Intermolecular interactions drive protein adaptive and co-adaptive evolution at both species and population levels

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Abstract

Proteins are the building blocks for almost all the functions in cells. Understanding the molecular evolution of proteins and the forces that shape protein evolution is an essential step in understanding the basis of function and evolution. Previous studies have shown that adaptation occurs frequently at the protein surface, such as in genes involved in host-pathogen interactions. However, it remains unclear whether adaptive sites are distributed randomly or at regions that are associated with particular structural or functional characteristics across the genome, since many of the proteins lack structural or functional annotations. Here, we seek to tackle this question by combining large-scale bioinformatic prediction, structural analysis, phylogenetic inference, and population genomic analysis of *Drosophila* protein-coding genes. By estimating and comparing the rate of adaptive substitutions at protein and residue level, we showed that adaptation is more relevant to function-related rather than structure-related properties. Among the function-related properties, we found that molecular interactions in proteins contribute to adaptive evolution, and putative binding residues exhibit higher rates of adaptation. We observed that physical interactions might play a role in the co-adaptation of fast-adaptive proteins. We found that strongly differentiated amino acids in protein coding genes are mostly adaptive, which may contribute to the long-term adaptive evolution. Our results suggest important roles of intermolecular interactions and co-adaptation in the adaptive evolution of proteins both at the species and population levels.

Introduction

Natural selection plays an important role in molecular evolution of protein sequences. Recent advances in genome sequencing and reliable inference methods at both phylogenetic and population levels have enabled fast and robust estimation of evolutionary rates and adaptation driven by natural selection. In addition, the increased availabilities of structural and functional data of proteins have made it possible to study how structural and functional constraints affect

protein sequence evolution and adaptation. It is now well established that different proteins and different sites within a protein have varying rates of evolution and adaptation due to both structural and functional constraints (Echave et al., 2016; Kosiol et al., 2008; Lindblad-Toh et al., 2011; Zhang and Yang, 2015). For example, genes that are highly expressed or perform essential functions are under strong purifying selection and tend to evolve slowly (Drummond et al., 2005; Moutinho et al., 2019; Pál et al., 2001; Zhang and He, 2005; Zhang and Yang, 2015); genes involved in host-pathogen interactions, e.g., immune responses and antivirus responses, show exceptionally high rates of adaptive changes (Enard et al., 2016; Nielsen et al., 2005; Obbard et al., 2009; Palmer et al., 2018; Sackton et al., 2007; Sironi et al., 2015; Uricchio et al., 2019); and residues that are intrinsically disordered or at the protein surface are fast evolving and has been proved to be hotspots of adaptive evolution (Afanasyeva et al., 2018; Goldman et al., 1998; Lin et al., 2007; Moutinho et al., 2019; Ramsey et al., 2011). More recently, Slodkowicz & Goldman (Slodkowicz and Goldman, 2020) employed genomic-scale integrated structural and phylogenetic evolutionary analysis in mammals and showed that positively selected residues are clustered near ligand binding sites, especially in proteins that are associated with immune responses and xenobiotic metabolism.

Although evidence have shown that adaptation is more likely to occur at intrinsically disordered regions and clustered at the surface of proteins, the functional properties of adaptation in the genomic scale remains unclear. Moreover, due to lack of structural and functional information of many proteins in the genome, the underlying mechanism derived from current studies might be incomplete. Here, we systematically investigated the evolution and adaptation of protein-coding genes in *Drosophila melanogaster* by comparing it to its closely related species, in order to distinguish the main factors that impact the evolution and adaption at the protein-coding level. We applied large-scale bioinformatic and structural analysis to obtain structural and functional properties of proteins. We then classified residues into different structural and functional sites. By comparing rates of sequence evolution and adaptation between different proteins and different sites, we were able to locate hotspots of adaptation at genome scale. We showed that, for *D. melanogaster* proteins, adaptation is more sensitive to functional properties rather than structural ones. Interestingly, we found that putative binding regions including allosteric sites at protein surface show higher rates of adaptation than other sites. For proteins that are under fast-adaptive evolution, we showed that they tend to interact with each other more frequently than random expectations and are often associated with reproduction, immunity, and environmental information processing in *D. melanogaster*. In addition, we showed that interacting proteins in *D. melanogaster* might undergo co-adaptive

evolution. Furthermore, we hypothesize that molecular interactions or physical interactions might be an important mechanism that contribute to the adaptive and co-adaptive evolution in *D. melanogaster* genome. At last, we showed that the accumulation of short-term adaptation to local environments could be a possible genetic mechanism that contribute to long-term adaptive evolution.

Results

Impact of gene properties on evolution of protein-coding genes in *D. melanogaster*

To uncover the main factors that impact the evolutionary rates of genes, we analyzed 13528 protein-coding genes in *D. melanogaster* using genome data from *melanogaster* subgroup species and *D. melanogaster* population genomics data from 205 inbred lines from Drosophila Genetic Reference Panel, Freeze 2.0, DGRP2 (Huang et al., 2014). We applied a maximum likelihood method (Yang, 2007) to compute dN/dS ratio (ω) using the protein-coding sequences of five closely related melanogaster subgroup species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba* and *D. erecta*). We estimated the proportions of adaptive changes (α) in each gene by applying an extension of MK test named asymptotic MK (Messer and Petrov, 2013; Uricchio et al., 2019) using *D. simulans* as outgroup. We then calculated the rate of adaptive changes (ω_a) of each gene by multiplying ω to α ($\omega_a = \alpha\omega$) (Moutinho et al., 2019) using *D. yakuba* as the outgroup species (See methods). The rate of nonadaptive changes can be further calculated by $\omega_{na}=\omega-\omega_a$. Finally, we successfully assigned ω to 12118 protein coding genes and ω_a and ω_{na} to 7192 genes.

For each of *D. melanogaster* genes subjecting the same pipeline of analysis, we further obtained 17 different structural or functional properties (see Methods), which can be further divided into two categories: structure-related properties and function-related properties. Specifically, structure-related properties include ratio of secondary structures (helix ratio, sheet ratio, helix+sheet ratio, coil ratio), intrinsic structural disorder (ISD), relative solvent accessibility (RSA); while function-related properties include gene pseudo-age, protein length, number of protein-protein interactions (PPI numbers), ratio of protein-binding sites (PPI-site ratio), ratio of DNA-binding sites (DNA-site ratio) and gene expression patterns such as male expression level, female expression level, mean expression level, male specificity and tissue specificity. The properties along with gene-specific protein evolution (ω, ω_a and ω_{na}) are available in supplementary file S1.

