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2 **Tissue-specific versus pleiotropic enhancers within the *bric-a-brac* tandem gene duplicates**
3 **display differential regulatory activity and evolutionary conservation**

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23 Running title: Differential enhancer conservation and activity within the *bric-a-brac* locus

24

25 **Abstract**

26 During animal evolution, de novo emergence and modifications of pre-existing transcriptional
27 enhancers have contributed to biological innovations, by implementing gene regulatory
28 networks. The *Drosophila melanogaster bric-a-brac (bab)* complex, comprising the tandem
29 paralogous genes *bab1-2*, provides a paradigm to address how enhancers contribute and co-
30 evolve to regulate jointly or differentially duplicated genes. We previously characterized an
31 intergenic enhancer (named LAE) governing *bab2* expression in leg and antennal tissues. We
32 show here that LAE activity also regulates *bab1*. CRISPR/Cas9-mediated LAE excision reveals
33 its critical role for *bab2*-specific expression along the proximo-distal leg axis, likely through
34 paralog-specific interaction with the *bab2* gene promoter. Furthermore, LAE appears involved
35 but not strictly required for *bab1-2* co-expression in leg tissues. Phenotypic rescue experiments,
36 chromatin features and a gene reporter assay reveal a large “pleiotropic” *bab1* enhancer (termed
37 BER) including a series of *cis*-regulatory elements active in the leg, antennal, wing, haltere and
38 gonadal tissues. Phylogenomics analyses indicate that (i) *bab2* originates from *bab1* duplication
39 within the Muscomorpha sublineage, (ii) LAE and *bab1* promoter sequences have been
40 evolutionarily-fixed early on within the Brachycera lineage, while (iii) BER elements have been
41 conserved more recently among muscomorphans. Lastly, we identified conserved binding sites
42 for transcription factors known or prone to regulate directly the paralogous *bab* genes in diverse
43 developmental contexts. This work provides new insights on enhancers, particularly about their
44 emergence, maintenance and functional diversification during evolution.

45 **Author summary**

46 Gene duplications and transcriptional enhancer emergence/modifications are thought having
47 greatly contributed to phenotypic innovations during animal evolution. However, how
48 enhancers regulate distinctly gene duplicates and are evolutionary-fixed remain largely
49 unknown. The *Drosophila bric-a-brac* locus, comprising the tandemly-duplicated genes *bab1-*
50 *2*, provides a good paradigm to address these issues. The twin *bab* genes are co-expressed in
51 many tissues. In this study, genetic analyses show a partial co-regulation of both genes in the
52 developing legs depending on tissue-specific transcription factors known to bind a single
53 enhancer. Genome editing and gene reporter assays further show that this shared enhancer is
54 also required for *bab2*-specific expression. Our results also reveal the existence of partly-
55 redundant regulatory functions of a large pleiotropic enhancer which contributes to co-regulate
56 the *bab* genes in distal leg tissues. Phylogenomics analyses indicate that the *Drosophila bab*
57 locus originates from duplication of a dipteran *bab1*-related gene, which occurred within the
58 Brachycera (true flies) lineage. *bab* enhancer and promoter sequences have been differentially-
59 conserved among Diptera suborders. This work illuminates how transcriptional enhancers from
60 tandem gene duplicates (i) differentially interact with distinct cognate promoters and (ii)
61 undergo distinct evolutionary changes to diversifying their respective tissue-specific gene
62 expression pattern.

63

64 **Introduction**

65 Gene duplications have largely contributed to create genetic novelties during evolution (1, 2).
66 Intra-species gene duplicates are referred to as “paralogs”, which eventually diverged
67 functionally during evolution in a phylogenetic manner. Gene family expansion has facilitated
68 phenotypic innovation through (i) acquisition of new molecular functions or (ii) the subdivision
69 of the parental gene function between the duplicate copies (3-5). Phenotypic novelties are
70 thought having originated mainly from evolutionary emergence or modifications of genomic
71 Cis-Regulatory Elements (CREs) or modules, most often dubbed as “enhancer” regions, which
72 regulate gene transcription in a stage-, tissue- and/or cell-type-specific manner (6-10). How
73 CRE (enhancers) within gene complexes (i) are distinctly interacting with their cognate
74 promoters and (ii) are differentially (co-)evolving remain largely unknown.

75 The *Drosophila melanogaster bric-a-brac (bab)* locus comprises two tandemly-duplicated
76 genes (Fig 1A), *bab1-2*, which encode paralogous transcription factors sharing two conserved
77 domains: (i) a Bric-a-brac/Tramtrack/Broad-complex (BTB) domain involved in protein-
78 protein interactions, and (ii) a specific DNA-binding domain (referred to as BabCD, for Bab
79 Conserved Domain), in their amino(N)- and carboxyl(C)-terminal moieties, respectively (11).
80 Bab1-2 proteins are co-expressed in many tissues (11, 12). In the developing abdominal
81 epidermal cells, within so-called histoblast nests, they jointly regulate directly *yellow*
82 expression in a sexually-dimorphic manner, thus controlling adult male versus female body
83 pigmentation traits (13-16). *bab1-2* co-expression in the developing epidermal histoblast nests
84 is partially governed by two CREs which drive reporter gene expression (i) in a monomorphic
85 pattern in the abdominal segments A2-A5 of both sexes (termed AE, for “Anterior Element”),
86 and (ii) in a female-specific pattern in the A5-A7 segments (DE, for “Dimorphic Element”) (Fig
87 1A) (14, 17). In addition to controlling male-specific abdominal pigmentation traits, *bab1-2* are
88 required, singly, jointly or in a partially-redundant manner, for embryonic cardiac development,

89 sexually-dimorphic larval somatic gonad formation, salivary glue gene repression, female
90 oogenesis, wing development as well as distal leg (tarsal) and antennal segmentation (11, 13,
91 17-24). In addition to abdominal AE and DE, two other *bab* enhancers, termed CE and LAE
92 (see Fig 1A), have been characterized, which recapitulate *bab2* expression in embryonic cardiac
93 cells and developing tarsal as well as distal antennal cells, respectively (17, 21, 25).

94 Adult T1-3 legs, on the pro-, meso- and meta-thoraces, respectively, are derived from distinct
95 mono-layered epithelial cell sheets, organized as sac-like structures, called leg imaginal discs
96 (hereafter simply referred to as leg discs) (26-28). Upon completion of the third-instar larval
97 stage (L3), each leg disc is already patterned along the proximo-distal (P-D) axis through
98 regionalized expression of the Distal-less (Dll), Dachshund (Dac) and Homothorax (Hth)
99 transcriptional regulators in the distal (center of the disc), medial and proximal (peripheral)
100 regions, respectively (26). The five (ts1-5) tarsal and the single pretarsal (distalmost) segments
101 are patterned through genetic cascades mobilizing transcription factors, notably the distal
102 selector protein Dll and the tarsal Rotund protein as well as nuclear effectors of Notch and
103 Epidermal Growth Factor Receptor (EGFR) signaling, i.e., Bowl and C15, respectively (26,
104 27).

105 While both *bab* genes are required for dimorphic abdominal pigmentation traits and somatic
106 gonad specification (13, 22), only *bab2* is critical for tarsal segmentation (11). While *bab1* loss-
107 of-function legs are apparently wild-type, a null allele (*bab*^{AR07}) removing *bab2* (and *bab1*)
108 activities causes segmental transformation along the P-D leg axis, notably sex comb teeth in
109 tarsal segments ts2-3 of male forelegs, normally only found in ts1, as well as ts2-5 tarsal fusions
110 in both genders (11). While the two *bab* genes are co-expressed within ts1-4 cells, *bab2* is
111 expressed more proximally than *bab1* in ts1, and in a graded manner along the P-D leg axis in
112 ts5 (11, 29). We previously showed that *bab2* expression in distal leg (and antennal) tissues is
113 governed by a 567-bp-long CRE/enhancer (termed LAE for “Leg and Antennal Enhancer”)

114 which is situated between the *bab1-2* transcription units (Fig 1A) (17, 25). However, LAE
115 enhancer contribution to *bab1-2* co-regulation in the developing distal legs remains to be
116 investigated in tarsal segments ts3-4 where expression levels of both paralogous BTB-BabCD
117 proteins are the highest (see Fig 1B) (11).

118 Here, we show that *bab1* expression in the developing distal leg also depends on the Rotund,
119 Bowl and C15 proteins, three transcription factors known to regulate directly *bab2* expression,
120 by binding to dedicated LAE sequences (17, 25). LAE excision by CRISPR/Cas9-mediated
121 genome editing indicates that this enhancer is partly involved in *bab1-2* co-regulation and, more
122 unexpectedly, is also required for their differential expression along the P-D leg axis.
123 Additionally, we show that LAE acts redundantly with a large enhancer signature region
124 (termed BER), located within the *bab1* transcription unit, which is bound by dedicated
125 transcription factors involved in diverse developmental processes and thus BER is prone to act
126 as a “pleiotropic” enhancer region. Our phylogenomics analyses indicate that LAE and *bab1*
127 promoter sequences have been fixed early on during dipteran evolution, well before *bab1*
128 duplication. Conversely, BER and *bab2* promoter sequences have been fixed much later. Lastly,
129 within *D. melanogaster* BER, we identified conserved binding sites for many transcriptional
130 regulators known or prone to regulate *bab1* and/or *bab2* expression in the developing leg and
131 antenna, but also in wing, haltere, mesodermal and gonadal tissues. This work illuminates how
132 transcriptional enhancers from tandem gene duplicates (i) differentially interact with distinct
133 cognate promoters and (ii) undergo distinct evolutionary changes to diversifying their
134 respective tissue-specific gene expression pattern.

135

136 **Results**

137 **The tandem *bab1-2* gene paralogs are co-regulated in the developing distal leg**

138 In addition to the distal selector homeodomain (HD) protein Distal-less, we and others have
139 previously shown that the C15 HD protein (homeoprotein) as well as Rotund and Bowl Zinc-
140 Finger (ZF) transcription factors (TFs) bind dedicated sequences within LAE to ensure precise
141 *bab2* expression in four concentric tarsal rings within the leg discs (Fig 1B) (17, 25). *bab1-2*
142 are co-expressed in ts2-4 tarsal segments, while *bab2* is specifically expressed in ts5 and more
143 proximally than *bab1* in ts1, both in a graded manner along the P-D leg axis (Fig 1C and S1A
144 Fig) (11). Given *bab1-2* co-expression in ts1-4, we first asked whether *C15*, *rotund* and *bowl*
145 activities are also controlling *bab1* expression in the developing distal leg. To this end, we
146 compared Bab1 expression with that of a X-linked *LAE-GFP* (or *LAE-RFP*) reporter gene
147 faithfully reproducing the *bab2* expression pattern there (17, 25), in homozygous mutant leg
148 discs for a null *C15* allele or in genetically-mosaic leg discs harboring *rotund* or *bowl* loss-of-
149 function mutant cells (Fig 1D-F).

150 *C15* is specifically activated in the distalmost (center) part of the leg disc giving rise to the
151 pretarsal (pt) segment (see Fig 1B) (30, 31). We have previously shown that the C15
152 homeoprotein down-regulates directly *bab2* to restrict its initially broad distal expression to the
153 tarsal segments (25). Bab1 expression analysis in a homozygous *C15* mutant leg disc revealed
154 that both *bab1* and *LAE-RFP* (*bab2*) are similarly de-repressed in the pretarsus (Fig 1, compare
155 panels C-D).

156 In contrast to *C15*, *rotund* expression is restricted to the developing tarsal segments (32) and
157 the transiently-expressed Rotund ZF protein contributes directly to *bab2* up-regulation in
158 proximal (ts1-2) but has no functional implication in distal (ts3-5) tarsal cells (17).
159 Immunostaining of genetically-mosaic leg discs at the L3 stage revealed that *bab1* is cell-
160 autonomously down-regulated in large *rotund* mutant clones in ts1-2, but not in ts3-4 segments
161 (Fig 1E), as it is the case for *LAE-GFP* reflecting *bab2* expression. Lastly, we examined whether
162 the Bowl ZF protein, a repressive TF active in pretarsal but not in most tarsal cells, is down-

163 regulating *bab1* expression there (33), like *bab2* (25). Both *bab1* and *LAE-RFP (bab2)* appeared
164 cell-autonomously de-repressed in *bowl* loss-of-function pretarsal clones (Fig 1F).

165 In addition to loss-of-function, we also conducted gain-of-function experiments for *bowl* and
166 *rotund*. Bowl TF gain-of-function was achieved by down-regulating *lines* which encodes a
167 related but antagonistic ZF protein (i) destabilizing nuclear Bowl and is specifically expressed
168 in the tarsal territory (33). As previously shown for *LAE-GFP* (and *bab2*) expression, nuclear
169 Bowl stabilization in the developing tarsal region appears sufficient to down-regulate cell-
170 autonomously *bab1* (S1C Fig). Prolonged expression of the Rotund protein in the entire distal
171 part of the developing leg disc, i.e., tarsal in addition to pretarsal primordia, induces ectopic
172 *bab1* expression-in the presumptive pretarsal territory, albeit with some differences with *bab2*
173 expression (S1B Fig, differentially-expressing cells are indicated with arrows), thus suggesting
174 differential sensitivity of the two gene duplicates to Rotund TF levels (see discussion).

175 Taken together, these data indicate that the C15, Bowl and Rotund transcription factors,
176 previously shown to interact physically with specific LAE sequences and thus to regulate
177 directly *bab2* expression in the developing distal leg, are also regulating *bab1* expression there.
178 These results suggest that the limb-specific intergenic LAE enhancer activity regulates directly
179 both *bab* genes.

180

181 **LAE activity regulates both *bab1* and *bab2* gene paralogs along the proximo-distal leg axis**

182 To test the role of LAE in regulating *bab1-2*, we deleted precisely the LAE sequence through
183 CRISPR/Cas9 genome editing (see Materials and Methods) (Fig 2A). Two independent
184 deletion events (termed Δ *LAE-M1* and *-M2*; see S2 Fig for deleted DNA sequences) were
185 selected for phenotypic analysis. Both are homozygous viable and give rise to fertile adults with
186 identical fully-penetrant distal leg phenotypes, namely ectopic sex-comb teeth on *ts2* (normally

187 only found on ts1) tarsal segment in the male prothoracic (T1) legs (Fig 2B), which are typical
188 of *bab2* hypomorphic alleles (11). The Δ LAE-M1 allele was selected for detailed phenotypic
189 analyses and is below referred to as *bab*^{ALAE}.

190 First, we quantified *bab1* and *bab2* mRNAs prepared from dissected wild-type and homozygous
191 *bab*^{ALAE} mutant leg discs. As shown in Fig 2C, both mRNAs were detected in mutant discs,
192 although *bab1* levels were two times lower than wild-type. Second, Bab1-2 expression patterns
193 were analyzed in homozygous *bab*^{ALAE} leg discs (Fig 3). To identify leg cells that should
194 normally express *bab2*, we used the X-linked LAE-GFP reporter. In homozygous *bab*^{ALAE}
195 mutant leg discs, *bab2* specific expression (see Fig. 1B) is no longer observed (Fig 3B-C), while
196 Bab1-2 shared expression is very low in ts3-4 to undetectable in ts1-2. Nevertheless, residual
197 *bab1-2* co-expression in homozygous *bab*^{ALAE} mutant discs indicates that additional *cis*-
198 regulatory region(s) within the *bab* locus act(s), at least partly, redundantly with the LAE
199 enhancer.

200 Taken together, our data indicate that LAE enhancer activity is (i) required for *bab1-2* co-
201 expression in the two proximal-most tarsal segments, particularly ts1, (ii) dispensable for their
202 co-expression in ts3-4, suggesting the presence of redundant *cis*-regulatory information and (iii)
203 critically required for *bab2*-specific tarsal expression both proximally and distally. Thus, LAE
204 activity governs both shared and paralog-specific expression of the *bab1-2* gene duplicates.

205

206 **LAE paralog-specific activity requires the *bab2* core promoter**

207 Whereas enhancer emergence has been proposed to account for acquisition of novel tissue- or
208 paralog-specific functions for gene duplicates (34-36), LAE regulatory function provides an
209 example of a single enhancer responsible both for shared and differential expression of two
210 tandemly-repeated gene paralogs. Previously tested LAE reporter constructs fused *bab2* core

211 promoter sequences to the minimal *Hsp70* promoter region (*pHsp70*) (17, 25). To examine the
212 contribution of the *bab2* promoter to LAE activity we compared the expression of two LAE
213 reporters containing (*LAE-RFP*) or not (*LAE-pHsp70only-GFP*) the *bab2* promoter sequence
214 (Fig 3D). Strikingly, the *LAE-pHsp70only-GFP* reporter was no longer activated in *RFP+*
215 (*bab2*-expressing) ts1 and ts5 cells (Fig 3E; see white brackets and arrows). These data indicate
216 that *bab2*-specific regulation by LAE activity requires the *bab2* core promoter sequences.

217

218 **In addition to the intergenic LAE, other leg-specific enhancer elements are present within**
219 **the *bab1* first intron**

220 Since LAE appeared dispensable for *bab1-2* co-expression in ts3-4 cells, our data suggested the
221 existence of other redundant *cis*-regulatory elements, presumably located also within the *bab*
222 locus. On one side, a X-linked Bacterial Artificial Chromosome (BAC) construct,
223 *BAC26B15^{ZH2A}*, encompassing the *bab2* gene and downstream intergenic sequence including
224 LAE (see Fig 4A), could rescue (i) Bab2 expression in the tarsal primordium and (ii), distal leg
225 phenotypes detected in homozygous animals for the null allele *bab^{AR07}* (17). On the other side,
226 a *BAC26B15* construct (*BAC26B15 Δ LAE^{ZH2A}*) inserted at the same genomic landing site and
227 specifically lacking LAE sequence did not (Fig 4B-D). These results confirmed that (i) in
228 absence of redundant *cis*-regulatory information, LAE is essential for *bab1-2* expression in the
229 tarsal segments and (ii) the *cis*-information redundant with LAE is located outside the genomic
230 region covered by *BAC26B15*.

231 To identify limb-specific redundant *cis*-regulatory information within the *bab* complex, we first
232 tested the capacity of another BAC, *BAC69B22*, which overlaps *bab1* and lacks LAE (see Fig
233 4A), to restore Bab1 expression in *bab^{AR07}* mutant leg discs. As shown in Fig 4E-F, the X-linked
234 *BAC69B22^{ZH2A}* could restore *bab1* expression in ts2-4, indicating that it contains *cis*-regulatory

235 information redundant with LAE activity in these segments. To test the capacity of *BAC69B22*
236 sequences to also regulate *bab2* expression in *ts2-4*, we placed *BAC69B22^{ZH2A}* across
237 *BAC26B15 Δ LAE^{ZH2A}*, to allow pairing-dependent *trans*-interactions (i.e., transvection) between
238 the two X chromosomes in females. This configuration partially restored Bab2 expression in
239 *ts2-4* cells from *bab^{AR07}* mutant L3 leg discs, albeit in salt and pepper patterns (Fig 4G),
240 diagnostic of transvection effects (37).

241 From these data, we predicted the existence of *cis*-regulatory information within the *69B22*
242 chromosomal interval capable to drive some *bab1-2* expression in distal leg tissues and acting
243 redundantly with the LAE enhancer.

244

245 **Chromatin features predict limb-specific *cis*-regulatory elements within *bab1***

246 Next, we sought to identify *cis*-regulatory information acting redundantly with LAE by taking
247 advantage of available genome-wide chromatin features and High-throughput chromosome
248 conformation Capture (Hi-C) experiments performed from L3 eye-antennal and/or leg discs
249 (Fig 5). *bab1-2* are indeed co-expressed in distal antennal cells within the composite eye-
250 antennal imaginal disc (11). A topologically-associating domain covering the entire *bab* locus
251 was detected in Hi-C data from eye-antennal discs (Fig 5A and S3A Fig) (38), revealing
252 particularly strong interactions between *bab1-2* promoter regions.

253 We then used published genome-wide data from Chromatin Immuno-Precipitation (ChIP-Seq),
254 Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE-Seq) and Assay for
255 Transposase-Accessible Chromatin (ATAC-Seq) experiments (38-41), looking for active
256 enhancer marks (H3K4me1 and H3K27Ac) and nucleosome-depleted chromatin regions (thus
257 accessible to transcription factors). Active enhancer signatures are mainly associated with a
258 ~15-kb-long genomic region that we termed BER, for “*bab1* Enhancer Region”, encompassing

259 the *bab1* promoter, first exon and part of its first intron (Fig 5B, lanes 1-2 and 5-6, respectively;
260 see also S3B Fig for peak calling data). Note that LAE is also accessible to transcription factors
261 and carries H3K4me1 marks, consistently with enhancer activity in distal antennal cells (17).

262 To more precisely locate putative enhancer element(s) within BER, we analyzed previously-
263 published ChIP-Seq data from L3 leg discs (42) for binding sites for Dll, Sp1 and Hth proteins,
264 known to regulate *bab1* and/or *bab2* expression in the developing legs (17, 42-44). Strong Dll
265 binding is detected throughout BER, including over the *bab1* promoter (Fig 5B, lane 10; see
266 also S3B Fig). In leg discs, Dll binding is detected over 8 out of 10 Open Chromatin Subregions
267 (OCS) within BER (Fig 6C and S3B Fig) and six of those eight are also bound by Sp1 ZF
268 protein. Of note, all nucleosome-depleted (i.e., OCS) BER subregions in the leg are also
269 accessible in the eye-antennal discs (40) (Fig 5B, compare lanes 2 and 9, and Fig 6C, two upper
270 lanes). Lastly, of six OCS sequences co-bound by Dll and Sp1, four are also bound by Hth
271 protein (Fig 5B, lane 8, and Fig 6C, bottom lane). FAIRE-Seq data indicate that, in addition to
272 LAE, only OCS7 is nucleosome-depleted in the leg and eye-antennal discs but not the wing
273 and/or haltere discs (Fig 5B, lanes 13, 16, 18, and Fig 6C, four upper lines; see also S3B Fig).
274 Importantly, ventral limb-specific OCS7 is co-bound by Dll, Sp1 and Hth transcription factors
275 (Fig 6C). Thus, within the entire *bab* locus, only LAE and BER OCS7 are specifically bound
276 by transcriptional regulators in the developing leg and antenna.

