malaria mosquito; Bmor: *Bombyx*
 590 *mori*, silkworm; Amel: *Apis mellifera*, Western honey bee; Dpul: *Daphnia pulex*, water flea; Lgig: *Lottia*
 591 *gigantea*, owl limpet; Hsap: *Homo sapiens*, human. *Homo sapiens* has ten Nav1 paralogs but no Nav2. As
 592 suggested by Fig. 2, regions with TCAI < -0.8 are regarded as rare-codon clusters. Red boxes highlight the
 593 DIII-IV linkers carrying rare-codon clusters. Black lines: TCAI = 0.8. Blue curves: TCAI calculated by
 594 using whole-genome codon usage as the background. Orange curves: TCAI calculated by using gene-
 595 specific codon usage as the background.

596 **[10. Supplementary Material List](#)**

597 **Fig. S1. Using sliding window with adaptive size to identify PFCCs.**

598 **Table S1. Identified PFCCs.**

599 **Table S2. Analysis of the association between genes carrying N-terminal PFCCs (RLI<0.1) and GO**
600 **terms.**

601 **Table S3. Analysis of the association between genes carrying PFCCs and GO terms.**

602 **Table S4. Analysis of the association between PFCCs and Pfam domains.**

603 **Table S5. Information of Nav genes.**