Molecular interactions contribute to the variations of protein sequence evolution and adaptation. In order to identify the determinants that drive protein evolution (ω, ω_a and

102 ω_{na}), we calculated the Pearson's correlations of ω, ω_a and ω_{na} with all the structure- and
103 function-related properties. The correlation coefficient (r) and corresponding p-values (p) of
104 each of the properties were listed in Table 1. Interestingly, we observed that for structure-related
105 properties (secondary structure ratios, ISD, and RSA), variation of ω is dominated by
106 nonadaptive changes (ω_{na}) (Figure S1). Taking RSA as an example, we observed that RSA
107 strongly correlates with both ω (r=0.16, p=1e-73) and ω_{na} (r=0.15, p=3e-35), while weakly
108 correlates with ω_a (r=0.06, p=1e-6). These correlations suggest that, under the constraints of
109 structure-related properties, relaxation of purifying selection may play a more important role in
110 determine protein evolution. These are in line with previous studies that proteins with less
111 structural constraints, i.e. those harboring more disordered, exposed sites display faster
112 evolutionary and nonadaptive evolutionary rate (Afanasyeva et al., 2018; Moutinho et al., 2019)
113 However, for function-related properties (gene pseudo-age, protein length, PPI number,
114 PPI-site ratio, DNA-site ratio and gene expression patterns), the importance of ω_a in shaping
115 protein evolution begin to emerge (Figure S1). For example, when considering tissue specificity,
116 the correlation efficient (r) of ω is 0.30 (p=2e-205), while r of ω_a and ω_{na} are 0.16 (p=3e-35) and
117 0.17 (p=2e-42), respectively. In such cases, the correlations of ω_a and ω_{na} almost contributed
118 equally to the variation of protein sequence evolutionary rates, ω. Interestingly, among the
119 function-related properties, we found that molecular interactions, i.e., protein interactions,
120 strongly positively correlates with ω, ω_a and ω_{na} (Table 1). We also noticed that for molecular
121 interactions, compared to other function-related properties, variations of ω_a contributes slightly
122 to variations of ω. This could be a result of intercorrelations of molecular interactions and ISD or
123 RSA (Table S1), since disordered regions and exposed regions are often responsible for
124 interacting with other molecules (Keskin et al., 2008; Van Der Lee et al., 2014). These results
125 highlight the non-neglected contributions of functional constraints, including molecular
126 interactions, on the adaptive evolution of protein-coding sequence.
127 ***Complex correlations of protein length and male expression level with protein***
128 ***evolutionary rates.*** To better clarify and visualize the correlations of ω, ω_a, and ω_{na} with gene
129 properties in a refined fashion, we divided *D. melanogaster* genes into 15 groups according to
130 the ascending orders of ω values and compared these properties of different gene groups, while
131 ensuring that each gene group contains the same total number of amino acids (Figure S2).
132 Overall, for most of the properties being investigated, we observed similar correlations as shown
133 in Table 1. For example, fast evolving genes are relatively young, short, lowly expressed, male
134 or tissue specific, abundant of disordered, exposed residues, excluded in protein-protein
135 network center hubs, and abundant of protein and DNA binding sites.

136 In contrast to previous observations, we found complex (nonlinear) correlations of ω
137 gene groups with protein length and gene expression levels (Figure 1). For protein length, our
138 Pearson correlation analysis (Table 1) and a number of previous studies have suggested a
139 strong negative correlations with ω (Lipman et al., 2002; Moutinho et al., 2019). However, we
140 observed that some proteins with the slowest evolutionary rates, i.e. with the smallest ω values,
141 are significantly shorter than other gene groups with intermediate evolutionary rates (Fig. 1A).
142 These include highly conserved genes such as eIF1A (ω=0.0001,148 a.a), rala (ω=0.0001, 201
143 a.a.), ctp (ω=0.0001, 89 a.a.), and Mlc-c (ω=0.0001, 153 a.a.).
144 Similar complex correlations were also observed in male expression level and mean
145 expression level (Fig. 1BC). We found that, when checking male expression level and mean
146 expression level, the gene group that shows the largest mean ω has higher expression than
147 those with intermediate ω. Such U-shape correlations were not observed in female expression
148 levels. Although protein length and mean expression levels of genes are known to be strongly
149 correlated with protein evolutionary rates as listed in Table 1 and also in other references
150 (Drummond et al., 2005; Lipman et al., 2002; Zhang and Yang, 2015), fast evolving genes can
151 also be moderately or highly expressed, especially in male *D. melanogaster*. For example,
152 many seminal fluid proteins show high ω values and are highly expressed, such as Sfp60F
153 (ω=0.77, 82 a.a.), EbpII (ω=0.68, 66 a.a), Acp36DE (ω=0.68, 912 a.a.,), and Dup99B (ω=0.63,
154 54 a.a.). These proteins evolve at very fast rates (Begun and Lindfors, 2005; Swanson et al.,
155 2001), contain various range of amino acids (54 in Dup99B to 912 in Acp36DE), and are
156 moderately or highly expressed in male *D. melanogaster* (TPM ranging from 440 for Acp36DE
157 to 3189 for Sfp60F), lowly expressed in female (TPM all around 1, presumably in spermatheca).
158 We listed all the genes and protein length and expression levels in each ω gene group, which
159 can be found in supplementary file S2.
160 Since tissue specificity and male specificity both strongly correlates with ω, ω_a, and ω_{na}
161 (Table 1), we asked whether male specificity would be a redundant property compared to tissue
162 specificity to indicate protein evolution due to the complex correlations of male expression
163 levels. To answer this question, we classified *D. melanogaster* genes into 15 groups according
164 to ascending values of male specificity. We then did similar classification to classify all the
165 genes into 15 groups according to ascending values of tissue specificity (Figure 1). As
166 expected, we found that tissue specificity positively correlates with ω, ω_a and ω_{na} (Fig. S3).
167 However, we observed complex correlations for male specificity gene groups. Specifically, gene
168 group with the lowest male specificity show significantly higher ω, ω_a and ω_{na} than its following

169 gene group (Fig. S3). This could be a result of fast evolving female-biased genes (Yang et al.,
170 2016) included in this gene group.
171
172 **Putative molecular interaction sites are hotspots for protein adaptive evolution**
173 Having established that molecular interactions positively correlates with the adaptation of
174 protein sequence, we next investigate whether residues involved in molecular interactions are
175 targets for adaptive evolution. To tackle this question, we predicted protein-protein interaction
176 sites (PPI-sites) and DNA binding sites (DNA-sites) for each of *D. melanogaster* protein
177 sequence (see Methods). In addition, we characterized allosteric residues as surface and
178 interior critical residues with STRESS model (Clarke et al., 2016) for all the structural models.
179 We also extracted putative binding sites from STRESS Monte Carlo (MC) simulations. We
180 calculated ω, ω_a and ω_{na} for residues in each of the putative molecular interaction category.
181 Strikingly, we observed that residues involved in protein-protein interactions, DNA binding and
182 ligand binding exhibited higher rates of adaptive evolution compared to their corresponding null
183 sites (Fig. 2A-C). In addition, allosteric residues at protein surface showed higher adaptation
184 rates than allosteric residues at protein interior or residues that are not involved in ligand binding
185 from STRESS simulations (Fig. 2C).
186 Since we observed significant positive intercorrelations between PPI and DNA binding
187 with ISD and RSA (Table S1), we next asked whether the increase of ω_a in protein-protein
188 interactions sites or DNA binding sites was caused by the increase of disorder or site exposure.
189 We calculated and compared ω, ω_a and ω_{na} for putative PPI and DNA binding sites with
190 different levels of ISD or RSA. Remarkably, we found that ω_a of these putative binding sites
191 remains similar among different levels of ISD or RSA (Fig. S4, left column). The results suggest
192 that putative PPI or DNA binding events in proteins can result in elevated adaptation rates
193 regardless their structural disorder or site exposure. While for residues that are not associated
194 with putative PPI or DNA binding, we also observed increase in ω_a when increasing ISD or RSA
195 (Fig. S4, right column), which could be the result of some other yet unknown underlying
196 mechanisms or inaccuracy of putative binding sites predictions.
197 In order to gain better understanding of adaptation in molecular interaction sites, we
198 further visualized positive selections that are associated with molecular interactions. We first
199 investigated whether adaptive evolution is associated with particular protein structures or protein
200 families. To do this, we looked into fast-adaptive proteins with the largest ~15% rates of
201 adaptation ($\omega_a > 0.15$) that are linked to high quality structural models. Interestingly, among
202 these proteins, we found 45 enriched as trypsin-like cysteine/serine peptidase domain and 17