277 In summary, data mining indicates that BER includes a cluster of enhancer elements bound by
278 Dll and Sp1 in leg discs and thus are good candidates for acting redundantly with LAE in
279 regulating *bab* genes in a ventral limb-specific manner.

280

281 **BER includes multiple *cis*-regulatory elements active in diverse developmental contexts**

282 To further ascribe regulatory roles to BER subregions, we took advantage of a systematic
283 analysis of Gal4 reporter lines (45). Out of six lines containing BER fragments (Fig 6B), only
284 two, *73C11* and *73C05*, overlapping OCS1-3, are active in the leg and eye-antennal discs
285 (FlyLight database; (<http://flweb.janelia.org/cgi-bin/flew.cgi>; S4B Fig). Nevertheless, none
286 reproduce the *bab1/2* leg or antennal expression patterns in four or two concentric distal rings,
287 respectively. These and other published data from reporter constructs including *bab1* first intron
288 sequences (14) indicate that OCS1-7 (see Fig 6B) are not sufficient to properly drive *bab1/2*
289 expression in the developing legs and suggest the requirement of additional BER elements,
290 particularly the *bab1* promoter region (i.e., OCS9). This hypothesis is consistent with binding
291 of the known *bab1-2* leg regulators Dll and Sp1 throughout BER, in addition to LAE (Fig 5,
292 lane 10; and S3B Fig).

293 The *73C05* BER fragment also drives reporter gene expression in the wing, haltere and genital
294 discs (S4 Fig, panels E and H-J) in patterns strikingly similar to those described for *bab2* (11,
295 44). Consistently, FAIRE- and ChIP-Seq data from haltere discs indicate respectively that
296 OCS1-3 are nucleosome-free and bind the Ultrabithorax (Ubx) Hox-type homeoprotein known
297 to activate directly *bab2* expression in haltere tissues (Fig 5B, lanes 16-17, and Fig 6C) (46,
298 47). Furthermore, ChIP-Seq data from whole L3 larvae (modENCODE), showed binding over
299 the entire BER region of the Hox Abd-B genital selector (Fig5B, lane 20) (48). Lastly, BER
300 includes (i) nucleosome-depleted sequences governing expression in adult muscles and bound
301 by the mesodermal transcription factors Mef2, Slp1 and Tinman in late embryos (14, 49) as
302 well as (ii) a sequence element (overlapping OCS5) which confers enhancer activity in ovarian
303 somatic cells (50) (see Fig 6D).

304 Taken together, our data indicate that BER sequences drive *bab1-2* expression in developing
305 limbs but also in other tissues such as wing, haltere, genitalia and mesoderm. Moreover, owing
306 to the presence of binding sites for transcription factors known to regulate *bab* gene expression

307 in these respective tissues, spread out over the whole BER sequence, the latter is thus proposed
308 to act as a pleiotropic enhancer region.

309

310 **Cross- and auto-regulations among the *bab* genes**

311 Bab proteins interact with A/T-rich DNA sequences through their BabCD DNA-binding
312 domain, including binding sites within their own locus (51). We therefore tested whether the
313 Bab1-2 proteins autoregulate and/or cross-regulate their own expression. Previous data
314 indicated that Bab2 protein expression is unaffected in *bab1* loss-of-function mutants (11).
315 Given that protein null *bab2* alleles are not available, we used RNA interference coupled to
316 flip-out (FO) Gal4 expression to down-regulate clonally *bab2* expression within developing
317 legs, and examine *LAE-RFP* and *bab1* expression in mosaic L3 leg discs (Fig 7). Strikingly,
318 both *LAE-RFP* and *bab1* were up-regulated cell-autonomously in most tarsal mitotic clones
319 (n=17/20) (Fig 7, panels A-A"). Moreover, *bab2* down-regulation in proximal-most RFP+ ts1
320 cells (expressing only *bab2*) activated cell-autonomously *bab1*, in addition to up-regulating
321 *LAE-RFP* expression (Fig 7, white arrows in panels B-B"). These results suggested to us that
322 the *bab2* paralog specifically down-regulates its own expression through partial repression of
323 LAE enhancer activity. To confirm these observations, we generated mutant clones for the
324 *bab^{AR07}* null allele, lacking both *bab2* and *bab1* activities. A slight cell-autonomous *LAE-GFP*
325 reporter up-regulation could be observed in all examined *bab^{AR07}* clones (detected with anti-
326 Bab2 antibodies; n>20) (Fig 7, panels C-C"), independently of their size and position within
327 *bab2*-expressing tarsal cells (see white arrows and arrowheads).

328 Altogether, we conclude that *bab2* down-regulates its own expression, likely via partial
329 repression of LAE activity, thus ensuring appropriate levels of both paralogous BTB-BabCD
330 transcription factors in distal leg tissues, and most likely in other appendages as well.

331

332 **The *bab* gene complex arose from *bab1* duplication in the Muscomorpha infraorder**

333 The different levels of *cis*-regulatory element redundancy within the *bab* locus led us to trace
334 back the evolutionary origin of the *bab* duplication found in *D. melanogaster* (*Dmel*). To start,
335 we identified proteins orthologous to *Dmel* Bab1 or Bab2, i.e., displaying an N-terminal BTB
336 associated to a C-terminal BabCD domain (collectively referred to as BTB-BabCD proteins)
337 (11) within highly diverse dipteran species (see Fig 8A). Two distinct BTB-BabCD proteins
338 strongly related to *Dmel* Bab1 and Bab2, respectively, were identified in the Muscomorpha
339 (higher flies, also known as Cyclorrhapha) superfamily, both within the Schizophora (in
340 Calyptratae, such as *Musca domestica* and *Glossina morsitans*, and in Acalyptratae, particularly
341 among Drosophilidae) and Aschiza subsections (Fig 8A-B and Supplementary data). In
342 contrast, a single BTB-BabCD protein could be identified in evolutionarily-distant dipteran
343 species within (i) the brachyceran Asilomorpha and Stratiomyomorpha superfamilies (such as
344 *Proctacanthus coquilletti* and *Hermetia illucens*, respectively), collectively referred to as
345 Orthorrhapha; (ii) the Nematocera suborder families (with rare exceptions, in Psychodomorpha
346 and Bibionomorpha, see below); (iii) other Insecta orders (e.g., Coleoptera, Hymenoptera and
347 Lepidoptera), and in crustaceans (e.g., *Daphnia pulex*) (see Supplementary data).

348 To analyze the phylogenetic relationships between these different Bab-related proteins, their
349 primary sequences were aligned and their degree of structural relatedness examined through a
350 maximum likelihood analysis. As expected from an ancient duplication, muscomorphan Bab1-
351 2 paralogs cluster separately, while singleton asilomorphan BTB-BabCD proteins are more
352 related to muscomorphan Bab1 than Bab2 (Fig 8B and S5 Fig), indicating that muscomorphan
353 *bab2* originated from *bab1* duplication.

354 Interestingly, contrary to most nematocerans, two or even three *bab1* paralogs are present in
355 the fungus gnat *Coboldia fuscipes* (Psychodomorpha) and the gall midge *Mayetiola destructor*
356 (Bibionomorpha), respectively. Significantly, *M. destructor* and *C. fuscipes bab1* paralogs (i)
357 cluster separately in our phylogenetic analysis (Fig 8B and S5 Fig) and (ii) two are arrayed in
358 the same chromosomal contexts for both species (S6 Fig), indicating that they have likely been
359 generated through independent gene duplication processes in the Bibionomorpha and
360 Psychodomorpha, respectively.

361 Taken together, and updating a previous work (13), our phylogenomics analysis (summarized
362 in Fig 9B-C) indicates that a single ancestral *bab* gene related to *bab1* has been duplicated to
363 give rise to *bab2* within the Muscomorpha (Cyclorrhapha) infraorder.

364

365 **LAE sequences have been fixed in the Brachycera, thus predating *bab1* duplication**

366 Having traced back the *bab* gene duplication raised the question of the evolutionary origin of
367 the LAE enhancer, which regulates both *bab1* and *bab2* expression (17) (this work). We have
368 previously shown that LAE includes three subsequences highly-conserved among twelve
369 reference Drosophilidae genomes (52), termed CR1-3 (for Conserved Regions 1 to 3; see S7A
370 Fig and Supplementary data), of which only two, CR1 and 2, are critical for tissue-specificity
371 (17, 25). The 68 bp CR1 includes contiguous binding sites for Dll and C15 homeoproteins,
372 while the 41 bp CR2 comprises contiguous binding sites for Dll as well as the ZF protein Bowl
373 (S7 Fig, panels B and C, respectively) (17, 25).

374 To trace back the LAE evolutionary origin, we then systematically searched for homologous
375 CR1-3 sequences (>50% identity) in dipteran genomes. Importantly, conserved LAE sequences
376 have not been yet reported outside drosophilids. Small genomic regions with partial or extensive
377 homologies to the CR1 (encompassing the C15 and Dll binding sites) and CR2 (particularly the

378 Dll and Bowl binding sites) could be detected in all examined Brachycera families but not in
379 any nematoceran (Fig 9B and S7B-C Fig). Contrary to closely-associated CR1-2 homologous
380 sequences, no CR3-related sequence could be identified nearby, in any non-Drosophilidae
381 species. Significantly, homologous LAE sequences are situated (i) in between the tandemly-
382 duplicated paralogs in muscomorphan species for which the entire *bab* locus sequence was
383 available to us, suggesting an evolutionarily-conserved enhancer role, or (ii) 20 kb upstream of
384 the *bab1*-related singleton in the asilomorphan *P. coquilletti* (see Fig 9C).

385 Taken together, as summarized in Fig 9A-C, these data suggest that a LAE-like enhancer with
386 CR1- and CR2-related elements emerged early on in the Brachycera suborder, 180-200 million
387 years ago, and has been since fixed within or upstream the *bab* locus in the Muscomorpha and
388 Asilomorpha infraorders, respectively.

389

390 **Like LAE elements, *bab1* promoter sequences have been fixed early on in the Brachycera**

391 Given their differential interplay with the long-lasting LAE enhancer, we next analyzed the
392 evolutionary conservation of *Dmel bab1-2* promoter core sequences (Fig 9B and S8 Fig). Both
393 *bab* promoters are TATA-less. Whereas *bab1* has a single transcriptional initiator (Inr) element
394 (TTCAGTC), its *bab2* paralog displays tandemly-duplicated Inr sequences (ATTCAGTTCGT)
395 (53, 54) (S8 Fig). Both promoters display 64% sequence identity over 28 base pairs, including
396 Inr (TTCAGT) and downstream putative Pause Button (PB; consensus CGNNCG) sequences
397 (55) (see S8A Fig). These data suggested that (i) the duplication process having yielded *bab2*
398 included the ancestral *bab1* promoter and (ii) PolII pausing ability previously shown for *bab2*
399 promoter (56-58) probably also occurs for *bab1* promoter.

400 Homology searches revealed that *bab1* promoter sequences have been strongly conserved in
401 the three extant Muscomorpha families and even partially in some asilomorphans (e.g., *P.*

402 *coquellitti*), for which a *bab1*-related singleton gene is present (Fig 9B and S8B Fig). In striking
403 contrast to *bab1*, sequence conservation of the *bab2* promoter could only be detected among
404 some Acalypratae drosophilids (Fig 9B and S8C Fig). In agreement with a fast-evolutionary
405 drift for *bab2* promoter sequences, the duplicated Inr is even only detected in *Drosophila* group
406 species.

407 Taken together, these evolutionary data (summarized in Fig 9B) indicate that, likewise for the
408 LAE enhancer, *bab1* promoter sequences have been under strong selective pressure among the
409 Brachycera, both in the Muscomorpha and Asilomorpha infraorders, while paralogous *bab2*
410 promoter sequences diverged rapidly among muscomorphans.

411

412 **Unlike LAE, other *bab* CRE sequences have not been conserved beyond the**
413 **Muscomorpha**

414 The broad LAE sequence conservation led us to also trace back the evolutionary origins of the
415 pleiotropic BER enhancer region as well as the cardiac CE, abdominal anterior AE and
416 sexually-dimorphic DE *cis*-regulatory elements (see Fig 9). Sequences homologous to half of
417 the BER OCS subregions could be detected among the 12 reference Drosophilidae genomes
418 (52), in Calypratae schizophorans and even in the Muscomorpha Aschiza subsection (e.g.,
419 OCS3) (S10-18 Fig). Unlike the LAE enhancer, homologous BER sequence elements (except
420 the *bab1* promoter) could not be detected in non-muscomorphan families. Cardiac CE and
421 abdominal DE are even less conserved given that related sequences could be only detected
422 within schizophoran (excepted in Calypratae) (Fig 9B and S9 Fig, panels B and C,
423 respectively), whereas abdominal AE sequences could be only identified among drosophilids
424 (Supplementary data) but not in aschizan, asilomorphan and nematoceran *bab* loci.

425 In conclusion, contrary to the LAE enhancer which among the Diptera emerged early on in the
426 Brachycera suborder, other so-far identified *bab* cis-regulatory sequences have not been
427 conserved beyond the Muscomorpha infraorder. Thus, as summarized in Fig 9B, and unlike the
428 long-lasting brachyceran LAE (CR1-2) sequences, these data suggest that other enhancer
429 sequences have been fixed within the Muscomorpha concomitantly (BER) or even after (CE,
430 DE and AE) the *bab2* paralog emergence. Moreover, as expected for a pleiotropic enhancer
431 region, BER sequence conservation allowed us to predict binding sites for transcription factors
432 known, or so far unsuspected, prone to regulate directly the two *bab* genes in many distinct
433 developmental contexts, and which are presented hereafter.

434

435 **Predictive TF combinatorial code governing *bab* gene expression in diverse tissues**

436 We gathered our data from TF binding site evolutive conservation (described in Supplementary
437 data and S10-19 Fig) with ChIP-Seq experiments from the literature (GEO datasets; see
438 Materials and Methods) (Fig 5B and S3B Fig). Associated with our precise knowledge of *bab*
439 locus enhancer sequences and with previous genetic data also gained from the literature, our
440 compilation presented in Fig. 10 allows to propose new models for the TF code involved in
441 *Dmel bab* locus regulation: (i) It provides new insights into limb-specific *bab1/2* regulation
442 proposing additional direct regulators such as Sp1 in the legs, Hth in the antenna, Scalloped
443 (Sd) in the wing and Ubx in the haltere (ChIP-Seq data shown in S3 Fig); (ii) It suggests that
444 BER also acts as an enhancer region for *bab* gene regulation in other developmental contexts
445 and that common TF sets (notably Abd-B together with Dsx) are acting through distinct cis-
446 regulatory elements within BER to drive *bab* gene expression in distinct tissues (e.g., in
447 abdominal histoblast nests versus genitalia); (iii) It proposes a direct Bab2 binding on LAE for
448 *bab* gene auto- and cross regulation (tested above); (iv) Finally, analysis of sequences conserved

449 among brachyceran *bab* loci identified predicted binding sites for more broadly-expressed
450 transcriptional regulators, i.e., GAF, Pho and CTCF (directly interacting with BER OCS; see
451 Fig 5B, lanes 3, 14 and 19, respectively, as well as S3 Fig for GAF), as well as Eip93F,
452 Eip74EF, Chinmo, all related to chromatin organization whose putative roles in *bab* locus
453 regulation are discussed hereafter.

454

455 **Discussion**

456 In this work, we have addressed the issue of the emergence and functional diversification of
457 enhancers and promoters from two tandem gene duplicates. Using the *Drosophila bab* locus as
458 a model, we showed that the paralogous genes *bab1-2* originate from an ancient *bab1*
459 duplication in the Muscomorpha/Cyclorrhapha. The early-fixed brachyceran *bab1* LAE has
460 been co-opted lately to regulate also *bab2* expression. Furthermore, this unique enhancer is also
461 responsible for paralog-specific *bab2* expression along the P-D leg axis presumably through
462 privileged interactions with the *bab2* promoter. Finally, LAE regulates only some aspects of
463 *bab1-2* expression in the developing limbs because redundant information has emerged within
464 a large pleiotropic enhancer driving *bab1* and/or *bab2* expression in highly-diverse tissues, by
465 binding common sets of developmental transcription factors. This work brings some cues about
466 (i) how a single enhancer can drive specificity among tandem gene duplicates, (ii) how
467 enhancers evolutionary adapt with distinct cognate gene promoters, and (iii) which functional
468 links can be predicted between dedicated transcription factors and chromatin dynamics during
469 development.

470

471 **A shared enhancer differentially regulating two tandem gene paralogs through distinct**
472 **promoter targeting specificities**

473 Here, we have shown that a single enhancer, LAE, regulates two tandem gene paralogs at the
474 same stage and in the same expression pattern. How can this work? It has been proposed that
475 enhancers and their cognate promoters are physically associated within phase-separated nuclear
476 foci composed of high concentrations of TFs and proteins from the basal RNA polymerase II
477 (PolII) initiation machinery inducing strong transcriptional responses (59, 60). Our Hi-C data
478 from eye-antennal discs show a strong interaction between *bab1-2* promoter regions (Fig 5),
479 suggesting that both *bab* promoters could be in close proximity within such phase separated
480 droplets, thus taking advantage of shared transcriptional regulators and allowing concerted gene
481 regulation. In contrast, no strong chromosome contacts could be detected between LAE and
482 any of the two *bab* promoter regions, indicating that this enhancer is not stably associated to
483 the *bab2* or *bab1* promoter in the eye-antennal disc (where only the antennal distal part
484 expresses both genes). It would be interesting to gain Hi-C data from leg discs, in which the
485 *bab1-2* genes are much more broadly expressed.

486 In addition to be required for *bab1-2* co-expression in proximal tarsal segments, we showed
487 here that the LAE enhancer is also responsible for paralog-specific *bab2* expression along the
488 proximo-distal leg axis. While it has been proposed that expression pattern modifications occur
489 through enhancer emergence, our present work indicates that differential expression of two
490 tandem gene paralogs can depend on a shared pre-existing enhancer (i.e., LAE). How this may
491 work? Relative to its *bab1* paralog, *bab2* expression extends more proximally within the Dac-
492 expressing ts1 cells (44) and more distally in the ts5 segment expressing nuclear Bowl protein,
493 whereas both Dac and Bowl proteins have been proposed to act as *bab2* (and presumably *bab1*)
494 repressors (25, 33, 61). CRISPR/Cas9-mediated LAE excision allowed us to establish that this
495 enhancer is critically required for paralog-specific *bab2* leg expression proximally and distally,
496 in ts1 and ts5 cells, respectively. In this context, we and others have previously proposed that
497 transiently-expressed Rotund activating TF may antagonize Bowl (and eventually Dac)

498 repressive activity to precisely delimit *bab2* expression among *ts1* cells (17, 61). Given that
499 *bab1-2* are distinctly expressed despite being both regulated by Bowl and Rotund, we propose
500 that paralog-specific LAE activity depends on privileged interactions with *bab2* promoter
501 sequences (discussed below). Thus, we speculate that the *bab2* promoter responds to Rotund
502 transcriptional activity differently from its *bab1* counterpart. Consistent with this view, ectopic
503 Rotund expression reveals differential regulatory impacts on the two *bab* gene promoters (S1B
504 Fig). Genetic together with Hi-C experiments indicate that this could occur through specific
505 interactions between LAE-bound TFs (e.g., Rotund) and dedicated proteins within the PolII
506 pre-initiation complex stably-associated to the *bab2* core promoter. We envision that the LAE-
507 bound ZF protein Rotund, the chromatin-remodeling ZF protein GAF (for GAGA-associated
508 Factor) and the PolII-associated TFIID subunit TAF3, the latter known to interact physically
509 with GAF and Bab2 BTB proteins (62, 63), are parts of the underlying promoter targeting
510 molecular mechanism.

511 In this context, despite that sequence homologies between both promoters (consistent with an
512 ancient duplication event mobilizing the ancestral *bab1* promoter) are still detectable, it is
513 significant that the *bab2* promoter evolves much faster than its *bab1* counterpart. While the
514 *bab1* promoter sequence has been strongly conserved among brachycerans, predating *bab2*
515 gene emergence in the Muscomorpha, the *bab2* promoter sequence has only been fixed recently
516 among Drosophilidae, notably through the Initiator (*Inr*) sequence duplication, indicating very
517 fast evolutionary drift after the gene duplication process which yielded the *bab2* paralog. We
518 envision that this evolutionary ability has largely contributed to allow novel expression patterns
519 for *bab2*, presumably through differential enhancer-promoter pairwise interplay.

520

521 **Differential evolutionary conservation of tissue-specific versus pleiotropic enhancers**

522 Our comprehensive phylogenomics analyses from highly diverse Diptera families indicate that
523 the *bab* gene complex has been generated through tandem duplication from an ancestral *bab1*-
524 related gene singleton within the Muscomorpha (Cyclorrhapha), about 100-140 years ago. This
525 result contrasts with published data reporting that the duplication process having yielded the
526 tandem *bab* genes occurred much earlier in the Diptera lineage leading to both the Brachycera
527 (true flies; i.e., with short antenna) and Nematocera (long horned “flies”, including mosquitos)
528 suborders (13). In fact, tandem duplication events implicating the *bab* locus did occur in the
529 Bibionomorpha, as reported (13)), and even in the Psychodomorpha with three *bab1*-related
530 gene copies (Figure 8 and S6 Fig), but our phylogenetic analysis supports independent events.
531 Thus, within the Diptera, the ancestral *bab1* singleton had a high propensity to duplicate locally.
532 In this study, we have shown a strong evolutionary conservation of LAE subsequences among
533 brachycerans, notably its CR2 element containing Dll and Bowl binding sites (S7C Fig). This
534 conservation suggests a long-lasting enhancer function in distal limb-specific regulation of
535 ancestral singleton *bab1* genes. In striking contrast, BER sequence conservation could only be
536 detected among extant muscomorphan *bab* loci. We assume that during evolution large
537 pleiotropic enhancers may better assimilate binding sites for gene-specific transcription factors,
538 thus generating regulatory novelties in distinct imaginal discs.
539 Gene duplication is a major source to generate phenotypic innovations during evolution,
540 through diverging expression and molecular functions, and eventually from single gene copy
541 translocation to another chromosomal site. Emergence of tissue-specific enhancers not shared
542 between the two gene duplicates, as well as of “shadow” enhancers, have been proposed to be
543 evolutionary novelty sources (64) (6). Our work indicates that the presumably long-lasting
544 brachyceran LAE enhancer has recently been co-opted in drosophilids to allow differential *bab*
545 gene expression. Conversely, the large BER region has apparently accumulated regulatory

546 sequence elements bound by diverse tissue-specific transcription factors (e.g., Dll, Hth, Abd-B
547 and Dsx) acting in different cellular contexts.

548

549 **A pleiotropic enhancer region overlapping with a PcG-response element**

550 ChIP-Seq analysis for histone H3 modifications (H3K4me3, H3K27Ac and H3K4me1
551 enhancer/promoter marks; H3K27me3 chromatin repressive mark) from eye-antennal discs has
552 revealed the pleiotropic BER enhancer region but also an overlapping repressive PcG
553 (Polycomb Group family) domain, indicating that BER encompasses a bivalent chromatin
554 domain, while another one is detected over the *bab2* promoter region. A dual enhancer/silencing
555 function for PcG-Response Element (PRE) during embryogenesis has recently been established
556 genome-wide (65), and the authors have proposed that reuse of enhancer regulatory elements
557 by PcG proteins may help fine-tune gene expression and ensure the timely maintenance of cell
558 identities throughout development. More recently, we have shown widespread enhancer-PcG
559 domain interplay during developmental gene activation through chromatin looping in eye-
560 antennal discs (38). Altogether these data suggest that the *bab1-2* genes might be poised for
561 activation throughout the eye-antennal disc, and possibly other imaginal discs as well.

562 The Pleiohomeotic (Pho) protein is a DNA-binding PcG complex recruiter, critical for gene
563 silencing maintenance during development (66). Pho interaction with several BER subregions,
564 as detected in ChIP-Seq experiments from L3 tissues, as well as the presence of many
565 evolutionarily-conserved predicted Pho binding sites, support a role for Pho in PcG repression
566 throughout BER. We thus propose that Pho-containing PcG repressive complex bound at PREs
567 within the *bab* bivalent locus is counteracted by one or several tissue-specific transcriptional
568 activators identified in this work, which remain(s) to be characterized.

569 In this context, it is significant that in the eye-antennal disc, the ZF protein CTCF, acting
570 redundantly with other chromatin insulator proteins, strongly interacts directly with the two
571 flanking regions of the TAD covering the *bab* locus and also with several BER OCSs (Fig 5B).
572 Significantly, two of these predicted CTCF interacting sites overlap with putative optimal
573 binding sites for the PcG-recruiter Pho (S18-19 Fig). These data suggest that the CTCF
574 architectural protein and the PcG-recruiter Pho may functionally interact to regulate the *bab*
575 locus chromosome topology. Interestingly, the human Pho homolog (YY1) is a structural
576 enhancer-promoter looping regulator (67) and orchestrates, together with the CTCF protein, a
577 3D chromatin looping switch during early neural lineage commitment (68). To our knowledge,
578 functional relationships between CTCF and Pho proteins have not been investigated genome-
579 wide in *Drosophila*.

580

581 **Dynamic *bric-a-brac* locus chromatin accessibility during development**

582 Recent data indicate that chromatin accessibility is dynamic during *Drosophila* larval
583 development, being triggered by the ecdysone hormone (69). Dynamic enhancer activity and
584 chromatin accessibility have been proposed to be regulated by the ecdysone-induced Eip93F
585 (Ecdysone-induced protein 93F, also called E93) transcriptional regulator (70). ChIP data from
586 early pupal wings indicate that Eip93F binds many BER OCS as well as the *bab1* promoter
587 region (70). Consistently, many putative Eip93F binding sites are present in these BER
588 subregions and several have been conserved beyond Drosophilidae (Fig 10C). Interestingly, the
589 human Eip93F homolog interacts with CtBP through a conserved motif (71), and *Drosophila*
590 CtBP is known to recruit diverse chromatin-modifying complexes, notably to participate in
591 Pho-mediated PcG recruitment to PREs (72). Thus, Eip93F binding to BER *cis*-regulatory
592 elements may impact the proposed dual PcG activity at the *bab* locus. In addition to Eip93F,

593 BER regulatory sequences include many evolutionarily-conserved putative binding sites for the
594 Eip74EF protein (Fig 10C), another ecdysone-induced TF, including one which overlaps with
595 a conserved putative Pho binding site, suggesting again functional correlation between
596 Ecdysone regulation and PcG activity.

597 Lastly, the *cis*-regulatory landscape within the *bab* locus (i.e., AE, DE, CE, LAE and BER)
598 includes one or several evolutionarily-conserved predicted binding sites for the Chinmo BTB-
599 ZF protein participating to developmental timing, notably through interplay with ecdysone
600 signaling (73, 74). Consistently, ChIP-Seq experiments from embryos (ModENCODE data;
601 <http://www.modencode.org/>) indicate Chinmo binding to BER sequences (75). Intriguingly, the
602 Chinmo ZF protein is an additional BTB-containing TF prone to regulate directly the *bab* genes,
603 possibly through molecular partnerships with the chromatin organizer GAF (another BTB-ZF
604 protein interacting directly with both *bab* promoter regions; Fig 5B and S3 Fig) and the twin
605 Bab BTB-BabCD proteins themselves. Thus, Chinmo implication in chromatin organization
606 and enhancer activity within the *bab* locus undoubtedly deserves to be investigated.

607 In summary, the *bab* locus offers a good paradigm to investigate molecularly in great details
608 how chromatin structure, particularly higher-order chromosome organization, impacts on
609 transcriptional memory during development and selective enhancer-promoter interplay in
610 diverse tissular contexts. Indeed, our comprehensive predictive combinatorial code for tissue-
611 specific, as well as broadly-expressed architectural transcription factors (e.g., CTCF, Pho and
612 GAF) regulating two tandem gene paralogs, offers the opportunity to dissect underlying
613 molecular mechanisms, which are prone to be conserved during animal evolution and thus to
614 be of broad biological significance.

615

616 **Material and Methods**

617 Fly stocks, culture and genetic manipulations

618 *D. melanogaster* stocks were grown on standard yeast extract-sucrose medium. The *vasa-*
619 *PhiC31 ZH2A attP* stock (kindly provided by F. Karch) was used to generate the *LAEpHsp70-*
620 *GFP* reporter lines and the *BAC69B22* construct as previously described (17). *LAE-GFP* and
621 *LAE-RFP* constructs (including both *Hsp70* and *bab2* core promoters) inserted on the ZH2A
622 (X chromosome) or ZH86Fb (third chromosome) *attP* landing platforms, and displaying
623 identical expression patterns, have been previously described (17, 25). *C15²/TM6B*, *Tb¹* stock
624 was kindly obtained from G. Campbell. Mutant mitotic clones for null alleles of *bowl* and
625 *rotund* were generated with the following genotypes: *y w LAE-GFP; DllGal4^{EM2012}, UAS-*
626 *Flp/+; FRT82B, Ub-RFP/FRT82B rn¹²* (i.e., *rn* mutant clones are RFP negative; Fig 1E) and *y*
627 *w LAE-RFP; DllGal4^{EM2012}, UAS-Flp/+; Ub-GFP, FRT40A/bowl¹ FRT40A* (i.e., *bowl* mutant
628 clones are GFP negative; Fig 1F), respectively. Rotund protein gain-of-function within the *Dll-*
629 expressing domain was obtained with the following genotype: *y w LAE-GFP; DllGal4^{EM2012};*
630 *UAS-Rn¹/+*. The *Dll^{EM212}-Gal4* line was provided by M. Suzanne, while the *UAS-Rn¹* line was
631 obtained from the Bloomington stock center. “Flip-out” (FO) mitotic clones over-expressing
632 dsRNA against *lines* were generated by 40 mn heat shocks at 38°C, in mid-late L2 to early-mid
633 L3 larvae of genotypes: *y w LAE-RFP hsFlp; UAS-dsRNAlines/pAct>y+>Gal4, UAS-GFP* (i.e.,
634 FO clones express GFP in S1C Fig). Mutant mitotic clones for the null *bab^{AR07}* allele were
635 generated by 30 mn heat shocks at 38°C, in early first to late second-instar larvae of genotypes:
636 *y w LAE-GFP, hsFlp; FRT80B/bab^{AR07}, FRT80B*. FO mitotic clones over-expressing dsRNA
637 against *bab1* or *bab2* were generated by 40 mn heat shocks at 38°C, in early to early-mid L3
638 larvae of genotypes: *y w LAE-RFP hsFlp; UAS-dsbab2 /Pact>y+>Gal4, UAS-GFP* and *y w*
639 *LAE-RFP, hsFlp; Pact>y+>Gal4, UAS-GFP/+; UAS-dsbab1/+* (i.e., FO clones express GFP
640 in Fig 7). *UAS-dsRNA* stocks used to obtain interfering RNA against *lines* (#40939), *bab1*
641 (#35707) and *bab2* (#37260) were obtained from the Bloomington stock Center.

642

643 **Immuno-histochemistry and microscopy**

644 Leg discs were dissected from wandering (late third instar stage) larvae (L3). Indirect immuno-
645 fluorescence was carried out as previously described (17) using a LEICA TCS SP5 or SPE
646 confocal microscope. Rat anti-Bab2 (11), rabbit anti-Bab1 (14), rabbit anti-Dll (76), rabbit anti-
647 Bowl (61), and rabbit anti-C15 (31) antibodies were used at 1/2000, 1/500, 1/200, 1/1000 and
648 1/200, respectively.

649

650 **CRISPR/Cas9-mediated chromosomal deletion**

651 Guide RNAs (gRNAs) were designed with CHOPCHOP at the Harvard University website
652 (<https://chopchop.cbu.uib.no/>). Four gRNA couples were selected that cover two distinct
653 upstream and downstream LAE positions: TCGGTGGAGCCTTCTTCGCCAGG or
654 TGGAGCCTTCTTCGCCAGGCCGG; and TATACTGTTGAGATCCCATGCGG or
655 TTAGGCGCACATAAGGAGGCAGG (the PAM protospacer adjacent motif sequences are
656 underlined), respectively. Targeting tandem chimeric RNAs were produced from annealed
657 oligonucleotides inserted into the pCFD4 plasmid, as described in
658 (<http://www.crisprflydesign.org/>). Each pCFD4-LAE-KO construct was injected into 50 *Vasa*-
659 *Cas9* embryos (of note the *vasa* promoter sequence is weakly expressed in somatic cells). F0
660 fertile adults and their F1 progeny, with possible somatic LAE-deletion events and candidate
661 mutant chromosomes (balanced with *TM6B*, *Tb*), respectively, were tested by polymerase chain
662 reactions (PCR) with the following oligonucleotides: AGTTTTTCATCCCCCTTCCA and
663 GTATTTCTTTGCCTTGCCATCG (predicted wild-type amplified DNA: 2167 base pairs).

664

665 **Quantitative RT-PCR analysis**

666 T1-3 leg imaginal discs were dissected from homozygous *white*¹¹¹⁸ and *bab*^{ALAE-M1} late L3
667 larvae in PBS 0.1% Tween. 50 discs of each genotype were collected and frozen in nitrogen.
668 Total messenger RNAs were purified using RNeasy kit (Qiagen) and reverse transcribed by
669 SuperScript II (ThermoFisher). *bab1*, *bab2* or *rp49* cDNA levels were monitored by
670 quantitative PCR using the following oligonucleotides: Bab1Fw:
671 CGCCCAAGAGTAACAGAAGC; Bab1Rev: TCTCCTTGTCCTCGTCCTTG; Bab2Fw:
672 CTGCAGGATCCAAGTGAGGT; Bab2Rev: GACTTCACCAGCTCCGTTTC; RP49Fw:
673 GACGCTTCAAGGGACAGTATCTG; RP49Rev: AAACGCGGTTCTGCATGAG. A
674 Wilcoxon test was performed to evaluate the difference between samples.

675

676 **Homology searches, sequence alignments and phylogenetic analyses**

677 Homology searches were done at the NCBI Blast site (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).
678 Protein or nucleotide sequence alignments were done using MAFFT (Multiple Alignment using
679 Fast Fourier Transform) (<https://mafft.cbrc.jp/alignment/server/>). Phylogenetic relationships
680 were inferred through a maximum likelihood analysis with W-IQ-Tree
681 (<http://iqtree.cibiv.univie.ac.at/>) and visualized with the ETE toolkit
682 (<http://etetoolkit.org/treeview/>).

683

684 **Transcription factor binding prediction**

685 DNA binding predictions were done using the motif-based sequence analysis tool TomTom
686 from the MEME suite (<http://meme-suite.org/tools/tomtom>) and the Fly Factor Survey database
687 (<http://mccb.umassmed.edu/ffs/>).

688

689 **Gene expression omnibus datasets**

690 The following gene expression omnibus (GEO) datasets were extracted from the NCBI website
691 (<https://www.ncbi.nlm.nih.gov/gds/>): GSE59078; GSM1261348; GSM1426265; GSE126985;
692 GSM3139658; GSM948715; GSE113574; GSM948718; GSM948717; GSE38594;
693 GSM948720; GSM948716; GSM659162; GSM948719; GSE102339; GSE50363.

694

695 **Hi-C and histone tail mark analyses from L3 eye-antennal imaginal discs**

696 Hi-C and histone mark ChIP-Seq analyses from L3 eye-antennal discs have been recently
697 published in (38).

698

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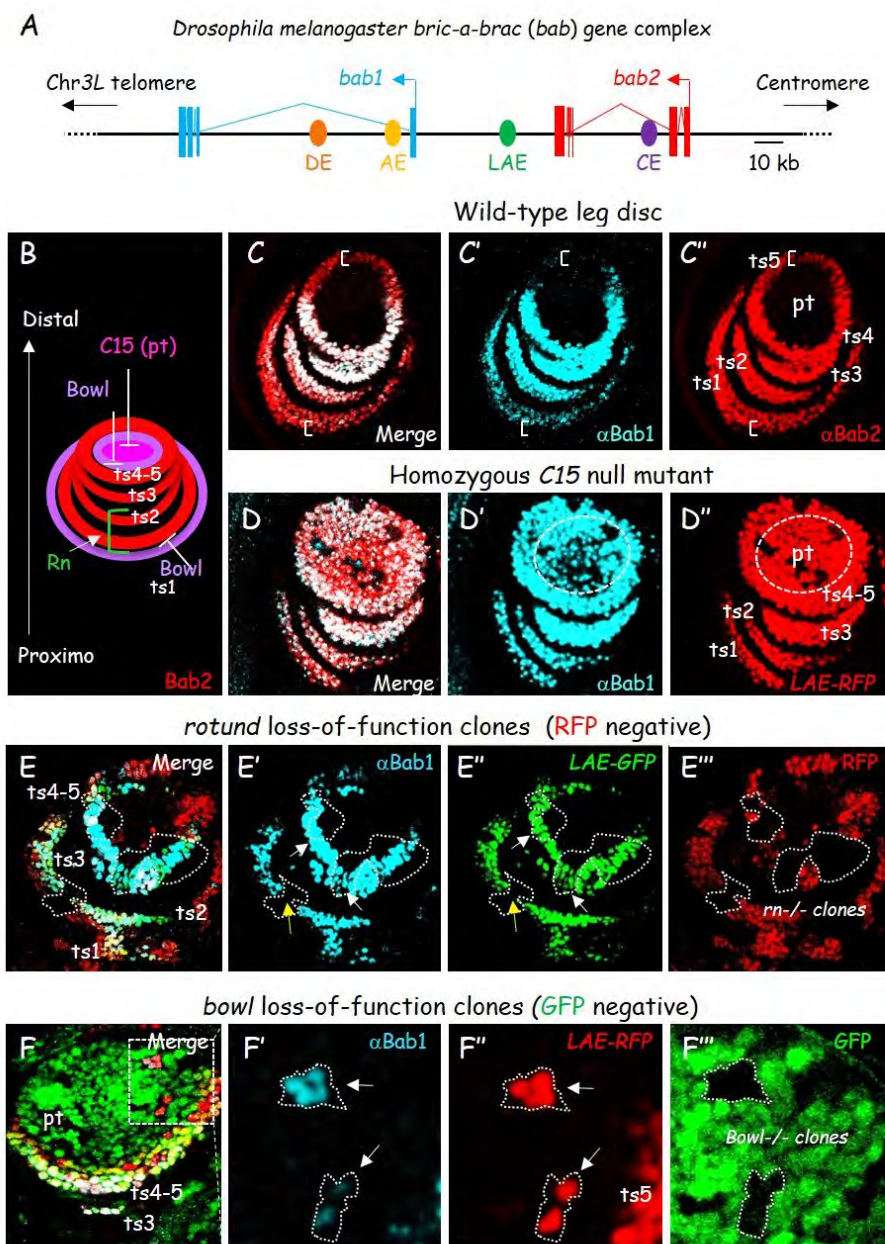


Fig 1. *C15*, *rotund* and *bowl* all regulate both *bab1* and *bab2*. (A) Schematic view of the *Dmel* *bab* locus on the 3L chromosomal arm (Chr3L). The tandem *bab1* (blue) and *bab2* (red) transcription units (filled boxes and broken lines represent exons and introns, respectively), the previously known CRE/enhancers are depicted by filled dots (abdominal DE and AE in dark and light orange, respectively; leg/antennal LAE in dark green and cardiac CE in purple), and the telomere and centromere directions are indicated by arrows. (B) A scheme depicted *C15*, Bowl and Rn TF activities in regulating *bab2* expression as a four-ring pattern within the developing distal leg, is shown. (C) Medial confocal view of a wild-type L3 leg disc. Merged Bab1 (blue) and Bab2 (red) immunostainings, as well as each marker in isolation in (C') and (C''), respectively, are shown. Positions of *bab2*-expressing ts1-5 cells and the pretarsal (pt) field are indicated in (C''). Brackets indicate paralogue-specific expression in proximalmost and distalmost *bab2*-expressing cells. (D) Distal confocal view of a homozygous *C15*² mutant L3 leg disc expressing *LAE-RFP*. Merged Bab1 immunostaining (in blue) and RFP fluorescence (red), and each marker in isolation in (D') and (D''), are shown. Bab2-expressing mutant pt cells are circled with a dashed line in (D') and (D''). (E) Medial confocal view of a mosaic L3 leg disc expressing *LAE-GFP* and harboring *rotund* mutant clones. Merged Bab1 (blue) immunostaining, GFP (green) and RFP (red) fluorescence, as well as each marker in isolation in (E'), (E'') and (E'''), respectively, are shown. Mutant clones are detected as black areas, owing to the loss of RFP. The respective ts1-5 fields are indicated in (E). White and yellow arrows indicate *bab1* (*bab2*) still- and non-expressing *rotund*^{-/-} clones, respectively. (F) Distal confocal view of a mosaic L3 leg disc expressing *LAE-RFP* and harboring *bowl* mutant clones. Merged Bab1 (blue) immunostaining, RFP (red) and GFP (green) fluorescence, as well as a higher magnification of the boxed area for each marker in isolation in (F'), (F'') and (F'''), respectively, are shown. Mutant clones are detected as black areas, owing to the loss of GFP. White arrows indicate pretarsal *bowl*^{-/-} clones ectopically expressing both *bab1* and *LAE-RFP* (*bab2*).