203 7TM chemoreceptors, suggesting widespread adaptive evolution acting on these protein
204 families or protein domains in *D. melanogaster* (Table S2). Many of the 7TM chemoreceptors
205 are olfactory and gustatory genes, which shows adaptive evolution in various species such as
206 *Drosophila* and mosquito (Hill et al., 2002; Lawniczak and Begun, 2007; McBride, 2007; Wu et
207 al., 2009). In addition to these two protein families, recurrent positive selections acting on some
208 other fast-adaptive proteins were identified in previous studies in *Drosophila* and mammals, and
209 the possible adaptive evolution mechanisms have been linked to exogenous ligand binding, for
210 example, serine protease inhibitors (serpin), Toll-like receptor 4 (TLR-4), and cytochrome P450
211 (Jiggins and Kim, 2007; Slodkowicz and Goldman, 2020).
212 In order to visualize the link between adaptive evolution and molecular interactions in the
213 two protein families with frequent adaptive evolution, we showed significant positive selections
214 and molecular interactions in two representatives: CG10232 and Or67a, each for trypsin-like
215 cysteine/serine peptidase domain and 7TM chemoreceptors, respectively. We observed that in
216 both cases, positively selected sites highly overlapped with predicted or inferred binding pockets
217 (Fig. 2D-E). Specifically, in CG10232, we found clusters of positive selected sites around NAG
218 binding sites that are inferred from a crystal structure of serine protease (PDB code: 2XXL) (Fig.
219 2D), while in Or67a, positively selected sites expand around the putative odorant binding
220 channel formed by helices S1-S6 in extracellular regions (Butterwick et al., 2018) (Fig. 2E).
221 Except for these examples that are associated with exogenous ligand or exogenous
222 peptide binding, we also identified two previously not described examples where adaptive
223 evolution might be linked to endogenous protein binding: Spaztle (spz, Fig. 2F) and Cul6 (Fig.
224 2G). Spaztle can bind to Toll-like receptors (TLR) and trigger humoral innate immune response.
225 We built the missing loop in Spaztle in the crystal structure of Toll/Spaztle complex (PDB code
226 4BV4) according to the dimeric crystal structure of Spaztle (PDB code 3E07). In this complex
227 structural model, we observed several positively selected sites in Toll-4/Spaztle interfaces (Fig.
228 2F). Cul6, another example, is a protein in cullins family in *D. melanogaster*. The cullins protein
229 family are known as scaffold proteins that assemble multi-subunit Cullin-RING E3 ubiquitin
230 ligase by forming SCF complex with F box and RING-box (Rbx) proteins (Zheng et al., 2002).
231 We constructed the putative Cul6 contained SCF complex by superimposition to the crystal
232 structure of the Cul1-Rbx1-Skp1-F box^{Skp2} SCF ubiquitin ligase complex (Zheng et al., 2002). In
233 the structural model, we observed positive selected sites in Cul6 clustered around the binding
234 sites of RING-box protein, Rbx1, and F-box protein, Skp1 (Fig. 2G).
235

236 **Frequent adaptive evolution and co-adaptative evolution in genes involved in**
237 **reproduction, immune system and environmental information processing**
238 To find out whether specific biological functions were associated with fast-adaptive genes, we
239 applied DAVID Go analysis with genes that have largest ~15% rates of adaptation ($\omega_a > 0.15$).
240 The significant Go terms are frequently linked to serine-type endopeptidase activity,
241 reproduction, protein lysis, chemosensory and other related biological functions (Table S3). As
242 these fast-adaptive genes tend to be enriched in similar biological functions, we asked whether
243 these genes are evolved co-adaptively, i.e., whether these proteins are interacting with each
244 other frequently. To test this possibility, we obtained PPI of *D. melanogaster* from STRING
245 database (Szklarczyk et al., 2019) and analyzed protein-protein interactions among fast-
246 adaptive proteins. We found that fast-adaptive proteins tend to interact with each other more
247 frequently than expected (PPI enrichment p-value < 1.0e-16). In the PPI network of fast-
248 adaptive proteins, we observed 7 strongly connected sub-clusters with at least 5 members (Fig.
249 3A, Table S4). Proteins in these sub-clusters are enriched in biological processes such as
250 reproduction, immune response, defense response to bacterium and virus, RNA interference,
251 chitin metabolic, etc., which are in line with the Go analysis of fast-adaptive genes (Table S5-
252 S10).
253 We next asked whether co-adaptation plays a role in the adaptive evolution of interacting
254 proteins to a broader extend, including both fast- and slow-adaptive proteins. To address this
255 question, we analyzed and compared adaptation rates of all PPIs available in STRING database
256 with high confidence in *D. melanogaster* and we found that protein partners of fast-adaptive
257 proteins (ω_a>0.15) have significantly larger maximum/average ω_a compared to slow-adaptive
258 proteins (Figure 4). We further analyzed and visualized adaptive evolutionary rates of proteins in
259 PPI networks of 9 different biological pathways extracted from KEGG pathways, including
260 immune system, xenobiotics biodegradation, response to environment, aging and development,
261 genetic information processing, sensory system, transport and catabolism, cell growth and
262 death and metabolism. We observed that, in these PPI networks, proteins with relatively large
263 ω_a tend to interact with each other (Figure S5A, S5B). We also noticed that, for pathways that
264 are previously known as adaptation-hotspots, e.g., immune system, fast-adaptive proteins can
265 act as central nodes and are co-adaptively evolved with other fast-adaptive proteins (Figure
266 S5C). While in pathways such as transport and catabolism, fast-adaptive proteins are mainly at
267 PPI periphery. In line with these findings, we found that ω_a are larger in pathways that harbor
268 fast-adaptive proteins as central nodes than other pathways (Figure S6).

Physical interactions contribute to co-adaptation of fast-adaptive genes. Having established that molecular interactions contribute to adaptive evolution of protein sequence, we then investigated whether these physical molecular interactions could drive protein-protein co-adaptation. To do this, we looked into interacting fast-adaptive protein pairs that are associated known or inferred complex structural models. For inferred complex structural models, we superimposed the structural models of the pair of proteins onto their high resolution homologous complex structures. Here we observed and illustrated co-adaptation at PPI interface in two examples: Toll-4/Spatzle and Spn28Db/CG18563 (Figure 3).

Toll-4/Spatzle. Toll-4 is a member of toll-like receptors. Previous studies have shown strong evidence of adaptive evolution of Toll-4 in *Drosophila* and mammals (Levin and Malik, 2017; Slodkowicz and Goldman, 2020). Toll-4 can bind to Spatzle and trigger further innate immune responses with high confidence (inferred from STRING database). In the previous section, we showed that several positively selected sites in Spatzle overlap with Toll-Spatzle interfaces. Here, we further showed that, in Toll-4, considerable number of significant positively selected sites were located at interface for Spatzle (Fig. 3B), which is in line with a previous study of Toll-4 in *D. willistoni* (Levin and Malik, 2017).

Spn28Db/CG18563. Spn28Db is one of the serine protease inhibitors in *D. melanogaster* that are expressed in male accessory glands, while CG18563 belongs to the protein family of trypsin-like cysteine/serine peptidase domain. The interactions between the two proteins were predicted with high confidence from STRING database, and the molecular interactions can be inferred from existing crystal structure of serpin and bacteria protease complex (PDB code 1EZX). We observed many positive selected sites at the molecular interface between the two proteins (Fig. 3C), suggesting that physical interactions might play a role in the co-adaptation of the two proteins.