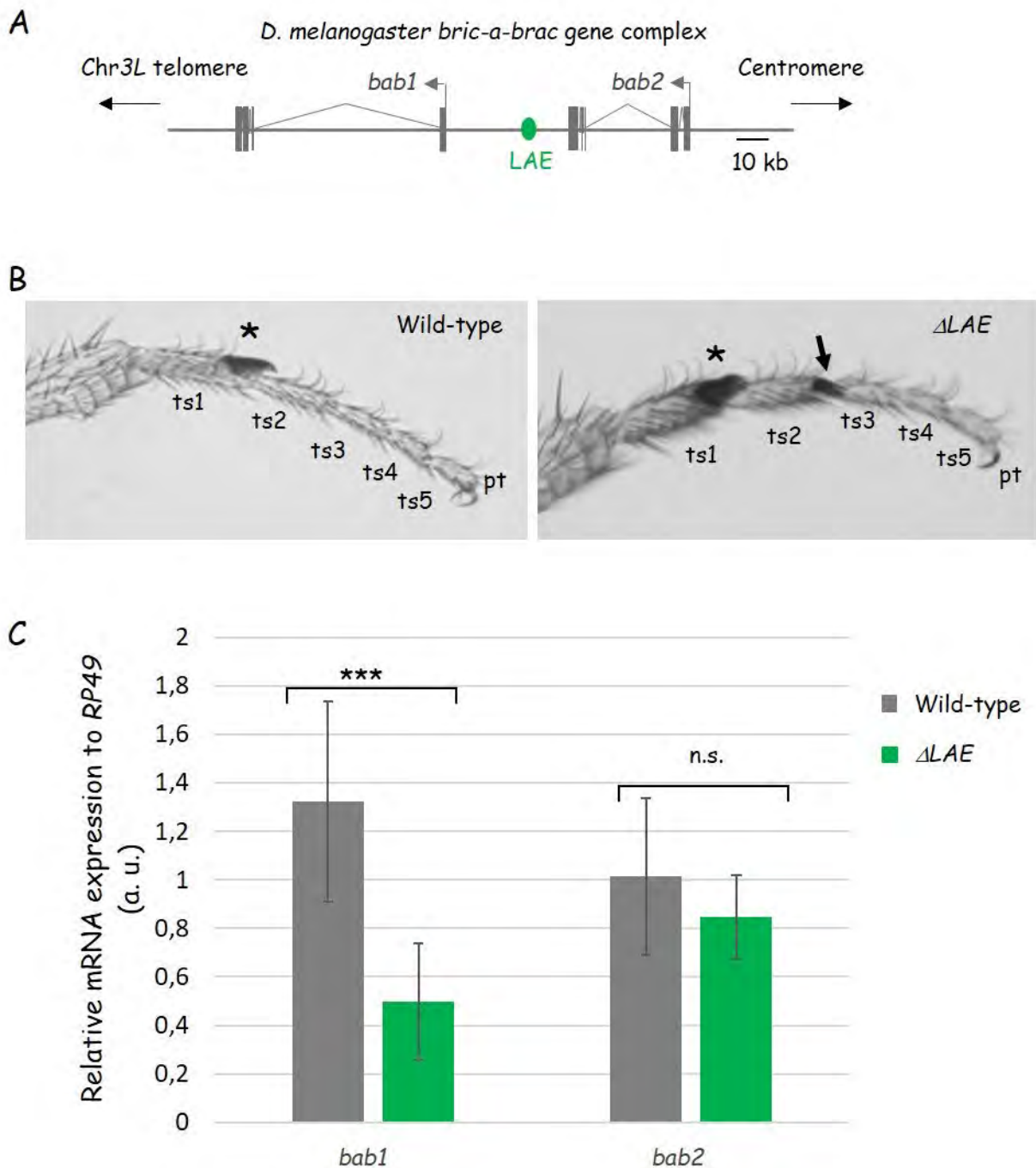


Fig 2. LAE is not critically required for tarsal segmentation and for overall *bab2* expression in the leg disc. (A) Schematic view of the *Dmel* *bab* locus on the 3L chromosomal arm (Chr3L). The tandem *bab1* and *bab2* transcription units (filled boxes and broken lines represent exons and introns, respectively), the intergenic LAE enhancer (in green), as well as the telomere and centromere directions, are depicted as in Fig 1A, except that both genes are depicted in grey. The small CRISPR/Cas9-mediated chromosomal deficiency (*bab^{ΔLAE}*) is shown in beneath (deleted LAE is depicted as a broken line). (B) Photographs of wild-type and homozygous *bab^{ΔLAE}* T1 distal legs from adult males. The regular sex-comb (an array of about 10 specialized bristles on the male forelegs) on distal ts1 is indicated with asterisks, while ectopic sex-comb bristles on distal ts2 from the mutant leg (right) is indicated by an arrow. Note that the five tarsal segments remain individualized in homozygous *bab^{ΔLAE}* mutant legs. (C) Overall *bab1-2* expression from wild-type and homozygous *bab^{ΔLAE}* L3 leg discs, as determined from reverse transcription quantitative PCR analyses. The *bab1-2* expression levels were quantified relative to *rp49* mRNA abundance.

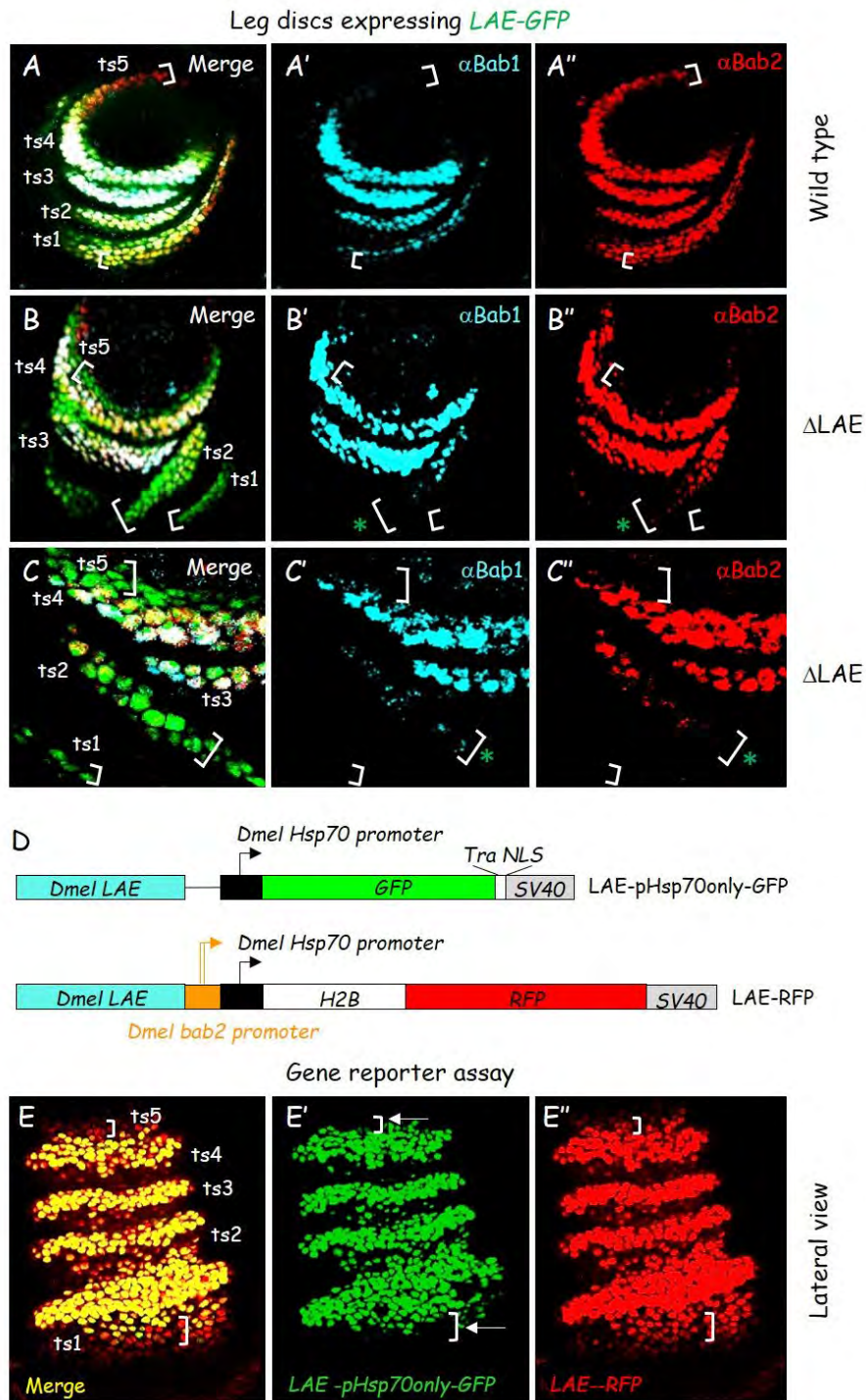


Fig 3. LAE is mostly critically required for paralog-specific *bab2* expression in the developing distal legs. (A-C) Medial (A-B) and distal (C) confocal views of wild-type (A) and homozygous *bab^{DLAE}* mutant (B and C) L3 leg discs expressing *LAE-GFP*. Merged GFP fluorescence (green), Bab1 (blue) and Bab2 (red) immunostainings, as well as the two latter in isolation in (A'-C') and (A''-C''), respectively, are shown. Brackets indicate positions of paralog-specific expression in proximalmost (*ts1*) and distalmost (*ts5*) *bab2*-expressing (GFP+) cells. Green asterisks in (B'-B'') and (C'-C'') indicate weaker expression of both *bab* paralogs in GFP+ *ts2* cells. (D) Modular structures of the *LAE-pHsp70onlyGFP* and *LAE-RFP* reporter constructs. GFP (green box) and RFP (red box) open-reading frames (ORFs) have been fused with ORFs for the Transformer (Tra) nuclear localization signal (NLS) and the histone H2B, respectively (see white boxes). The *SV40* polyadenylation signal region is boxed in grey. The *Dmel LAE* sequence is boxed in blue. The classical non-heat-inducible basal *Hsp70* promoter sequence is boxed in black, while the *bab2* core promoter sequence is depicted in orange. Note that both promoters are juxtaposed in the *LAE-RFP* construct. (E) LAE activity requires functionally the *bab2* promoter to ensure paralog-specific expression in the developing legs. A lateral confocal view of merged GFP (green) and RFP (red) fluorescence, as well as each marker in isolation in (E') and (E''), respectively, of the distal part of an early pupal leg expressing both the *LAE-pHsp70onlyGFP^{ZH2A}* and *LAE-RFP^{ZH86Fb}* reporter constructs (depicted in (D)), are shown. Brackets indicate tarsal RFP+ cells expressing *bab2* in a paralog-specific manner, which never express the *LAE-pHsp70onlyGFP^{ZH2A}* reporter which lacks *bab2* core promoter sequences (see white arrows).

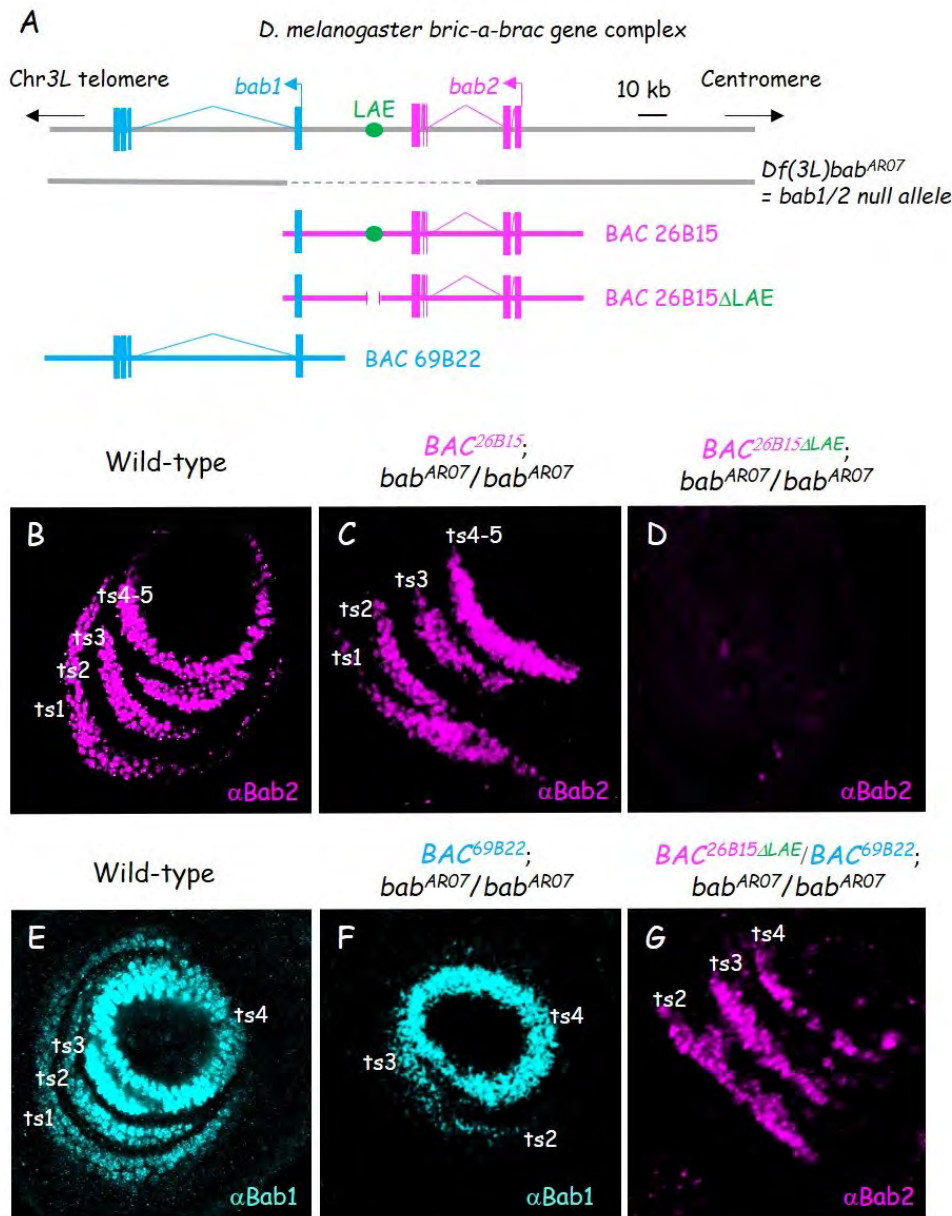


Fig 4. *bab1* includes partially-redundant limb-specific *cis*-regulatory information. (A) Chromosomal deficiency and BAC constructs covering the *bab* locus. The tandem gene paralogs and intergenic LAE are depicted as shown in Fig. 1A, except that *bab2* is depicted in pink instead of red. The *bab^{AR07}* 3L chromosomal deficiency is shown in beneath, with known deleted portion indicated by a dashed line. Note that the breakpoints have not been precisely mapped. The two overlapping BAC constructs *69B22* and *26B15*, as well as a mutant derivative of the latter specifically-deleted for LAE, are shown further in beneath. (B-G) Medial confocal views of wild-type (B-E) and homozygous *bab^{AR07}* mutant (C-D and F-G) L3 leg discs, harboring singly or combined X-linked BAC construct(s) shown in (A), as indicated above each panel. Bab2 (pink) and Bab1 (blue) immunostainings are shown. Positions of *bab1*- and *bab2*-expressing ts1-4 cells are indicated. Note stochastic *bab2* expression in (G).

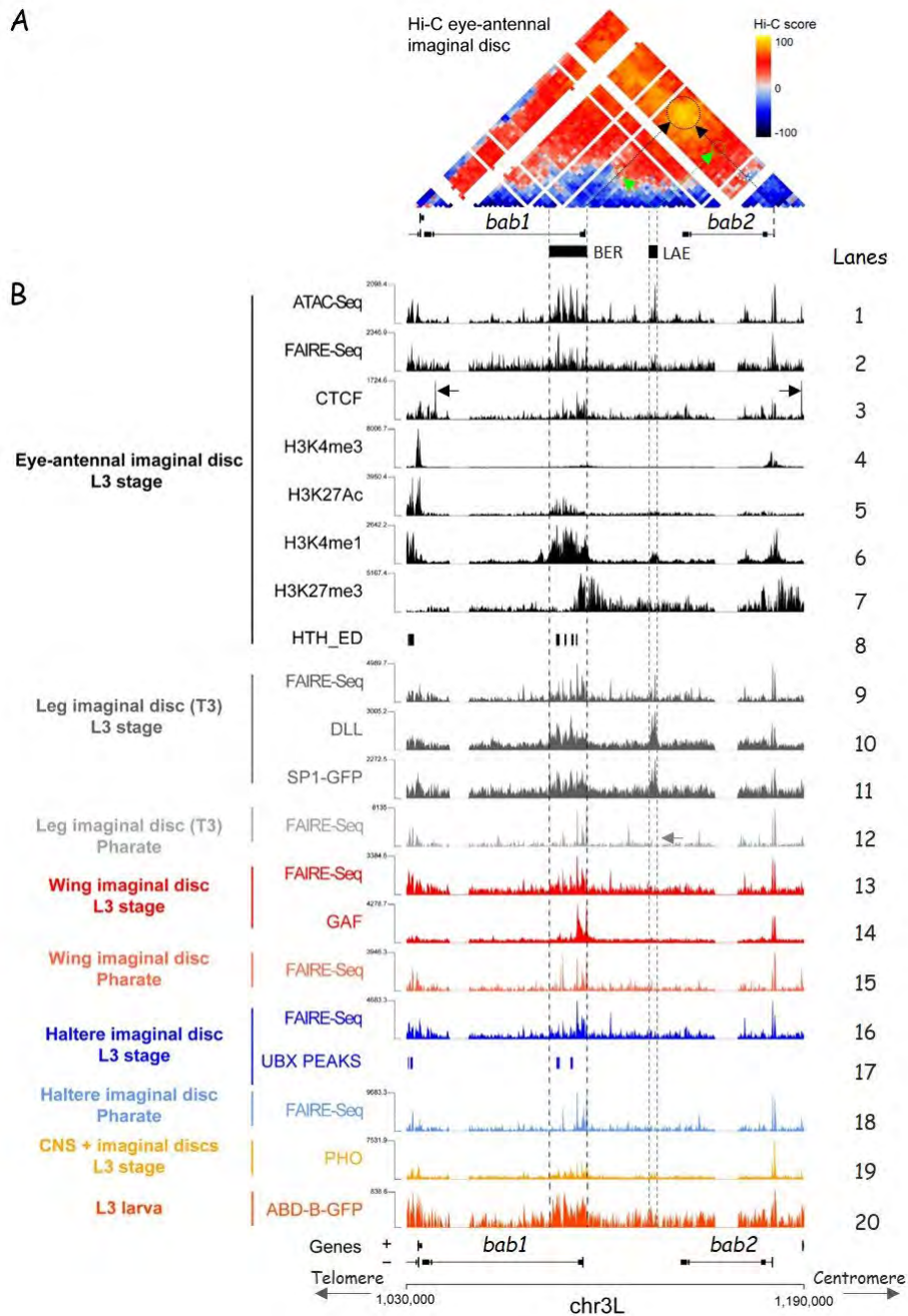


Fig 5. A topologically-associating domain encompasses the *bab* locus in the eye-antennal disc and genome-wide chromatin features identify an enhancer signature region (BER) within *bab1*. (A) Hi-C screenshot of a 160 kb region covering the *Dmel bab* gene complex. Score scale is indicated on the right (yellow to dark blue from positive to negative). (B) ATAC-, FAIRE- and/or ChIP-Seq profiles from L3 eye-antennal (ED), leg, wing and haltere discs as well as from adult pharate appendages (leg, wing and haltere) and from whole larval tissues, as indicated on the left side. As referred in the main text, lanes are numbered on the right side. ChIP-Seq peak calling data are shown in lanes 8, 17-18. Otherwise, normalized open chromatin, histone H3 post-translational modifications and TF binding profiles are shown. Positions of the tandem *bab1-2* genes are indicated on the bottom. The respective locations of the BER and LAE sequences are highlighted with vertical dashed lines. Of note, according to normalized FAIRE-Seq signals, LAE is not fully accessible in the pharate T3 leg (see grey arrow in lane 12). Strongest CTCF ChIP-Seq signals are indicated by horizontal black arrows (lane 3).

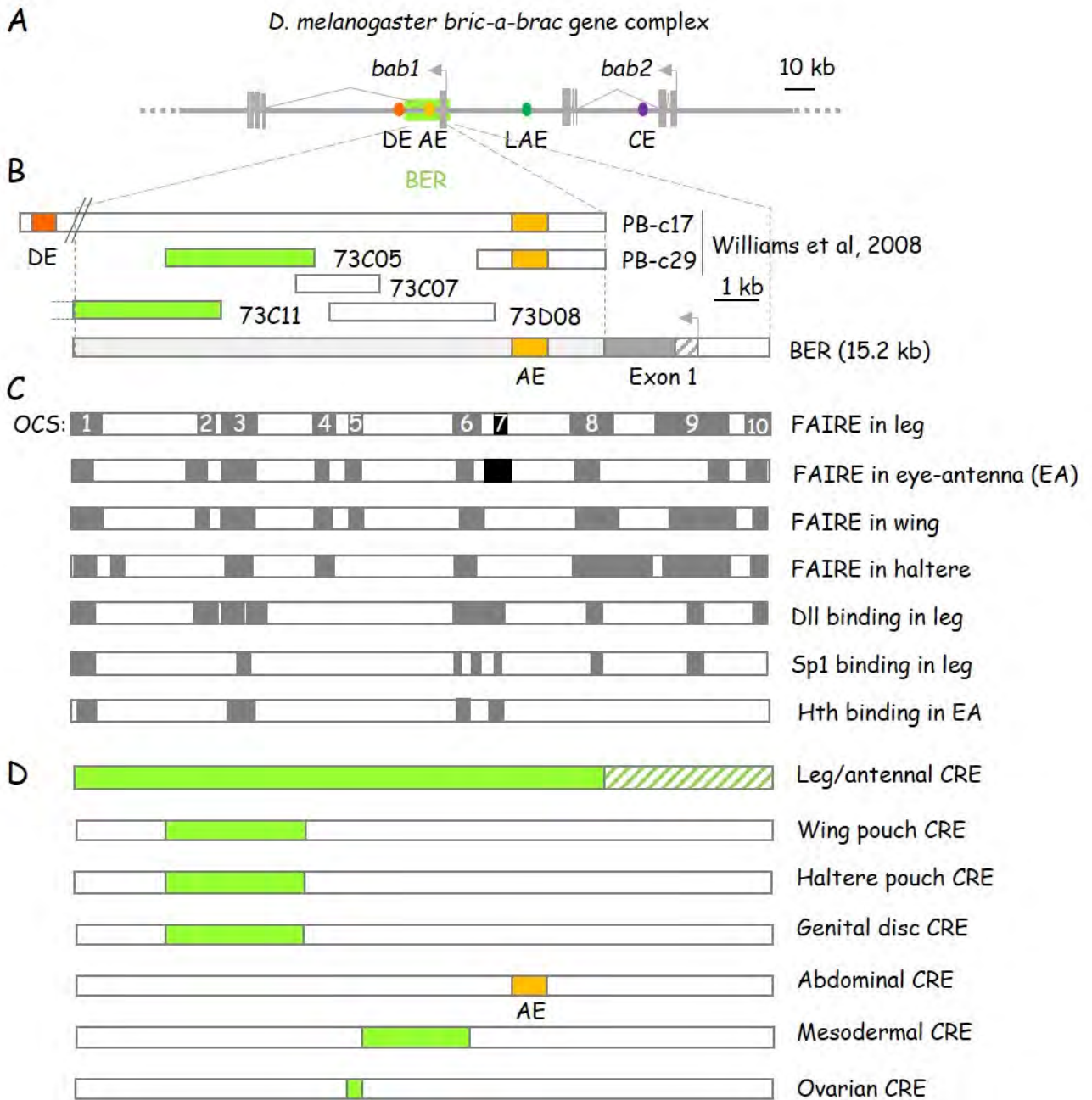


Fig 6. BER behaves as a composite pleiotropic enhancer. (A) The BER enhancer signature region includes the abdominal AE enhancer. Organization of the *Dmel bab* locus, with the tandem gene paralogs as depicted as in Fig. 2A. The characterized enhancers are depicted by filled dots (abdominal DE and AE in dark and light orange, respectively; leg/antennal LAE in dark green and cardiac CE in purple). BER is boxed in light green. The genomic portions of the overlapping *69B22* and *26B15* BAC constructs are shown in beneath. (B) Transgenic lines covering BER identify *cis*-regulatory elements driving reporter gene expression in diverse larval imaginal tissues. Genomic fragments covered by relevant Janelia Farm FlyLight reporter lines [77] and the DE- and/or AE-containing PB-c17 and PB-c29 genomic constructs, described in [14], are shown above a scheme of the BER region. *bab1* protein coding and 5'-untranslated sequences within the first exon are filled or hatched in dark grey, respectively, while the intronic region is in light gray. The AE sequence is in orange, as depicted in (A). FlyLight reporter lines driving reporter expression in diverse imaginal discs (see S4 Fig) are filled in light green. (C) BER includes open chromatin sequences (OCS) and is bound by Dll, Sp1, Hth TFs in diverse developing appendages (leg, eye-antenna, wing and haltere). OCS and TF-bound sequences are depicted by filled grey/black boxes. Numbers refer to OCSs detected in the leg discs (see main text). The black boxes represent OCSs detected in the eye-antennal (EA) and leg but not in wing and haltere discs. OCS and Dll or Sp1-bound regions, as determined from peak calling (FAIRE-Seq GSE38727 and ChIP-Seq GSE113574 GEO dataset series, respectively), are from [40] and [42], respectively. ChIP-Seq data for Hth are from [78]. (D) BER includes pleiotropic *cis*-regulatory elements (CREs). Locations of predicted CREs (see text) are indicated by light green boxes. The hatched part of the predicted leg/antennal CRE is inferred from data obtained with the PB-c17 construct reported in [14].

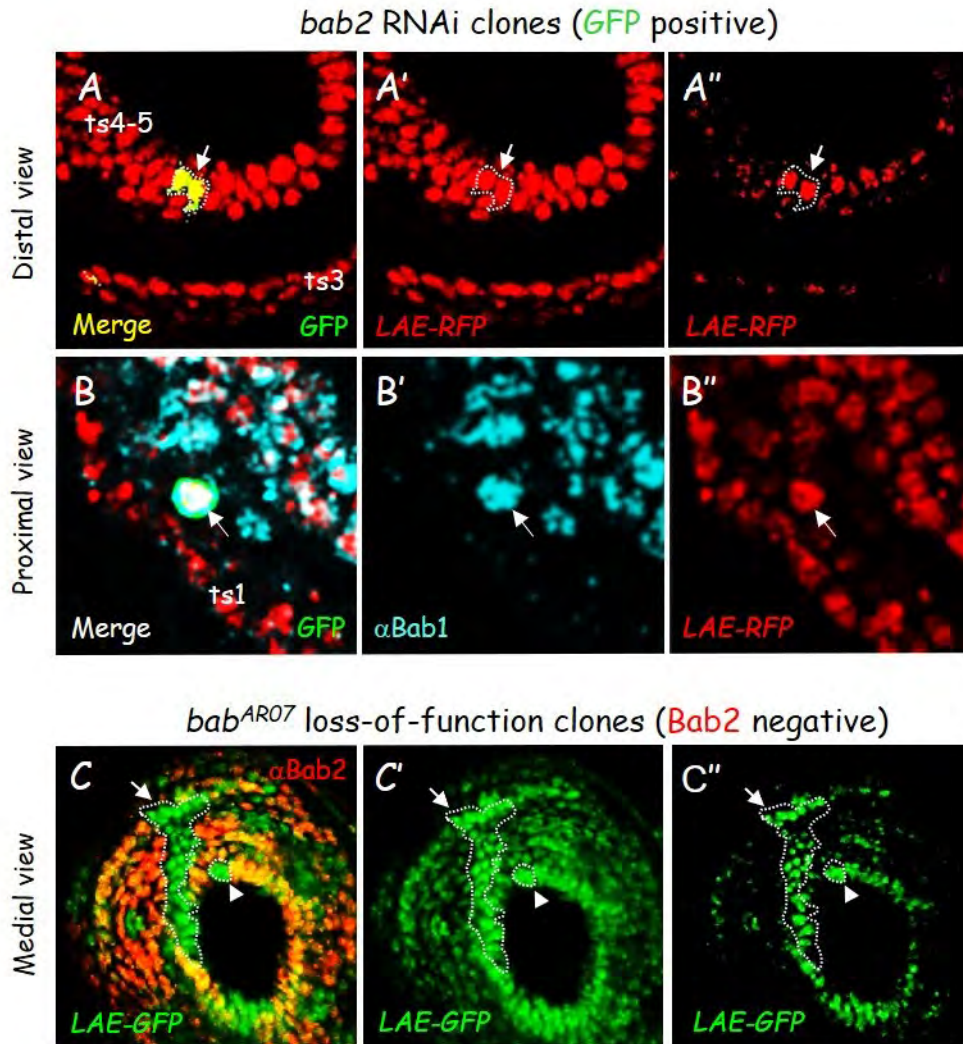


Fig 7. Auto- and cross-regulation among the two *bab* paralogs in the developing legs. (A) Distal confocal view of a L3 leg disc expressing *LAE-RFP* and harboring flip-out (FO) clones expressing interfering RNA against *bab2*. Merged RFP (red) and GFP (green) fluorescence, as well as the former in isolation and under two distinct signal magnification (A' and A''), are shown. A FO clone (GFP+) within *LAE-RFP* (*bab2*)-expressing ts4 cells is circled with a dashed line (see arrow). (B) Proximal confocal view of a L3 leg disc expressing *LAE-RFP* and harboring FO clones expressing interfering RNA against *bab2*. Merged Bab1 (blue) immunostaining, RFP (red) and GFP (green) fluorescence, as well as the two formers in isolation, in (B') and (B''), respectively, are shown. Note that a single-cell FO clone (GFP+), within *LAE-RFP* (*bab2*)-expressing ts1 cells, is sufficient to upregulate *bab1* (see arrows). (C-D) L3 leg (C) and eye-antennal (D) discs expressing *LAE-GFP* and harboring mutant clones for the protein null allele *bab^{AR07}*. Merged Bab2 (red) immunostaining and GFP (green) fluorescence as well as the latter in isolation under two distinct signal magnifications (C'-C'' and D'-D''), are shown. Tiny and larger clones (Bab2 negative) are circled with dashed lines (arrowheads and arrows, respectively).

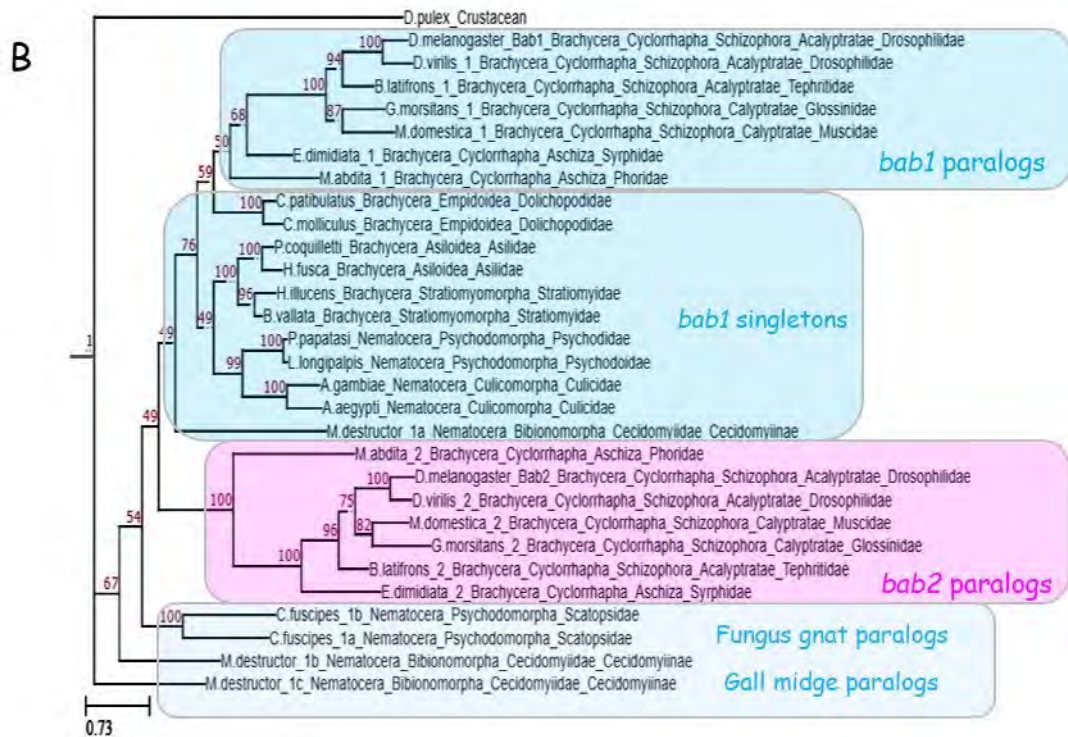
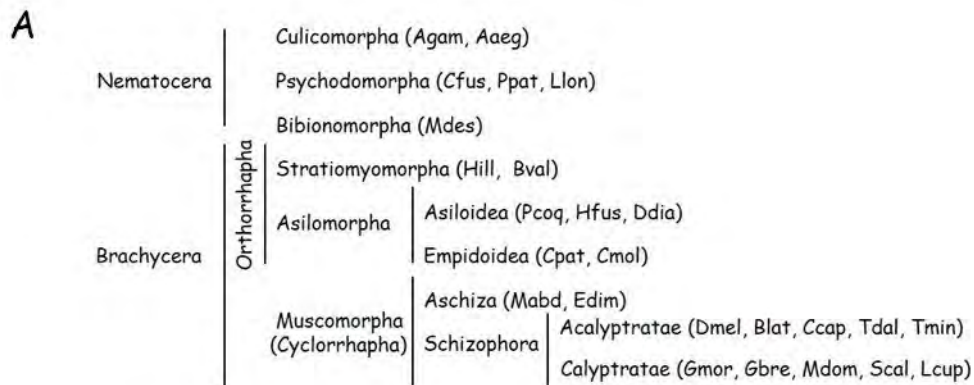


Fig 8. Phylogenetic relationships among dipteran *bab* paralogs and orthologs. (A) Dipteran families studied in this work. Species abbreviations are described in Supplementary data. (B) Phylogenetic relationships of the *bab* paralogs and orthologs inferred from a maximum likelihood consensus tree constructed from 1000 bootstrap replicates. Support values (percentage of replicate trees) are shown in red. Scale bar represents substitution per site. Clustered positions of *bab2* paralogs and *bab1* paralogs/orthologs are shown in pink and light blue, respectively.

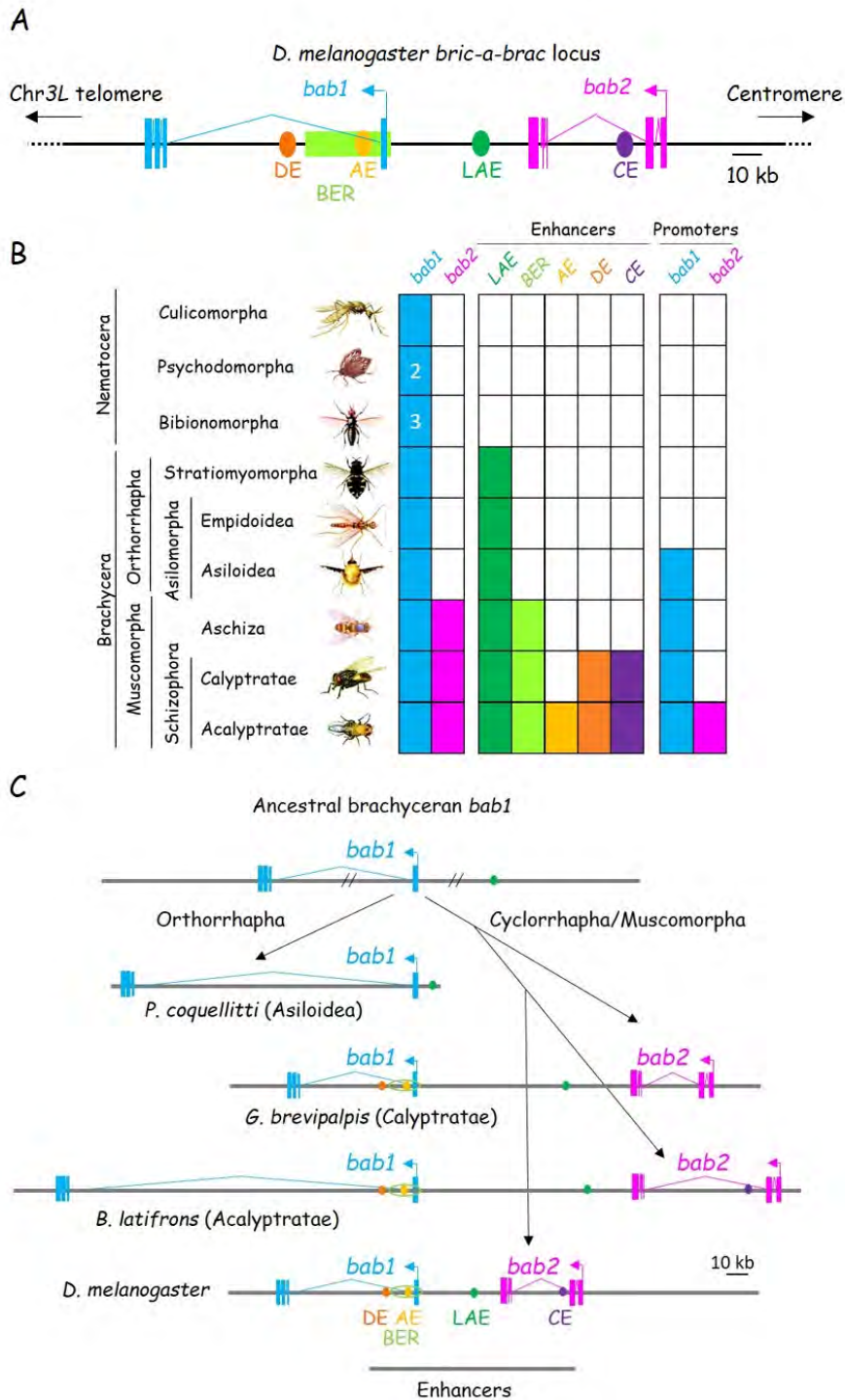


Fig 9. Conservation of enhancer/promoter sequences and evolutionary history of the *bab* locus among the Brachycera. (A) Organization of the *Dmel* *bab* gene paralogs and enhancers. The locus is depicted as in Fig 1A, except that *bab2* is represented in pink instead of red. (B) Evolutionary conservation of the *bab* gene paralogs, enhancers and promoters among diverse dipterans. Infraorders, sections, subsections and superfamilies are indicated on the left, arranged in a phylogenetic series from the “lower” Nematocera to the “higher” Brachycera suborders. Presence of *bab1* and/or *bab2* paralogs and conservation of enhancer and promoter sequences are indicated by filled or hatched boxes colored as depicted in (A). (C) Evolutionary scenario for the *bab* locus within the Brachycera suborders. A scheme depicting chromosomal fate of an ancestral *bab1*-like gene which gives rise to derived extant orthorrhaphan singletons (Asilomorpha) and Muscomorpha-specific paralogous (Calypttratae and Acalypttratae) genes. Locations of conserved enhancer sequences are shown, as depicted in (A).

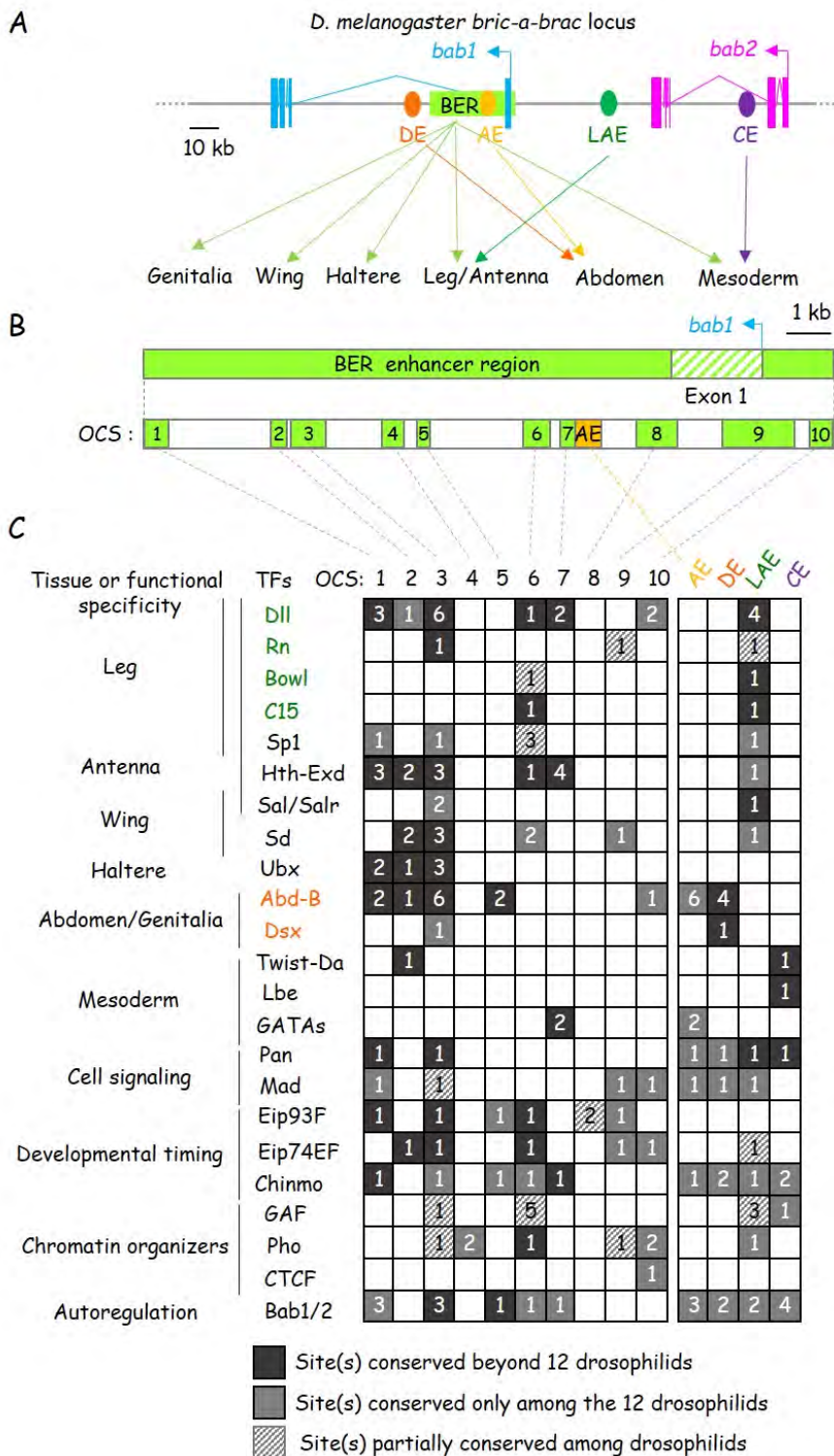
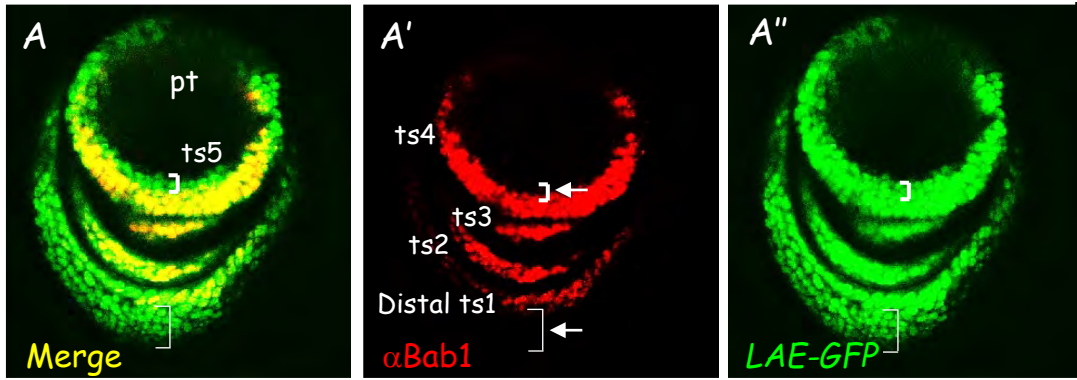
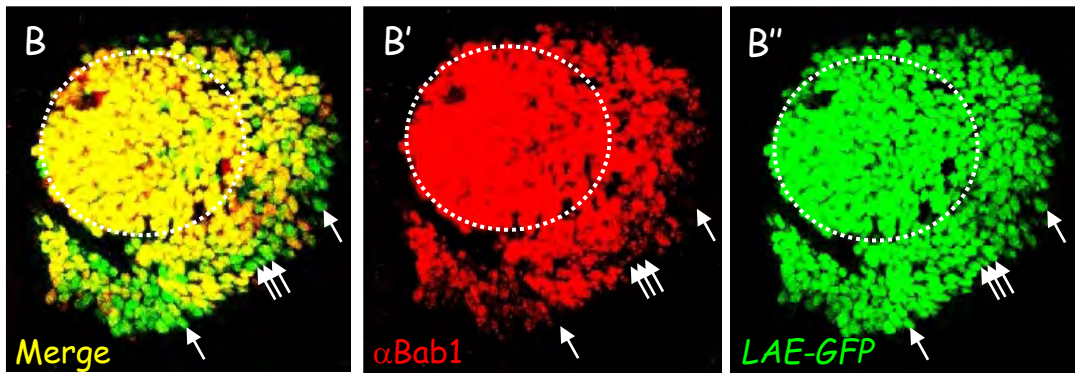


Fig 10. A comprehensive predictive tissue-specific TF code governing *bab* paralog expression. (A) Organization of the *Dmel bab* gene paralogs and enhancers, as depicted in Fig 9A. Expression tissue specificities conferred by each enhancer are shown in beneath. (B) BER structural and chromatin state organizations. The *bab1* first exon is hatched. OCS regions (see Fig 6C), as defined in leg tissues; are represented by light green boxes. The abdominal AE CRE (not detected in FAIRE-Seq data from leg and eye-antennal discs) is depicted as a light orange box. (C) Evolutionary conservation of predicted TF binding sites within *Dmel bab* cis-regulatory sequences. Site conservation among and beyond drosophilids of transcriptional regulators involved in tissue-specific morphogenetic processes, cell signaling, developmental timing and chromatin organization. Predicted/validated TF site numbers are indicated within colored or hatched boxes reflecting their relative conservation are indicated in beneath. Experimentally-validated direct *bab* regulators are colored according to their well characterized bound-enhancer sequences (see A).