Most clinally differentiated SNPs in protein-coding genes are adaptive

To find out the relations between short-term adaptation to local environments and long-term adaptive evolution, we extracted residues with significant F_{ST} SNPs from clinal variations (Svetec et al., 2016). We then computed evolutionary rates (ω), adaptation rates (ω_a) and non-adaptation rates (ω_{na}) of these residues as in previous section. We observed that these residues have much higher ratio of adaptation rates over non-adaptation rates than genome-wide random expectations (Figure 5), suggesting that these residues have higher proportions of adaptive changes, and that they can be hotspots for adaptive evolution. To further characterize structural and functional properties of short-term genetic variations, we mapped significant

303 nonsynonymous F_{ST} residues to different structural and functional characteristics, such as ISD,
304 RSA, PPI-sites, DNA-sites and ligand-binding sites. We found that these nonsynonymous SNPs
305 follow the patterns of adaptive changes. For example, they were enriched disordered regions
306 and protein surfaces, and were significantly more likely to be involved in protein-protein
307 interactions and ligand-binding than expectation (Table S11-S15).
308

309 **Discussion**

310 In this study, we systematically studied the impact of structure- and function-related gene
311 properties on protein sequence evolution and adaptation in *D. melanogaster* genome. We found
312 that, compared to protein structure-related properties, such as intrinsic structural disorder (ISD)
313 and relative solvent accessibility (RSA), function-related properties, such as tissue specificity
314 and male specificity, contribute more extensively to protein sequence adaptive evolution.
315 Especially, we noticed that molecular interactions in proteins contribute to the variation of
316 protein sequence adaptive evolution. In line with this result, we detected that molecular
317 interaction sites are hotspots for adaptative evolution. We confirmed that proteins that are fast
318 adaptive are enriched in GO terms that are associated reproduction, immunity and
319 environmental information processing. Furthermore, we revealed that fast-adaptive proteins
320 tend to interact with each other frequently and protein partners of these fast-adaptive proteins
321 tend to have higher adaptation rates, suggesting that co-adaptive evolution might be common in
322 *D. melanogaster*. By looking at interacting fast-adaptive proteins, we further demonstrated that
323 physical interactions may contribute to the mechanisms of co-adaptative evolution of fast-
324 adaptive proteins.

325 Extensive studies have been conducted to uncover the main drivers that govern protein
326 sequence evolutionary rate (Zhang and Yang, 2015). Gene expression level was proved to be a
327 major determinant (Zhang and Yang, 2015) through mechanisms such as the pressure for
328 translational robustness, i.e., robustness to translational missense errors (Drummond et al.,
329 2005). Here, we showed that caveat exists when we looked at gene expression levels in male
330 *D. melanogaster*. Previous studies have revealed that male biased or female biased genes can
331 be fast evolving (Yang et al., 2016). On the other hand, many male biased genes can be highly
332 expressed in testis, which results in a complex correlation between protein sequence
333 evolutionary rate and male expression level or even mean expression level of *D. melanogaster*.
334 The unique evolutionary property of these male biased or specific genes could be caused by the
335 unique transcriptional scanning mechanism in testis (Xia et al., 2020). We propose that tissue
336 specificity might be a better quantity when considering the impact of gene expression profile on

337 protein sequence evolution in *D. melanogaster*. In addition to male expression level, a similar
338 complex correlation was observed for protein length. It has been the notion that short proteins
339 tend to evolve faster than long proteins, which may be biologically relevant or byproduct of other
340 factors such as selection on buried and exposed sites (Moutinho et al., 2019). Here, we
341 demonstrated that, in *D. melanogaster*, although protein length is strongly negatively correlated
342 with protein sequence evolutionary rate, genes that have the slowest evolutionary rates tend to
343 be relatively short. This could be caused by the fact that under essential functional constraint,
344 genes can undergo strong purifying selections, while essential genes such as secreted proteins
345 are constrained to be smaller, and that essential genes could be shorter than other genes (Chen
346 et al., 2020).
347 It has been recognized that protein surface and intrinsic disorder regions are frequent
348 targets for adaptive evolution and contribute to the variations of protein sequence adaptive
349 evolution (Afanasyeva et al., 2018; Moutinho et al., 2019). However, the detailed mechanisms
350 underlying these observations remains unclear. One possible explanation would be that these
351 regions are frequently linked to intermolecular interactions (Afanasyeva et al., 2018; Moutinho et
352 al., 2019). For example, Moutinho et al hypothesized that molecular interactions involved in
353 host-pathogen coevolution were the major driver of protein adaptation (Moutinho et al., 2019).
354 Here, we further identified that proportions of possible molecular interaction sites inside proteins
355 contribute to the variations of protein sequence adaptive evolution and that these molecular
356 interaction sites or regulatory sites at protein surface can be hotspots of protein adaptation.
357 Indeed, some specific molecular interactions have been linked to adaptive evolution in several
358 case studies (Bachtrog, 2008; Hughes and Nei, 1988; Levin and Malik, 2017; Schott et al.,
359 2014) and large-scale studies based on proteins with high quality structural models (Slodkowicz
360 and Goldman, 2020). In the latter study, the authors showed that positive selections in
361 mammals tend to cluster closer to binding sites of exogenous ligands than expected by chance
362 (Slodkowicz and Goldman, 2020), suggesting an important role of function important regions in
363 adaptive evolution. Here, we extend the conclusion *to D. melanogaster* genome, including
364 proteins with or without high resolution structural models. We also showed that except for
365 exogenous ligands, endogenous ligands might also contribution to adaptive evolution, while the
366 latter might explain why interacting proteins tend to evolve co-adaptively.
367 Notably, previous studies have revealed that multi-interface proteins tend to be evolving
368 more slowly than single-interface proteins (Kim et al., 2006), which seems to be contradictory to
369 our results that proteins with more interaction sites evolve faster and have faster adaptation
370 rates. Here, we argue that, in our study, we used sequence profile to predict molecular

371 interaction sites in proteins at a genomic scale, rather than only looking into proteins with high
372 resolution structures. In this way, we may capture many weak or transient interactions, which
373 are thought to be evolving faster than obligate and conserved interactions (Mintseris and Weng,
374 2005). Meanwhile, we did not exclude intrinsic disordered regions (IDR) or intrinsic disordered
375 proteins (IDP) in our study, which are widespread in *D. melanogaster* genome. It has been
376 suggested that IDR/IDP tend to evolve fast due to lack of structural restraints (Echave et al.,
377 2016). In the functional aspect, IDR/IDP are thought to be promiscuous binders through many
378 multiple binding mechanisms, including forming static, semi-static, and fuzzy or dynamic
379 complexes (Uversky, 2019), suggesting that the evolution of IDR/IDP cannot be explained
380 merely by the lack of structural restraints. Actually, IDP and IDR in human genome were found
381 to be undergoing extensive adaptive evolution (Afanasyeva et al., 2018). At last, it has been
382 recognized that, except for allosteric regulations, encounter complexes (Gabdoulline and Wade,
383 1999) might also play an important role in mediating intermolecular interactions, such as
384 protein-protein association (Tang et al., 2006) and protein-ligand binding (Re et al., 2019). Since
385 encounter residues that are responsible for encounter complexes do not reside in conserved
386 binding interfaces, these residues could be under relaxed purifying selections or even positive
387 selections, which could be another yet-to-identify mechanism that contribute to protein
388 sequence adaptive evolution.
389 In consistent with previous studies in *D. melanogaster* (Begun and Lindfors, 2005;
390 Begun and Whitley, 2000; Lazzaro et al., 2004), we showed that fast-adaptive proteins are
391 enriched in molecular functions such as reproduction, immunity and environmental information
392 processing. We further demonstrated that fast-adaptive proteins tend to interact with each other
393 more frequently than random expectations, suggesting co-adaptation might be common among
394 fast-adaptive proteins. Mechanisms that contribute to the co-adaptation could be: (1) interacting
395 fast-adaptive proteins are often enriched in similar molecular functions and under similar
396 selective pressure; (2) interacting fast-adaptive undergo co-evolution through physical
397 interactions. In this study we showed two examples that adaptive evolution could occur at
398 protein-protein interface, which suggest that physical interactions could contribute to the co-
399 adaptation of fast-adaptive proteins in *D. melanogaster*. Moreover, we showed that co-
400 adaptation might exist to a broader extend rather than only among fast-adaptive proteins.
401 Specifically, proteins that interact with fast-adaptive proteins tend to have higher adaptation
402 rates. Since molecular interactions contribute to adaptive evolution, it is reasonable to
403 hypothesize that co-adaptation at this broader extend could be regulated by these interactions.
404 Actually, it has been suggested that interacting proteins tend to have similar evolutionary rates

405 and the possible mechanism would be the co-evolution of physical interactions (Pazos and
406 Valencia, 2008).
407 It has been suggested that populations in different local environments can have genetic
408 variances that result in local adaptations. In this study, we found that loci with significant genetic
409 variance among populations harbor higher proportions of long-term adaptive changes and these
410 loci follow similar patterns as adaptive changes, i.e. they are enriched in disordered regions,
411 protein surfaces, and functionally important regions. These results suggest that population
412 differentiation of protein-coding genes can be an important basis for long-term adaptive
413 evolution. Importantly, our results indicate that most of the clinal amino-acid changes are
414 adaptive, suggesting that non-selective forces play a non-essential role in the SNPs that show
415 strong geographic differences. Our results also support a large effect of spatially varying
416 selection on protein sequence and structures (Storz and Kelly, 2008).
417 It should be noted that studies at the genomic scale that aim to uncover the function- or
418 structure-related constraints imposed on protein sequence evolution and adaptation share
419 similar limitations that for most of the proteins or residues, structural or functional information
420 would be incomplete or even missing. Thus, in this study, we used highly accurate neural-
421 network based tools to predict molecular interactions, secondary structures, intrinsic structural
422 disorder, relative solvent accessibility for each of the protein in *D. melanogaster* genome. In this
423 way we were able to identify key factors that impact protein sequence evolution and adaptation
424 in a less accurate but rather systematic fashion. We hope that with the availability of more and
425 more curated structural, functional information and complex structural models of proteins in the
426 near future, we will be able to uncover the precise role of molecular interactions in protein
427 sequence adaptive evolution.
428
429 **Material and Methods**
430 ***d_N/d_S* ratio (ω).** We used a maximum likelihood method to infer d_N/d_S ratio (ω) of *D.*
431 *melanogaster* protein-coding genes using the genome sequences of five species in
432 *melanogaster* subgroup (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, and *D. erecta*).
433 The protein-coding sequences were extracted from the alignments of 26 insects, which were
434 obtained from UCSC Genome Browser (http://hgdownload.soe.ucsc.edu/downloads.html). The
435 sequences were further processed by GeneWise (Birney et al., 2004) to remove possible
436 insertions and deletions using the longest isoforms of the corresponding *D. melanogaster*
437 protein sequences as references (FlyBase version r6.15) (Thurmond et al., 2019). The
438 processed sequences were then realigned by PRANK -codon function (Löytynoja, 2014). We

439 used codeml in PAML (Yang, 2007) to compute gene-specific ω using M0 model. We removed
440 sequences that have more than 15% of their nucleotides not aligned (gaps) to *D. melanogaster*
441 genes in more than 2 species. To further avoid numeric errors and ensure reasonable
442 estimations, we only retained relatively divergent sequences that are: (1) divergent with dS
443 larger than 0.3, (2) less divergent with dS larger than 0.1 and dN smaller than 0.001 (dS>>dN).
444 At last, there were 12118 genes in total passed all the criteria and were assigned gene specific
445 ω, containing 6,538,872 amino acids. We also calculated site-specific ω by using likelihood ratio
446 tests (LRT) comparing M7 model against M8 model (Yang et al., 2005).
447 ***Rate of adaptive and nonadaptive changes.*** We recalled all SNPs of 205 inbred lines from
448 the Drosophila Genetic Reference Panel (DGRP), Freeze 2.0 (Huang et al., 2014)
449 (http://dgrp2.gnets.ncsu.edu). We then generated 410 alternative genomes using all monoallelic
450 and bi-allelic SNP data sets. We extracted the coding sequences of *D. melanogaster* genes
451 from the generated alternative genomes, removed all possible insertions and deletions using
452 GeneWise (Birney et al., 2004) as described above. We then align all the coding sequences to
453 their corresponding aligned CDS sequences using PRANK -codon function (Löytynoja, 2014).
454 We removed polymorphisms segregating at frequencies smaller than 5% to reduce possible
455 slightly deleterious mutations (Charlesworth and Eyre-Walker, 2008). In order to avoid possible
456 effects of low divergence between *D. simulans* and D melanogaster (Keightley and Eyre-
457 Walker, 2012), we used *D. yakuba* as outgroup to estimate nonsynonymous polymorphisms
458 (Pn), synonymous polymorphisms (Ps), nonsynonymous substitutions (Dn) and synonymous
459 substitutions (Ds) by MK.pl (Begun et al., 2007; Langley et al., 2012). Similar as Begun et al.
460 (Begun et al., 2007), we only analyzed genes with at least six variants for each of substitutions,
461 polymorphisms, nonsynonymous changes and synonymous changes. We used an extension of
462 MK test, asymptotic MK (Messer and Petrov, 2013; Uricchio et al., 2019), to estimate the
463 proportions of adaptive changes (α). The rate of adaptive changes (ω_a) was then calculated as
464 $\omega_a = \omega\alpha$ and the rate of non-adaptive changes as $\omega_{na} = \omega - \omega_a$. Details of the asymptotic MK
465 test were as following:
466 (1) Classical McDonald–Kreitman test. According to Smith and Eyre-Walker (Smith and Eyre-
467 Walker, 2002), the proportions of adaptive changes for protein-coding genes can be calculated
468 as following:

469 $$\alpha = 1 - \frac{DsPn}{DnPs}$$

470 According to this equation, we could estimate the proportion of adaptive changes and carried
471 out classical MK test by applying Fisher's exact test.

472 (2) Asymptotic estimation of α. A known problem of the classical estimation of α above is the
473 accumulation of slightly deleterious mutations at low frequencies. We therefore used an
474 extension of MK test, asymptotic MK test approach (Messer and Petrov, 2013) to estimate the
475 proportions of adaptive changes. As in original aMK, we defined α(x) as a function of derived
476 allele frequency (x):

477 $$\alpha(x) = 1 - \frac{DsPn(x)}{DnPs(x)}$$

478 where Pn(x) and Ps(x) are number of non-synonymous and synonymous polymorphisms at
479 frequency x, respectively. However, the original approach may suffer from numeric errors when
480 there were very few polymorphic sites, which is quite common in many of *D. melanogaster*
481 genes. To make the estimations more robust while preserving the same asymptote, we further
482 define Pn (x) and Ps(x) as total number of Pn and Ps above frequency x as described in
483 Uricchio et al (Uricchio et al., 2019). We fitted α(x) to an exponential curve of α(x) ≈ exp(-bx)+c
484 using lmfit (Newville et al., 2014) and determined the asymptotic value of α at the limit of x, 1.0.
485 We then estimate the rate of adaptive changes (ω_a) as

486 $$\omega_a = \frac{N_a/L_N}{dS} = \frac{dN_a}{dS} = \frac{dN_a}{dN} \cdot \frac{dN}{dS} = \alpha\omega$$

487 where N_a is the number of adaptive changes and $dN_a=N_a/L_N$ is the number of adaptive changes
488 per nonsynonymous site. Finally, we calculated the rate of nonadaptive changes (ω_{na}) as
489 $\omega_{na}=\omega-\omega_a$. The final dataset contains 7192 protein-coding genes, with smallest ω_a being 0.00
490 and largest being 1.29.