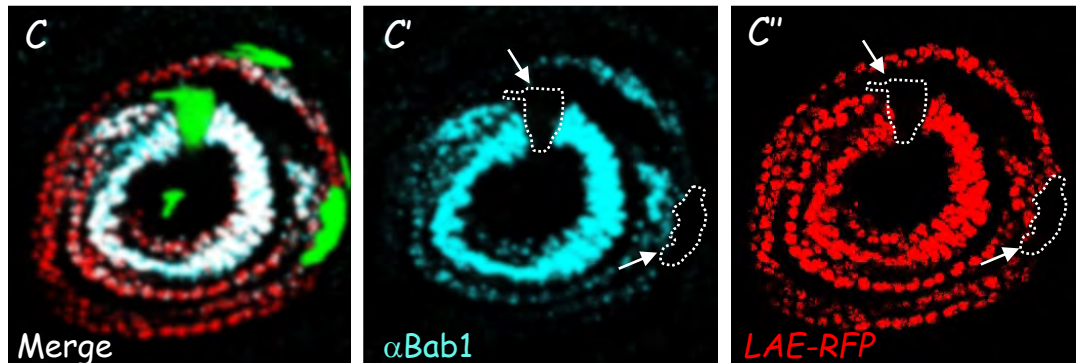
Wild-type leg disc



rotund gain-of-function (Dll domain)

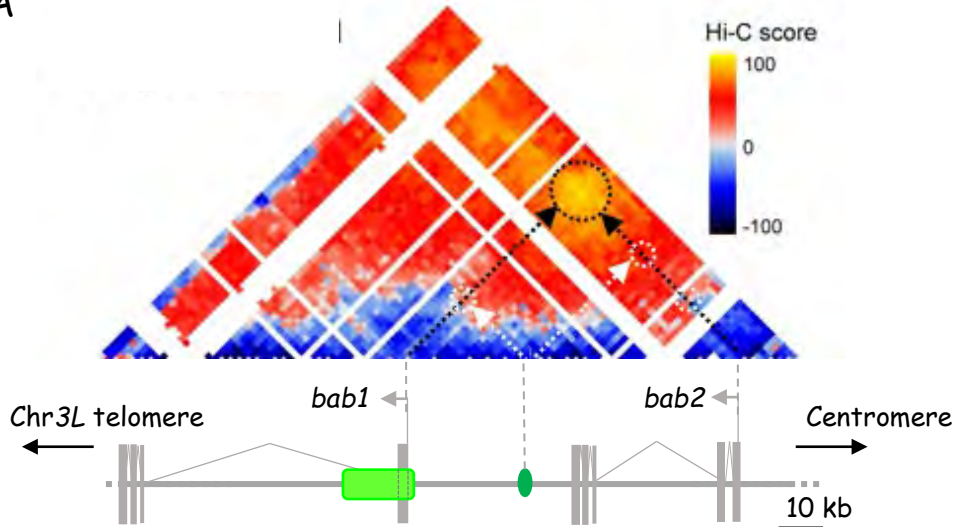


bowl gain-of-function clones (FO lines RNAi; GFP+)