491 ***Structure-/function- related properties of D. melanogaster proteins.*** We obtained function-
492 related properties mentioned in main text as following. We derived *D. melanogaster* gene ages
493 (Kondo et al., 2017; Zhang et al., 2010) for genes that are specific to *Drosophila*, and from
494 GenTree (Shao et al., 2019) for genes that are beyond *Drosophila* clade. We then assigned a
495 pseudo-age to each of the genes. Specifically, there are 11 age groups from "cellular
496 organisms", assigning to a pseudo age value of 0, to "melanogaster", assigning a pseudo age
497 value of 10. We downloaded *D. melanogaster* protein-protein interaction (PPI) from STRING
498 database (Szklarczyk et al., 2019). A cut-off of combined score larger than 0.7 was used to
499 retain high confident PPI for further analysis. We then used BSpred (Mukherjee and Zhang,
500 2011) to predict protein-protein interaction (PPI) sites and DRNApred (Yan and Kurgan, 2017)
501 to predict DNA binding sites. For each protein, we calculated ratios of protein interaction
502 residues (PPI-site ratio) and ratios of DNA binding residues (DNA-site ratio) by dividing total
503 predicted protein interaction sites and DNA binding sites over protein length, respectively. For
504 structure-related properties, we used DeepCNF (Wang et al., 2016) to predict these properties

505 for each gene, including three-state secondary structures (helix, sheet, and coil), structural
506 disorder, relative solvent accessibility (RSA). Further, we calculated the ratios of helix, sheet,
507 helix+sheet, and coil residues of each gene from predicted secondary structures. For each
508 gene, we computed intrinsic structural disorder (ISD) and relative solvent accessibility (RSA), as
509 protein-length normalized summations of the probabilities of each residue being disorder and
510 exposed, respectively.

511 ***Gene expression patterns.*** We downloaded gene expression profile from FlyAtlas2 (Leader et
512 al., 2018). We converted FPKM to TPM by normalizing FPKM against the summation of all
513 FPKMs as following:

514 $$\mathrm{TPM_i} = \frac{\mathrm{FPKM_i}}{\sum \mathrm{FPKM_j}} \times 10^6$$

515 After TPM conversion, we only retained genes with expression level larger than 0.1 TPM for
516 further analysis. We treated male and female whole-body TPM as male and female expression
517 levels. We calculated mean expression level by averaging male and female TPM. We used
518 following Z-score to describe male specificities of *D. melanogaster* genes:

519 $$zscore = \frac{TPM(male\ expression) - TPM(female\ expression)}{\sqrt{sd^2(male\ expression) + sd^2(female\ expression)}}$$

520 We calculated tissue specificities of genes using tau values (Yanai et al., 2005) based on the
521 expression profiles of 27 different tissues.

522 ***High quality 3D structures of D. melanogaster proteins.*** We downloaded high-quality
523 structures or structural models of *D. melanogaster* proteins from protein data bank (PDB)
524 (Burley et al., 2019), SWISS-MODEL Repository (Bienert et al., 2017), and MODBASE (Pieper
525 et al., 2011), with descending priorities. For example, if there were 3D structures of a same
526 protein or protein region in multiple databases, we first considered high-resolution structures
527 from PDB; if no structures were found in PDB, we then considered SWISS-MODEL Repository;
528 and at last from MODBASE. In addition, we used blastp (Camacho et al., 2009) to search
529 homologs of each *D. melanogaster* protein against all PDB sequences with E-value threshold of
530 0.001. We further carried out comparative structural modeling using RosettaCM (Song et al.,
531 2013) to model high-quality structural models of proteins or protein regions that were not
532 available in PDB, SWISS-MODEL Repository and MODBASE. For each RosettaCM simulation,
533 we used no more than 5 most significant hits from blastp search. For proteins that are in
534 complex forms, we only extracted monomers for further analysis. At last, we obtained 14543

535 high quality structural models, corresponding to 11284 genes. These structural models contain
536 2,691,913 unique amino acids, 41.2% of all the residues in genes that were assigned ω.
537 ***Evolutionary rates of different structural/functional sites.*** We classified amino acids into
538 different classes of structural/functional properties. Specifically, we classified three classes for
539 both ISD and RSA according the probability of residues being disordered or exposed: ordered
540 or buried (0.00 to 0.33), medium (0.33 to 0.67), disordered or exposed (0.67 to 1.00). For both
541 PPI and DNA binding, we classified two classes: PPI-site or DNA-site (binding sites), None-PPI
542 or None-DNA (corresponding null sites for PPI or DNA binding). For residues that have 3D
543 structures, we used STRESS (Clarke et al., 2016) to predict putative ligand binding sites and
544 allosteric sites from all the high-quality structures or structural models. The allosteric sites were
545 further classified as surface critical or interior critical according to their locations. We then
546 classified these residues into four groups: LIG (ligand binding sites), Surf. Crit. (surface critical
547 sites), Interior Crit. (interior critical sites) and Others (other sites). For each of the site classes,
548 we randomly sampled 1,00 sequences, each containing 10,000 amino acids. We computed ω,
549 ω_a, and ω_{na} for the randomly sampled sequences similar as the steps described in the above
550 sections.
551
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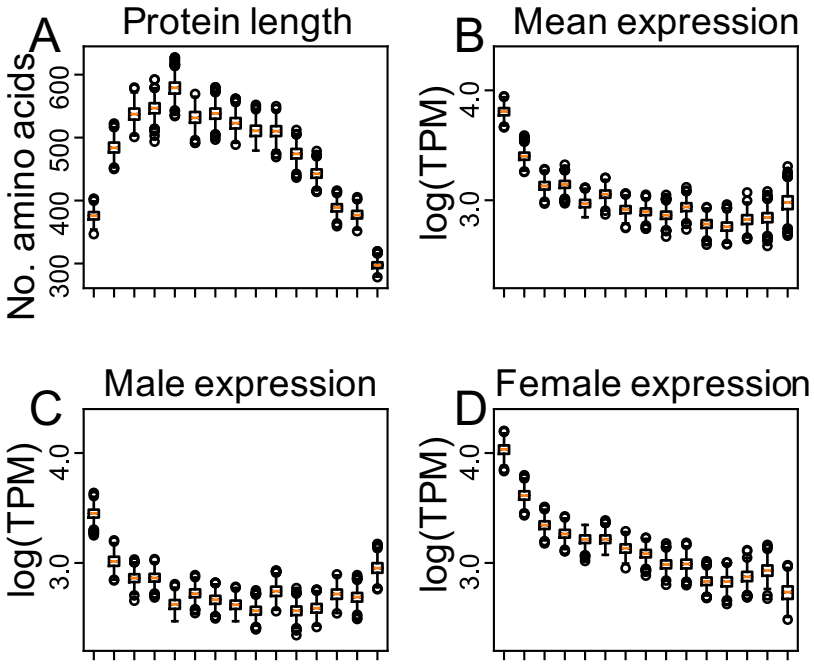

Figure 1. Protein length (A), mean (B), male (C) and female expression (D) levels in each gene groups divided by ascending ω values. Values for each gene group and each gene property were computed through 1,000 bootstrapping steps. Obvious complex U-shaped correlations with ω were observed for protein length (A) and male expression level (C).

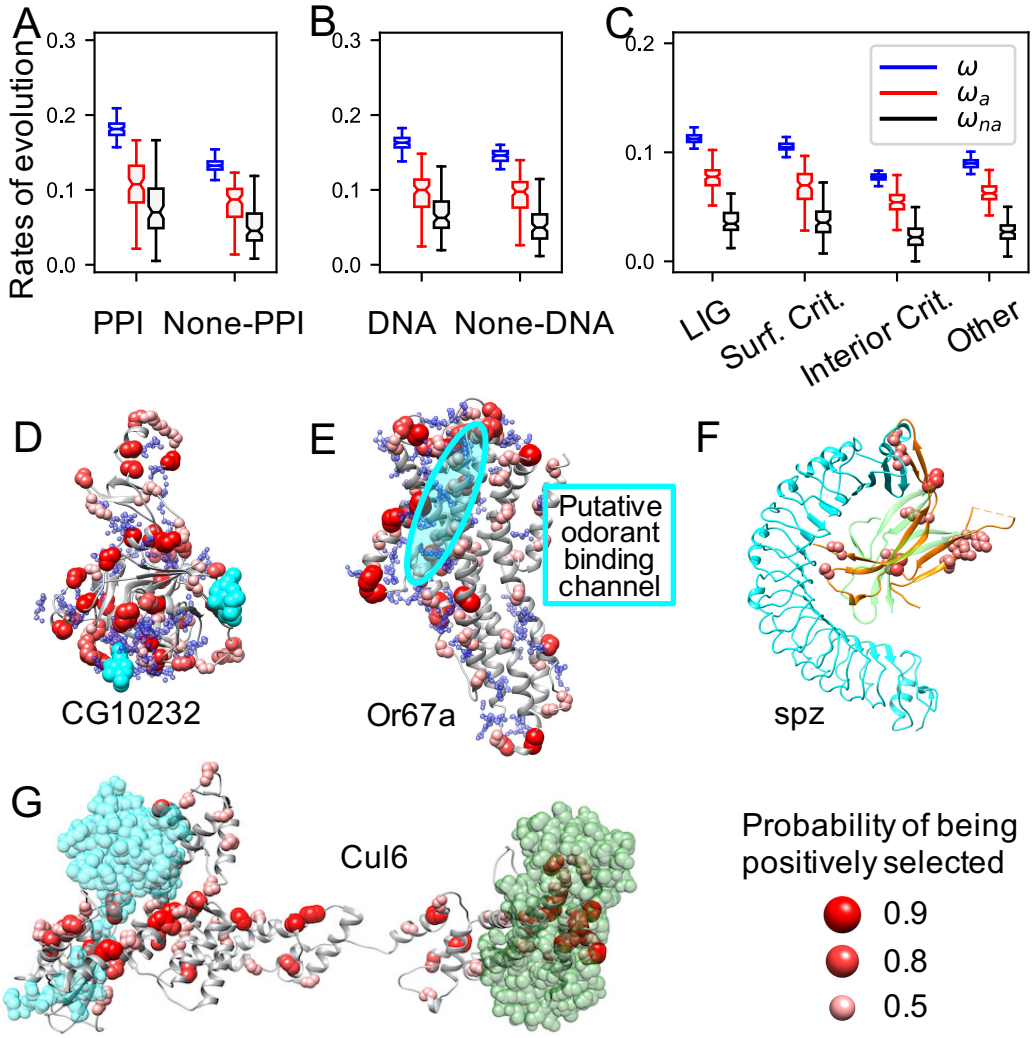

Figure 2. Adaptive evolution in molecular interaction sites. Protein-protein interaction sites (A), DNA binding sites (B) and putative ligand binding sites (C) show higher adaptation rates than none binding sites. Examples of positive selection around molecular interaction sites in high quality structural models of CG10232 (D), Or67a (E), spz (F), and Cul6 (G). Except for spz (PDB code 3e07), the other proteins are obtained from SWISS model repository. Putative ligand binding pockets of CG10232 (D) and Or67a (E) are shown in blue spheres. Ligands including interacting proteins are shown in cyan or green: NAG of CG10232 in cyan (D), Toll receptor of spz in cyan (F), RING-box protein in cyan and F-box protein in green for Cul6 (G). The putative odorant binding channel of Or67a is highlighted in cyan circle (E). The ligand poses in (D, F and G) are obtained by superimposition from structure 2XXL, 4BV4 and 1LDK, respectively.

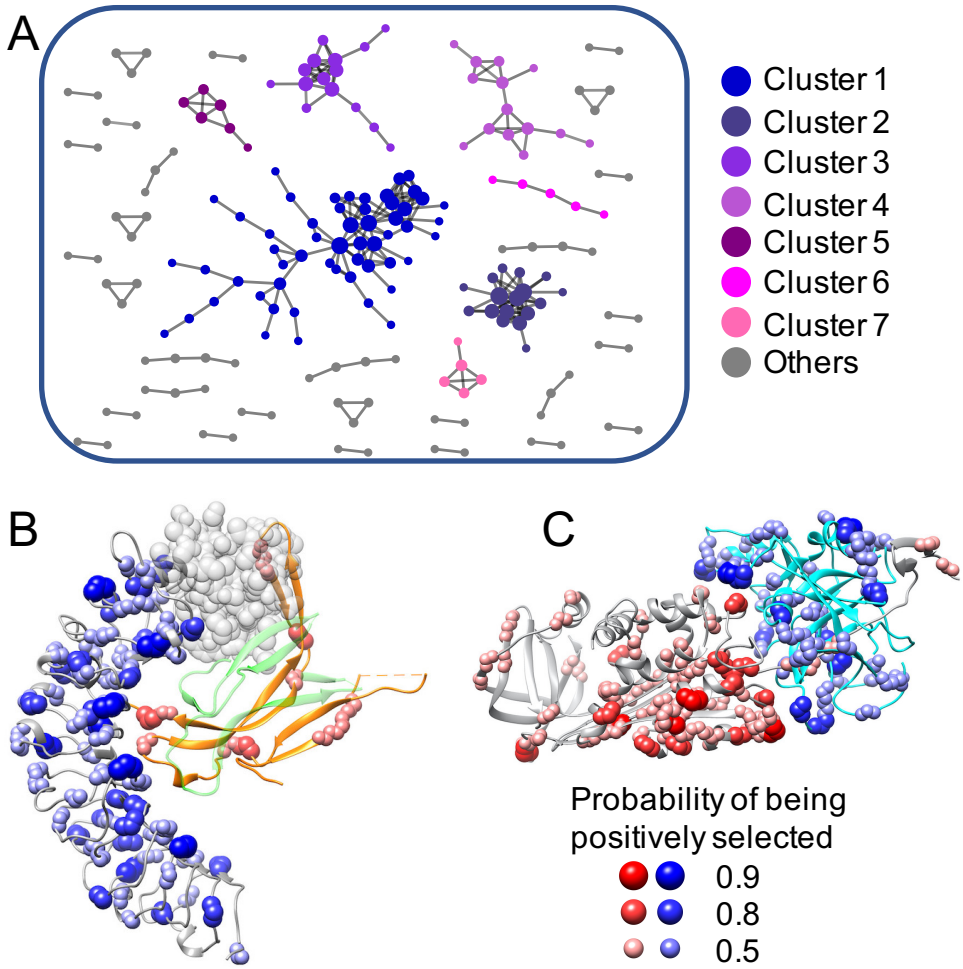

Figure 3. Co-adaptation of fast-adaptive proteins. (A) Sub-clusters of PPI networks of fast-adaptive proteins. Only proteins with at least one partner were shown. Examples of molecular interactions that might regulate co-adaptation in fast-adaptive proteins: (B) Toll-4 (gray) and spz (orange, with green representing the other spz monomer), (C) Spn28Db (gray, serine protease inhibitor 28Db) and CG18563 (cyan, with Go term “serine-type endopeptidase activity”). A putative N-terminus (transparent beads) of Toll-4 were built by superimposition from 4LXR, since the N-terminus were missing in the structural model. Complex structural model of Spn28Db and CG18563 was inferred from 1EZX.