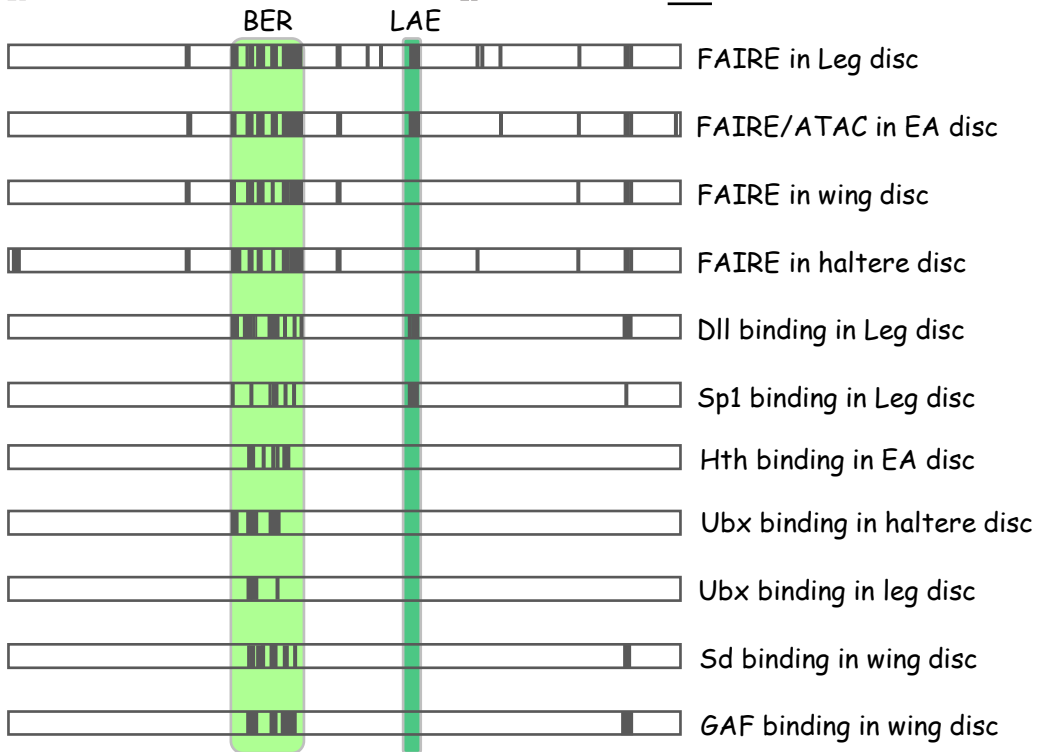


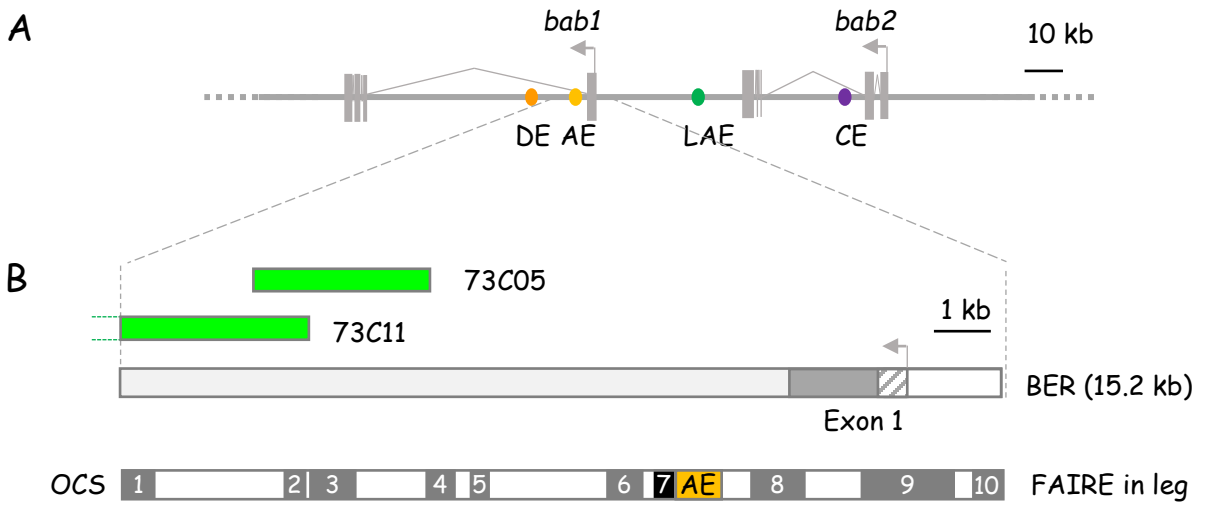


A

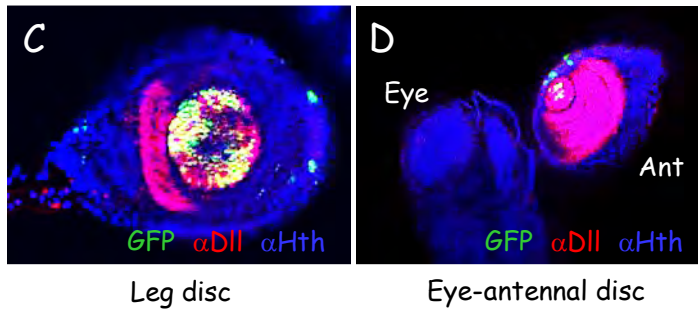


B

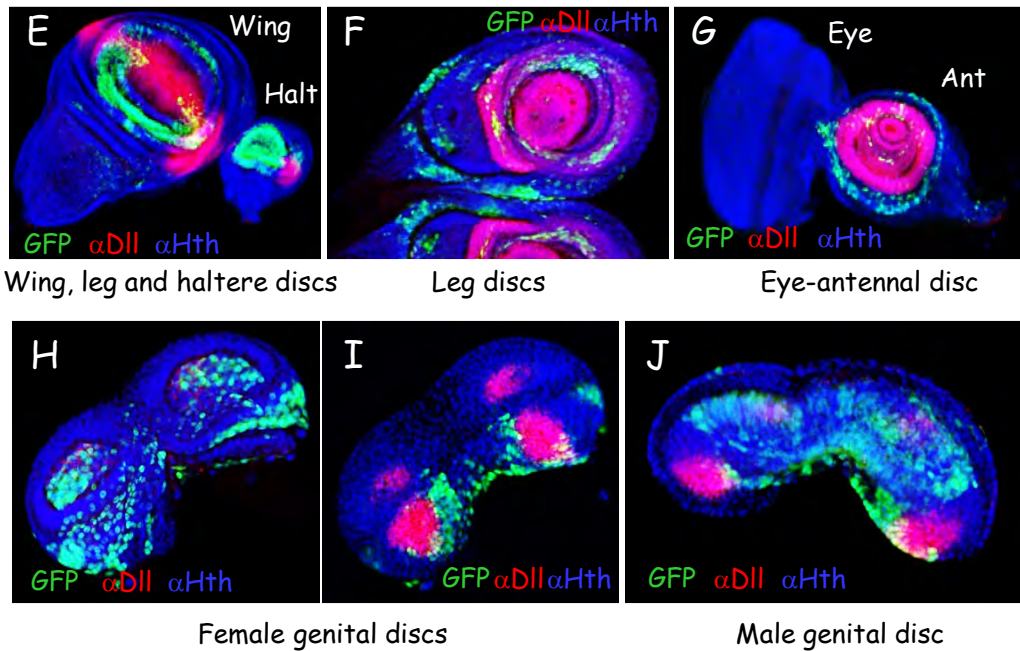


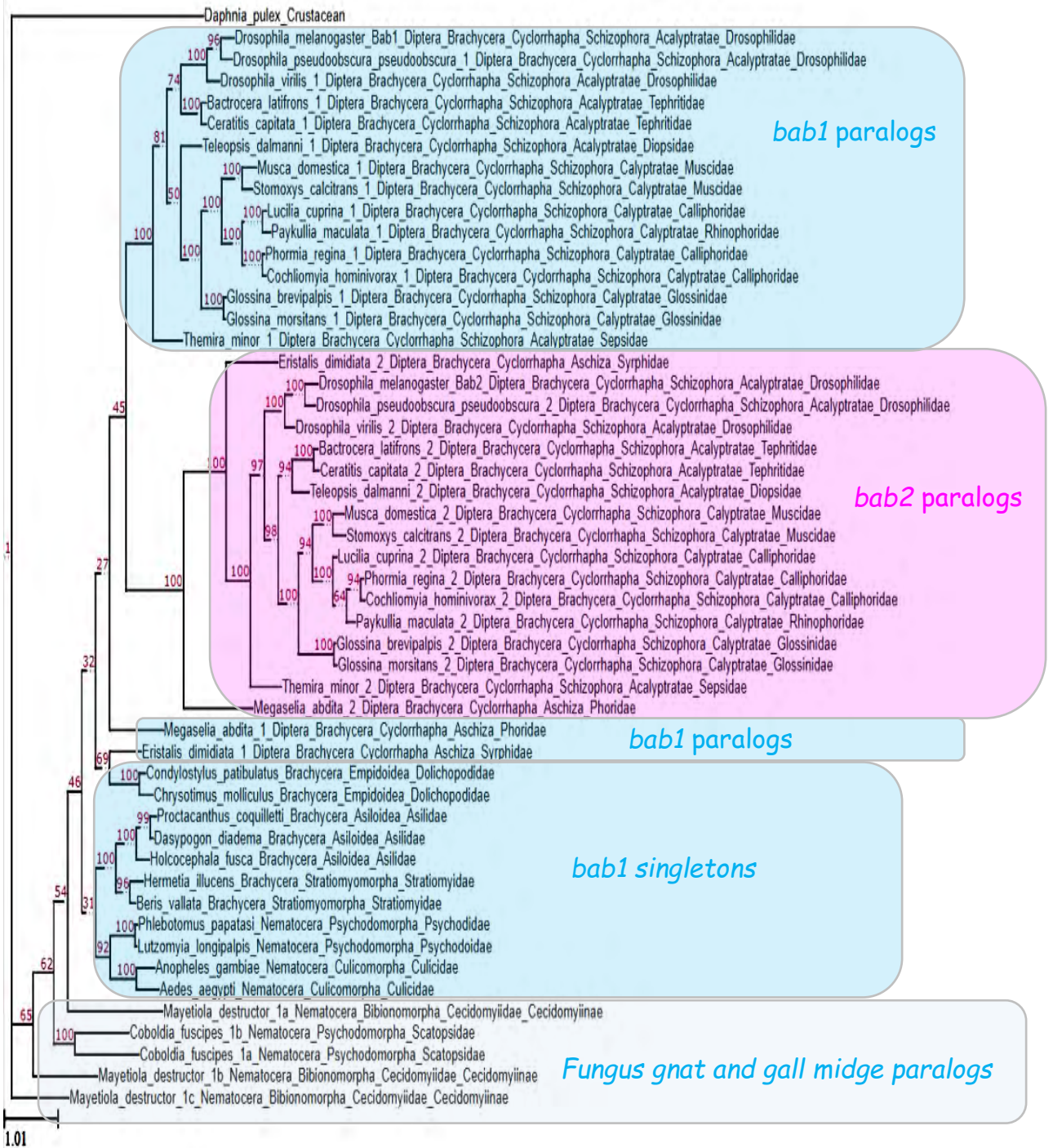


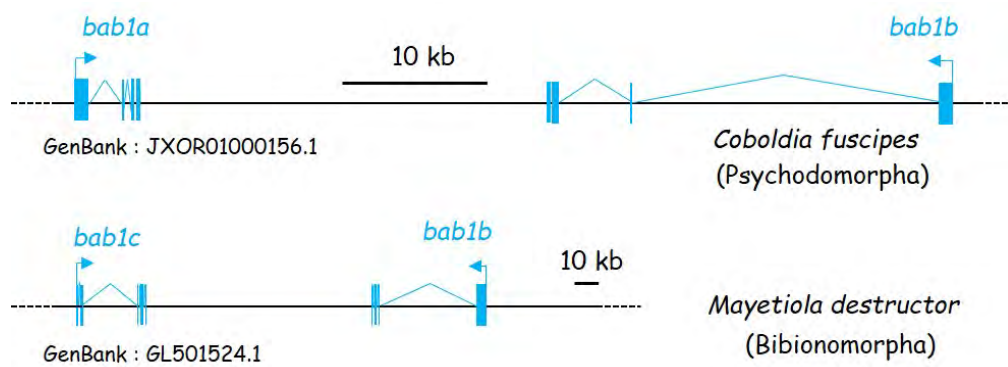
73C11-Gal4 + UAS-GFP



73C05-Gal4 + UAS-GFP



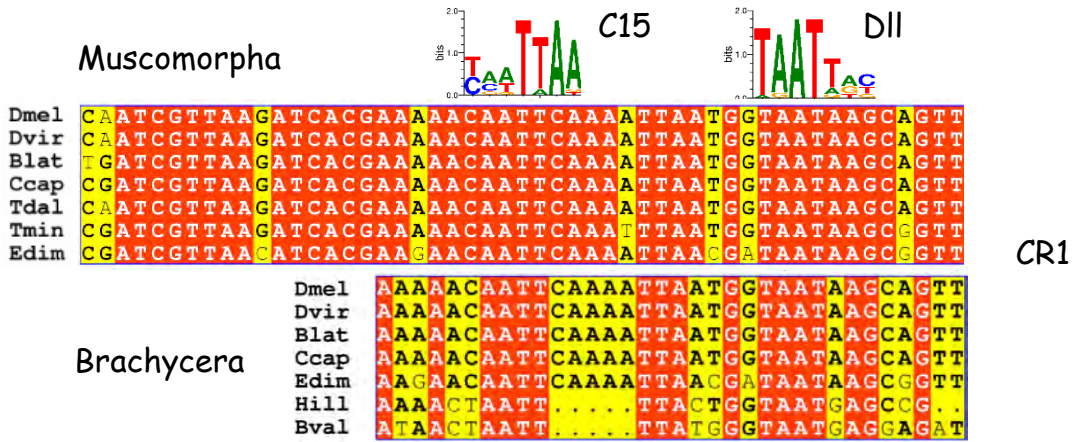




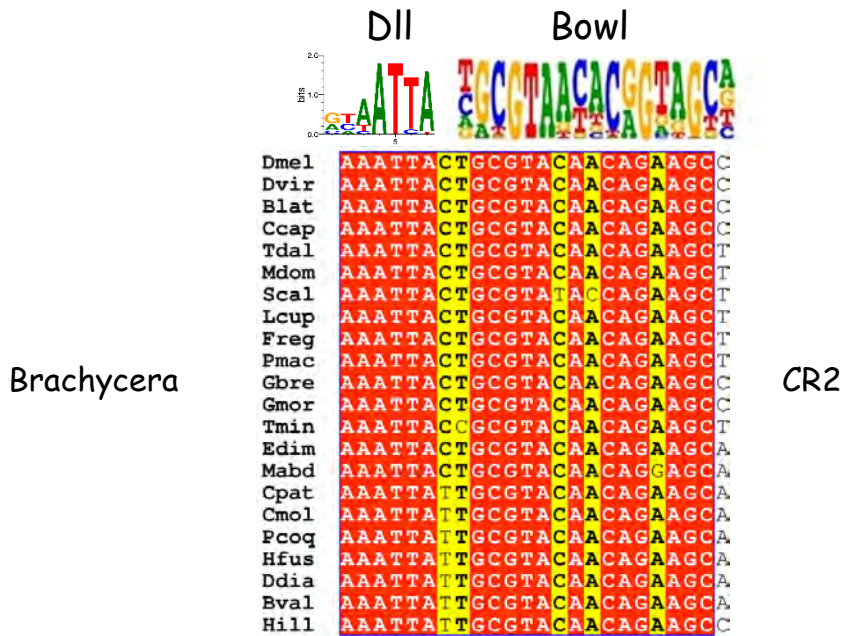
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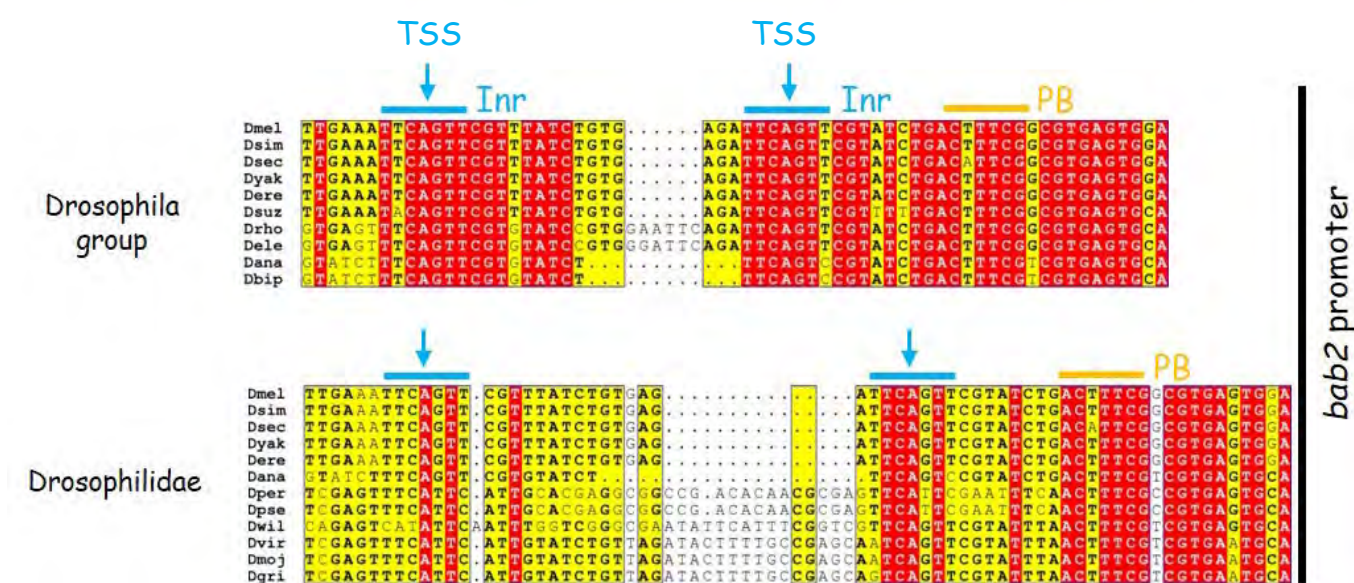
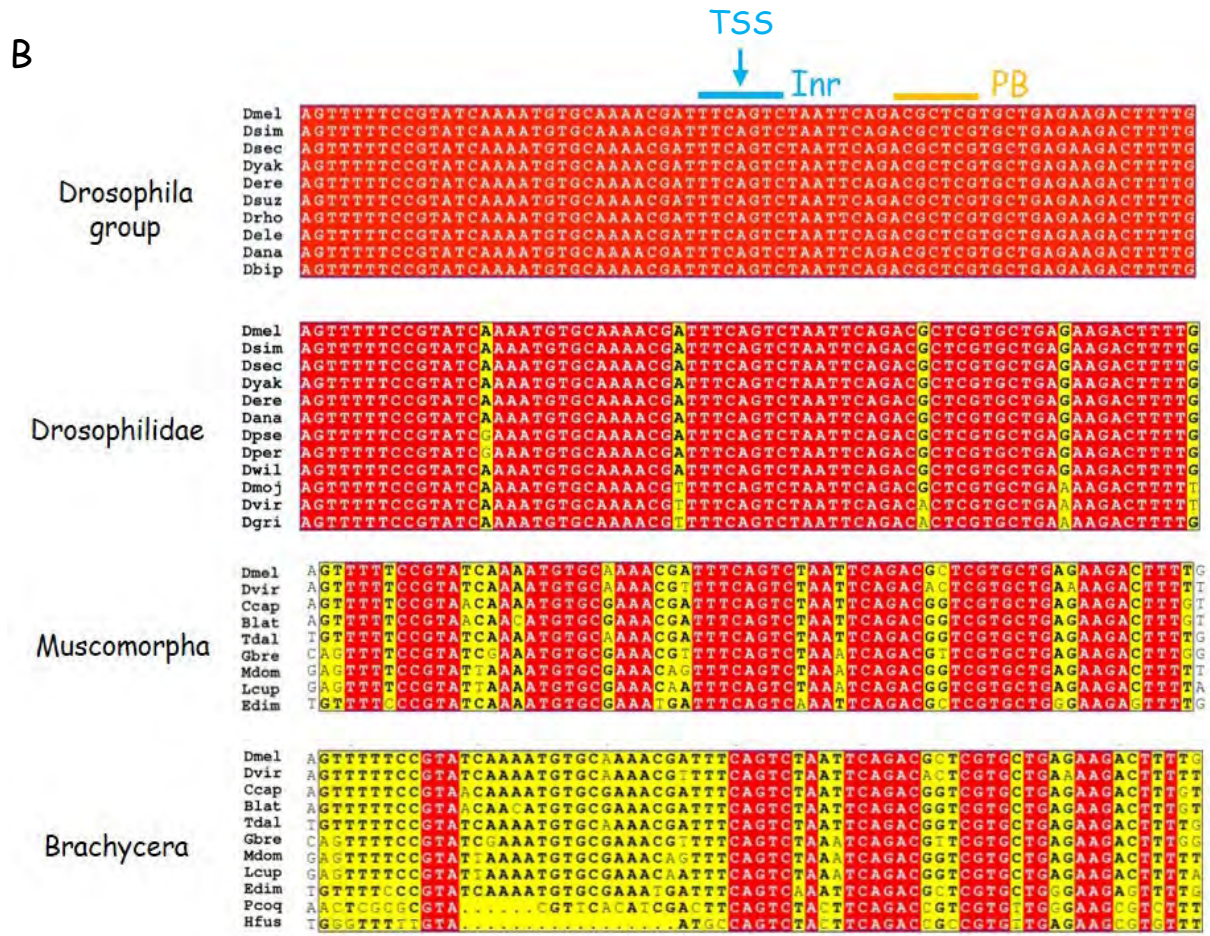
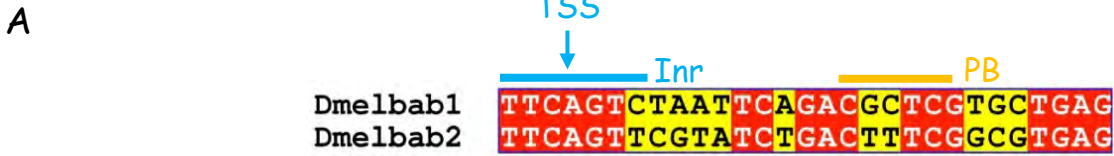


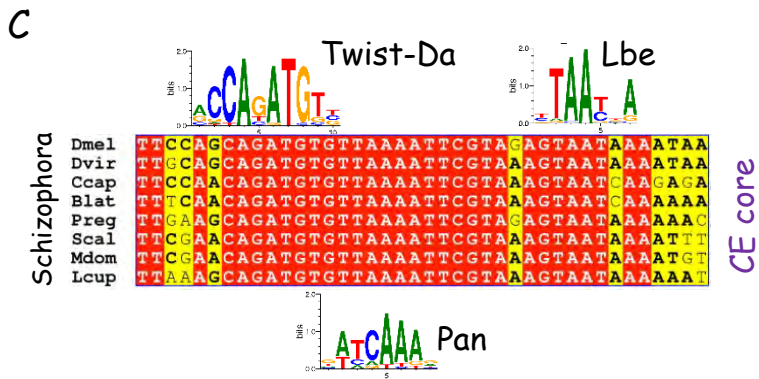
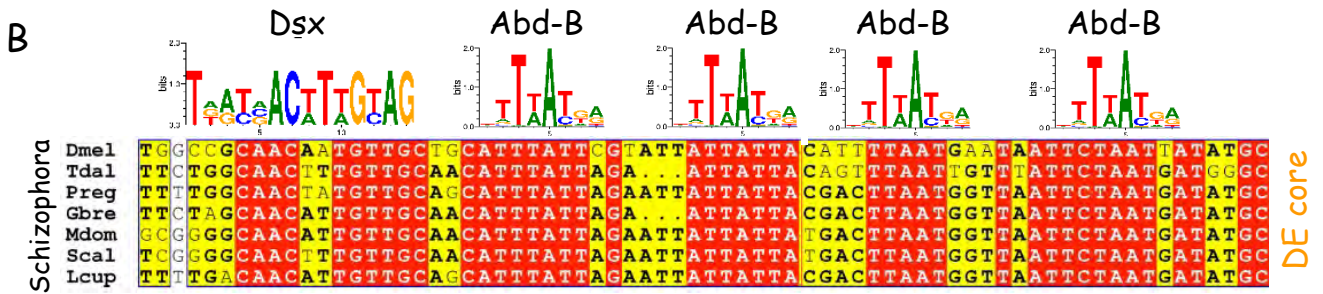
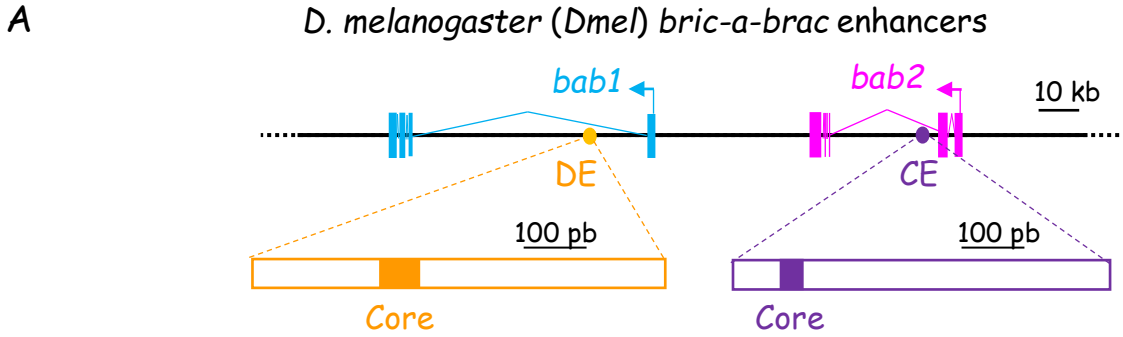
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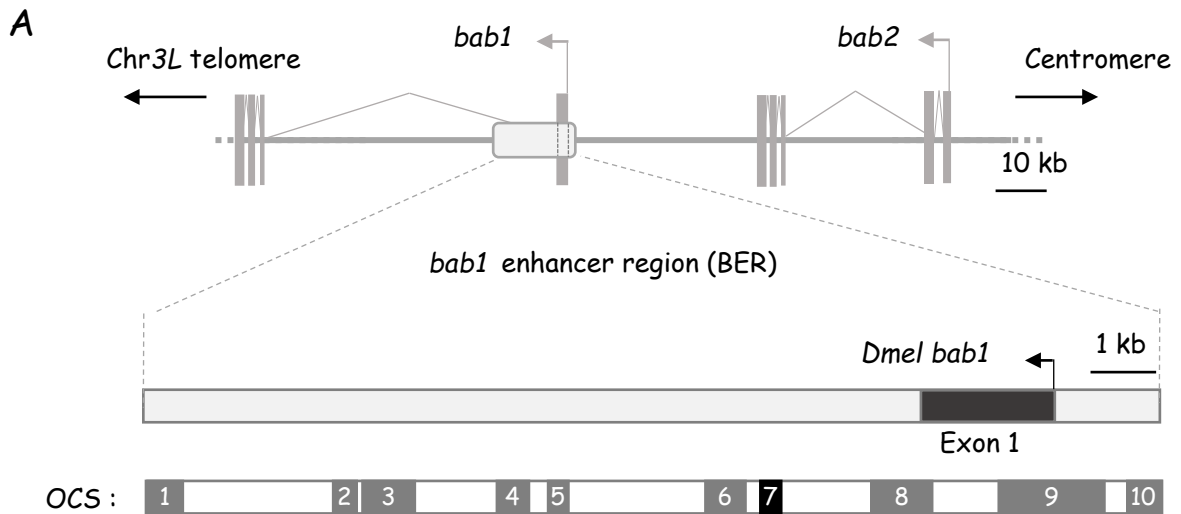


C








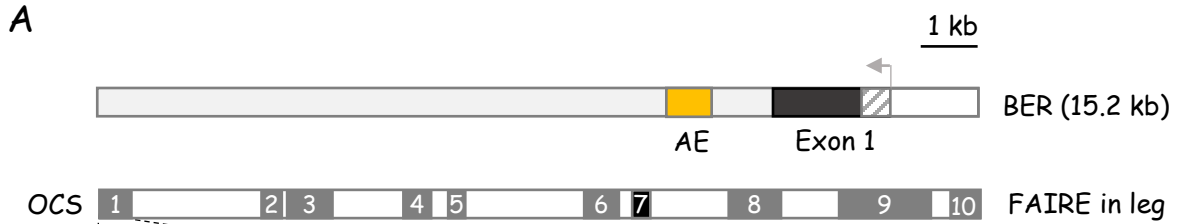


B Muscomorphan families OCS : 1 2 3 4 5 6 7 8 9 10

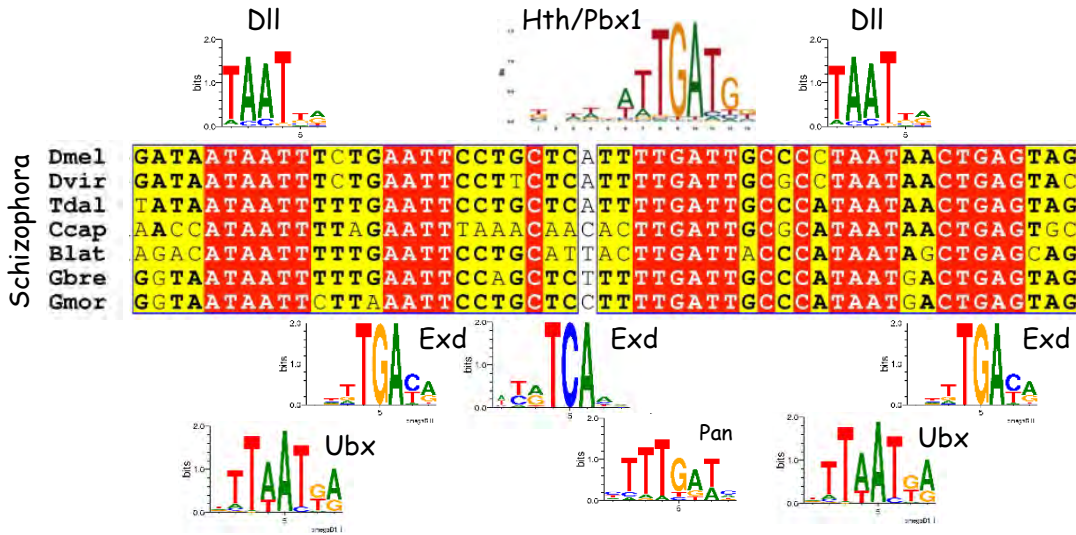
Schizophora	Acalypttratae	<i>Drosophilidae</i>	4	2	8	1	4	3	6	2	4	3
		<i>Tephritidae</i> (<i>Blat</i> , <i>Ccap</i>)	2	1	5		1	1	5		1	
		<i>Diopsidae</i> (<i>Tdal</i>)	2	1	5		1	1	5		1	
		<i>Sepsidae</i> (<i>Tmin</i>)	1		2			1	1		1	
	Calypttratae	<i>Glossinidae</i> (<i>Gbre</i> , <i>Gmor</i>)	1		3			1	3		1	
		<i>Muscidae</i> (<i>Mdom</i> , <i>Scal</i>)		1	1			1	1		1	
		<i>Calliphoridae</i> (<i>Lcup</i> , <i>Preg</i>)		1	2			1	3		1	
		<i>Rhinophoridae</i> (<i>Pmac</i>)		1	2			1	2		1	
		Aschiza	<i>Syrphidae</i> (<i>Edim</i>)			1						1
<i>Phoridae</i> (<i>Mabd</i>)				1						1		

 Sequence element(s) conserved among 12 *Drosophilids* (dubbed CS)

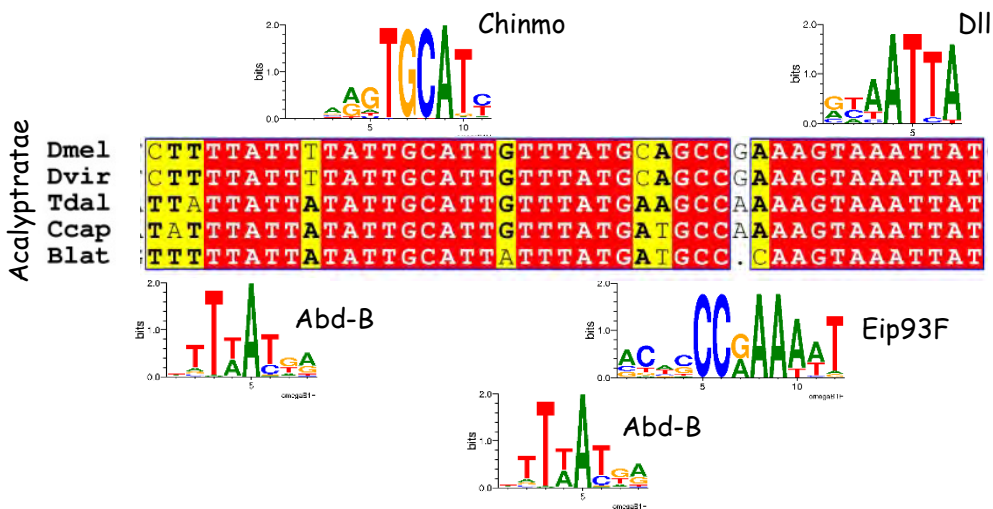
 CS element(s) conserved beyond *Drosophilidae*

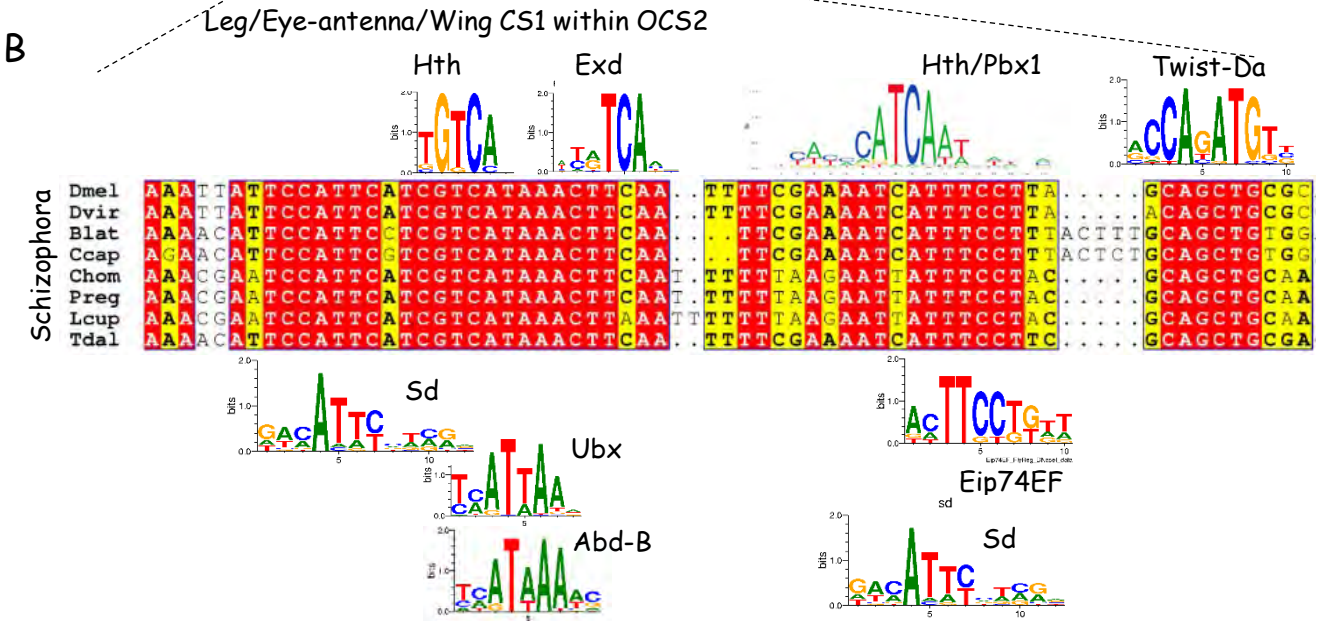
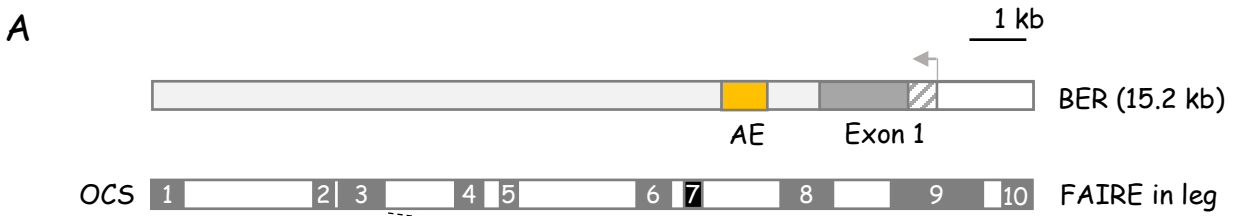