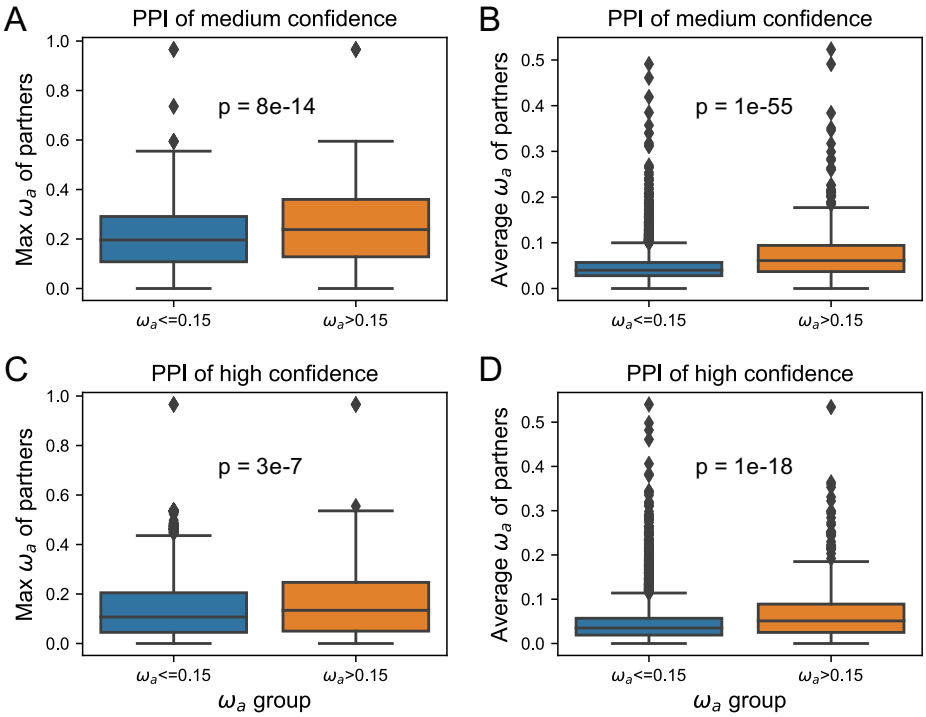

Figure 4. Co-adaptation of PPIs in *D. melanogaster*. For fast-adaptive proteins, adaptation rates of their partners (orange box plot) are significantly larger compared to slow adaptive proteins (blue box plot). Max ω_a of protein partners are shown in (A and C) and averaged ω_a, of protein partners are shown in (B and D). PPI from STRING with median confidence (combined score larger than 0.4) are shown in (A and B), and PPI with high confidence (combined score larger than 0.7) are shown in (C and D).

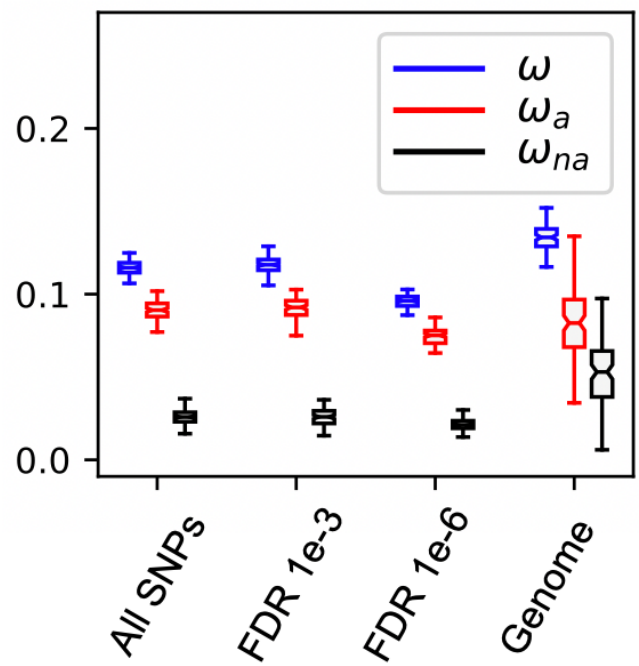

Figure 5. Adaptive evolution in FST SNPs. The significant SNPs at different FDR cutoffs all show much higher proportions of adaptation than genome-wide expectation.

Table 1. Pearson correlation coefficients between ω, ω_a and ω_{na} and gene properties [a]

Categories	Properties	ω		ω_a		ω_{na}	
Function-related properties	Gene age	0.55	(0)	0.34	(7e-154)	0.41	(2e-224)
	Protein length [b]	-0.22	(2e-136)	-0.13	(1e-25)	-0.30	(2e-142)
	Mean expression [b]	-0.21	(2e-109)	-0.11	(2e-18)	-0.11	(1e-18)
	Male expression [b]	-0.06	(5e-10)	0.00	(8e-1)	-0.03	(6e-2)
	Female expression [b]	-0.29	(2e-205)	-0.17	(2e-42)	-0.16	(3e-35)
	Male specificity	0.21	(2e-104)	0.12	(3e-22)	0.10	(3e-14)
	Tissue Specificity	0.30	(4e-226)	0.18	(1e-45)	0.19	(3e-48)
	PPI number [b]	-0.28	(1e-217)	-0.14	(4e-29)	-0.19	(4e-58)
	PPI-site ratio	0.14	(1e-50)	0.05	(7e-6)	0.10	(2e-16)
	DNA-site ratio	0.25	(8e-164)	0.12	(3e-23)	0.23	(3e-79)
Structure-related properties	Helix ratio	-0.05	(4e-7)	-0.01	(3e-1)	-0.05	(4e-5)
	Sheet ratio	-0.04	(2e-5)	0.00	(9e-1)	-0.01	(3e-1)
	Helix+sheet ratio	-0.09	(3e-25)	-0.02	(1e-1)	-0.07	(1e-9)
	Coil ratio	0.10	(8e-27)	0.01	(2e-1)	0.08	(8e-11)
	ISD	0.17	(8e-82)	0.04	(1e-3)	0.12	(2e-24)
	RSA	0.16	(7e-87)	0.06	(1e-6)	0.15	(3e-35)
Protein evolution	ω	1.00	(0)	0.65	(0)	0.78	(0)
	ω_a	0.65	(0)	1.00	(0)	0.03	(7e-3)
	ω_{na}	0.78	(0)	0.03	(7e-3)	1.00	(0)

[a] Pearson correlation coefficient R were listed along with corresponding P-values in parentheses.

790 [b] To better estimate the correlations for sequence length, expression levels and PPI numbers,
791 we used logarithmic scales rather than absolute values, which could vary dramatically from near
792 zero to thousands.
793 Abbreviations in this table: ISD, intrinsic structural disorder; RSA, relative solvent accessibility;
794 PPI number, protein-protein interaction number; PPI-site ratio, ratio of protein-protein interaction
795 sites; DNA-site ratio, ratio of DNA-binding sites.
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