B Leg/Eye-antenna/Wing/Haltere CS2 within OCS1

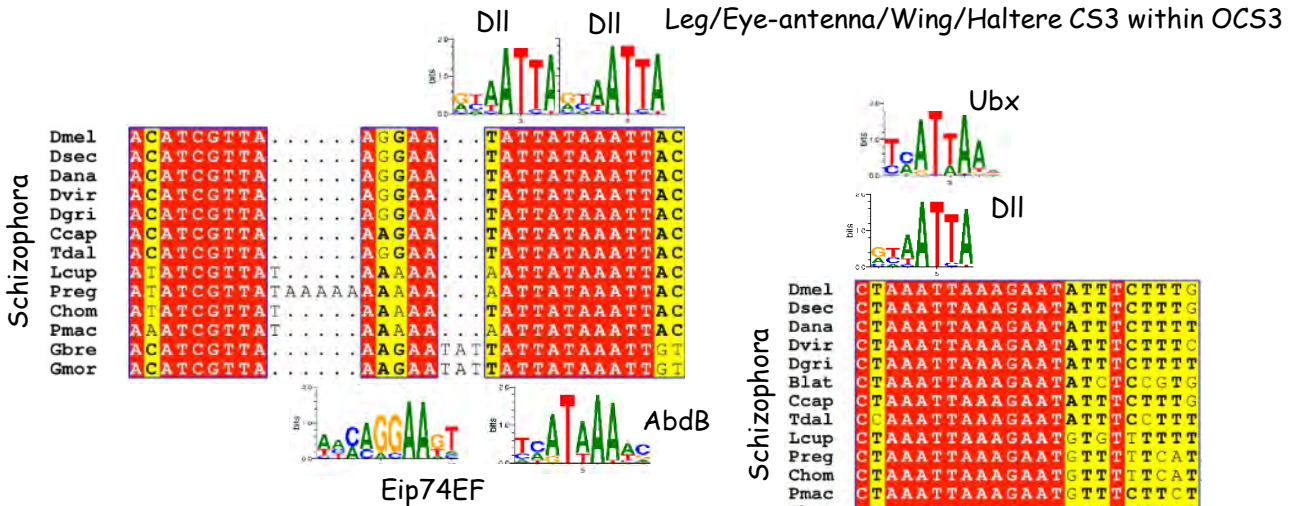


Leg-specific CS4 within OCS1

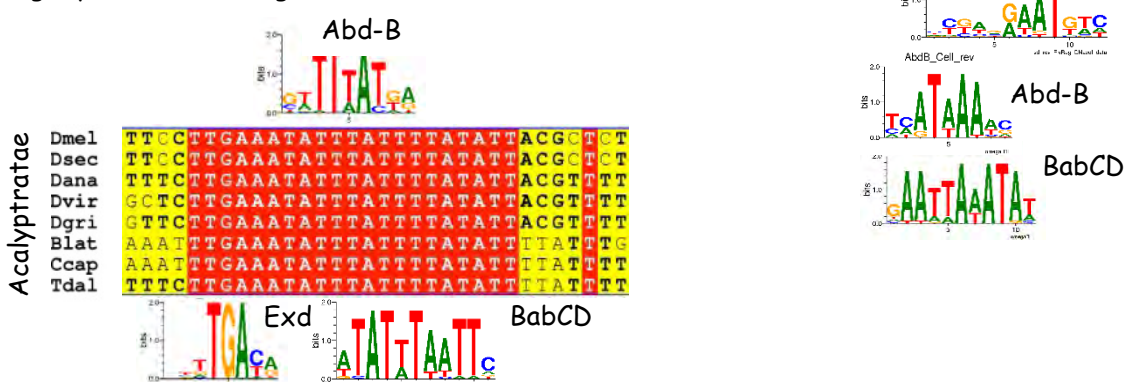


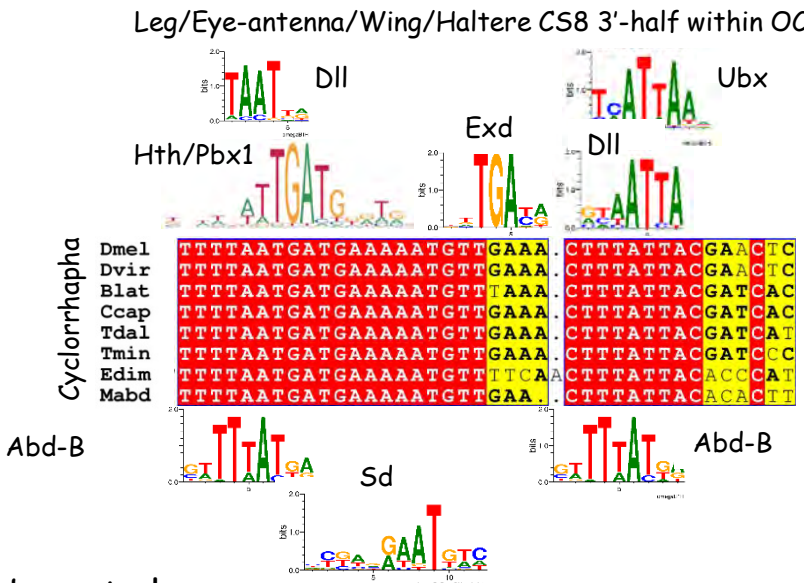
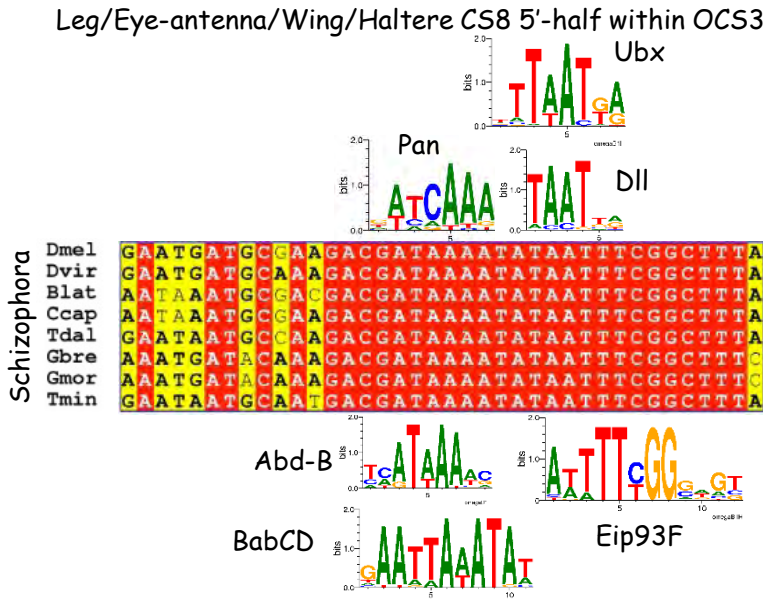
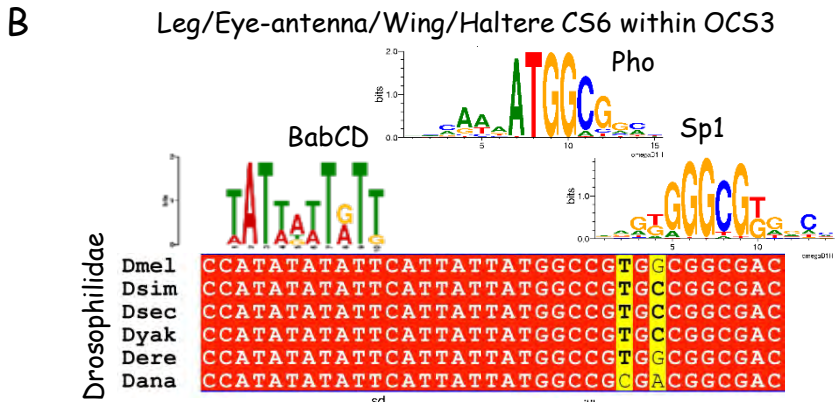
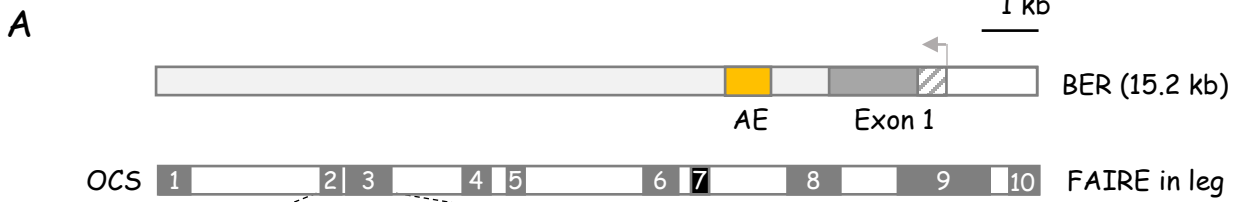


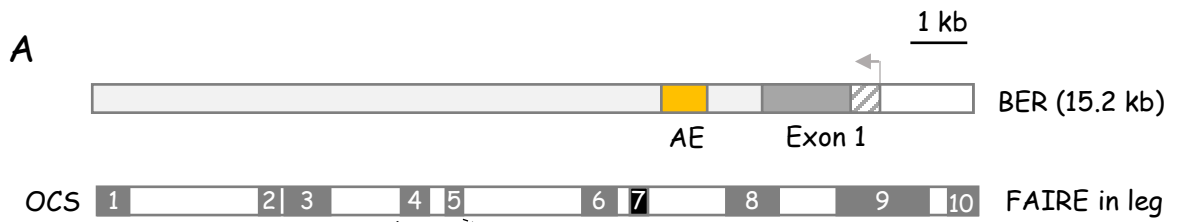
Leg/Eye-antenna/Wing/Haltere CS1 within OCS3



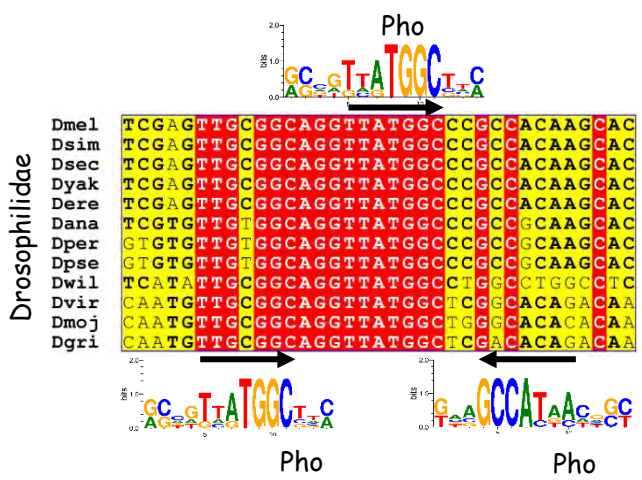
Leg/Eye-antenna/Wing/Haltere CS2 within OCS3



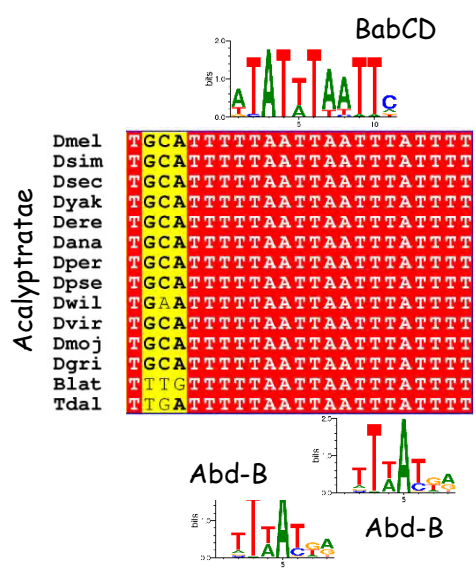


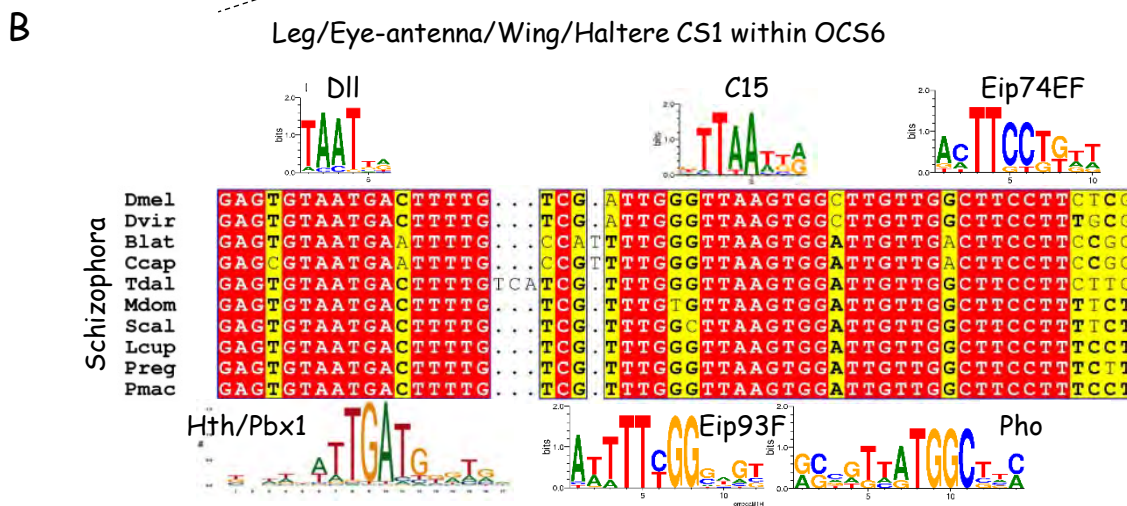
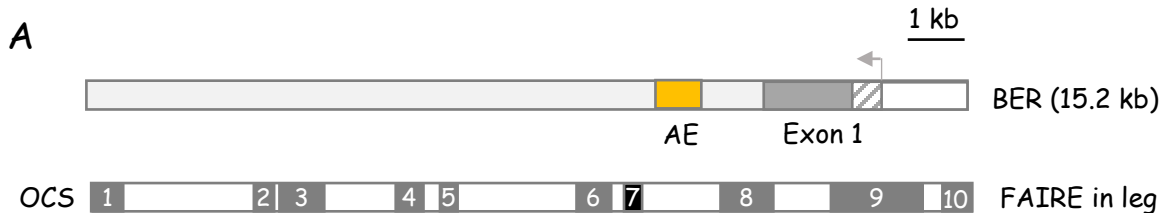


B Leg/Eye-antenna/Wing/Haltere CS1 within OCS4

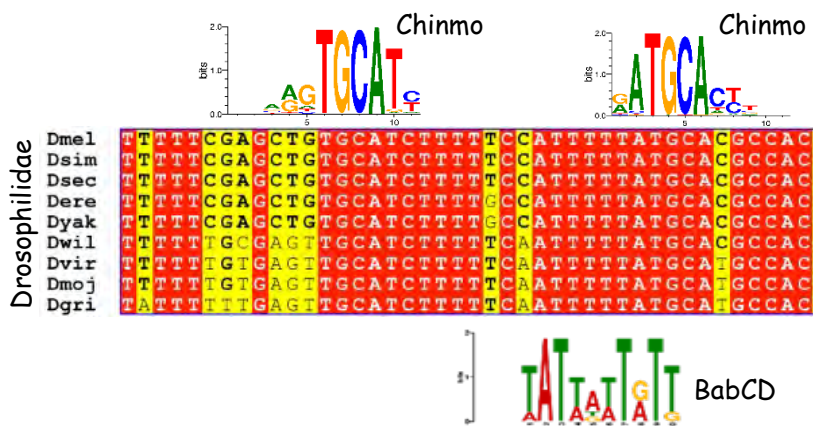


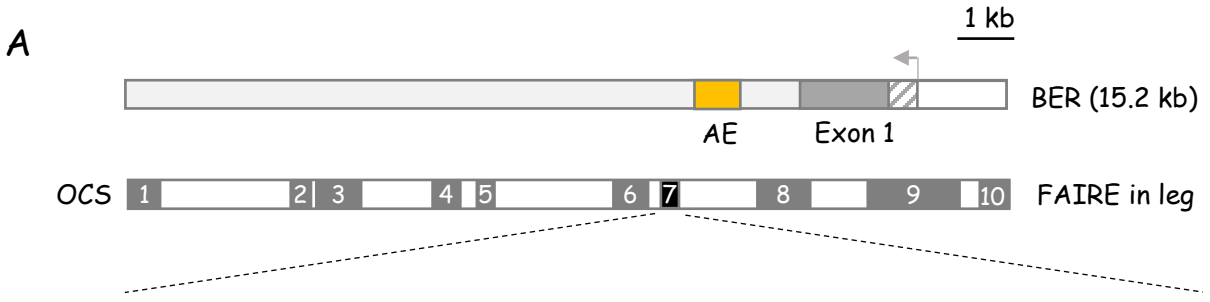
Leg/Eye-antenna/Wing CS1 within OCS5





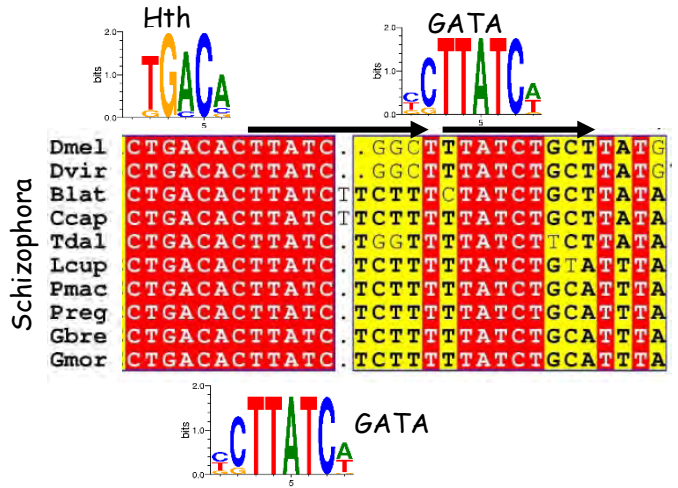
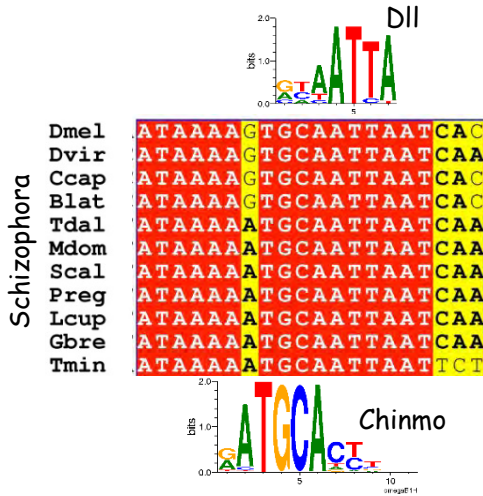
Leg/Eye-antenna/Wing/Haltere 3' next-to-CS1 within OCS6





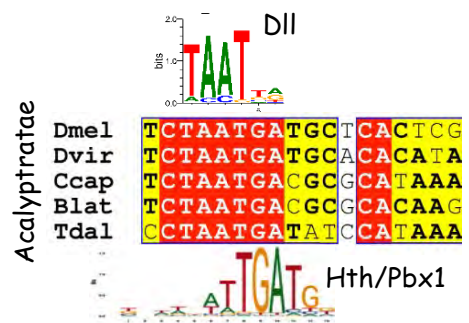
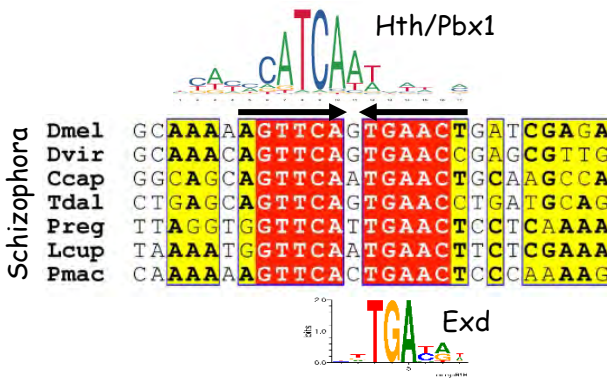
B Leg and eye-antenna-specific CS4 within OCS7

Leg and eye-antenna-specific CS5 within OCS7

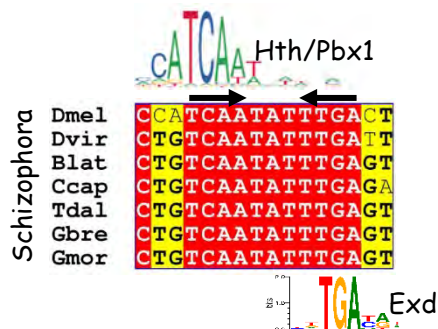


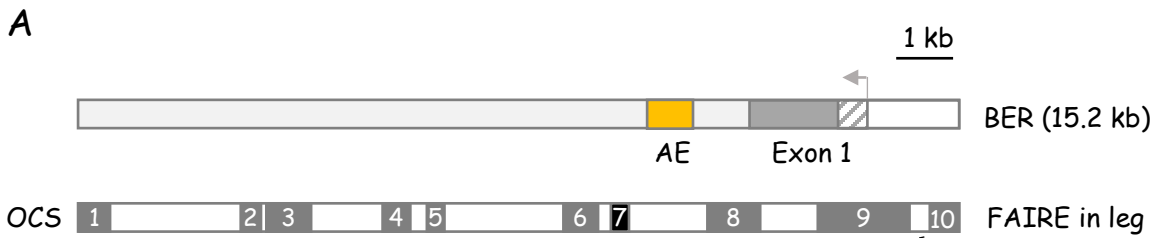
Eye-antenna-specific CS1 within OCS7

Eye-antenna-specific CS2 within OCS7



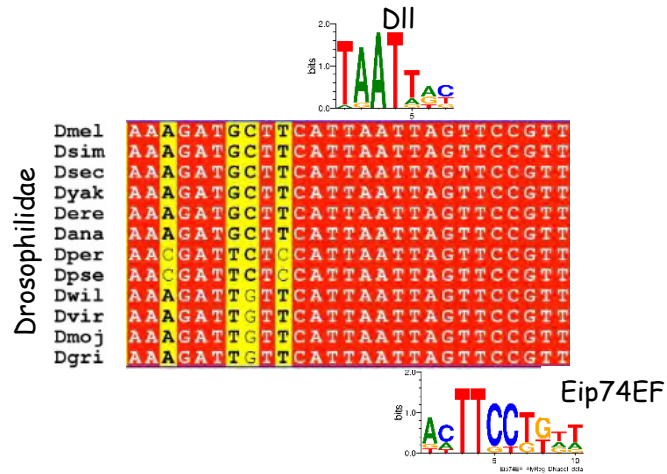
Eye-antenna-specific CS6 within OCS7



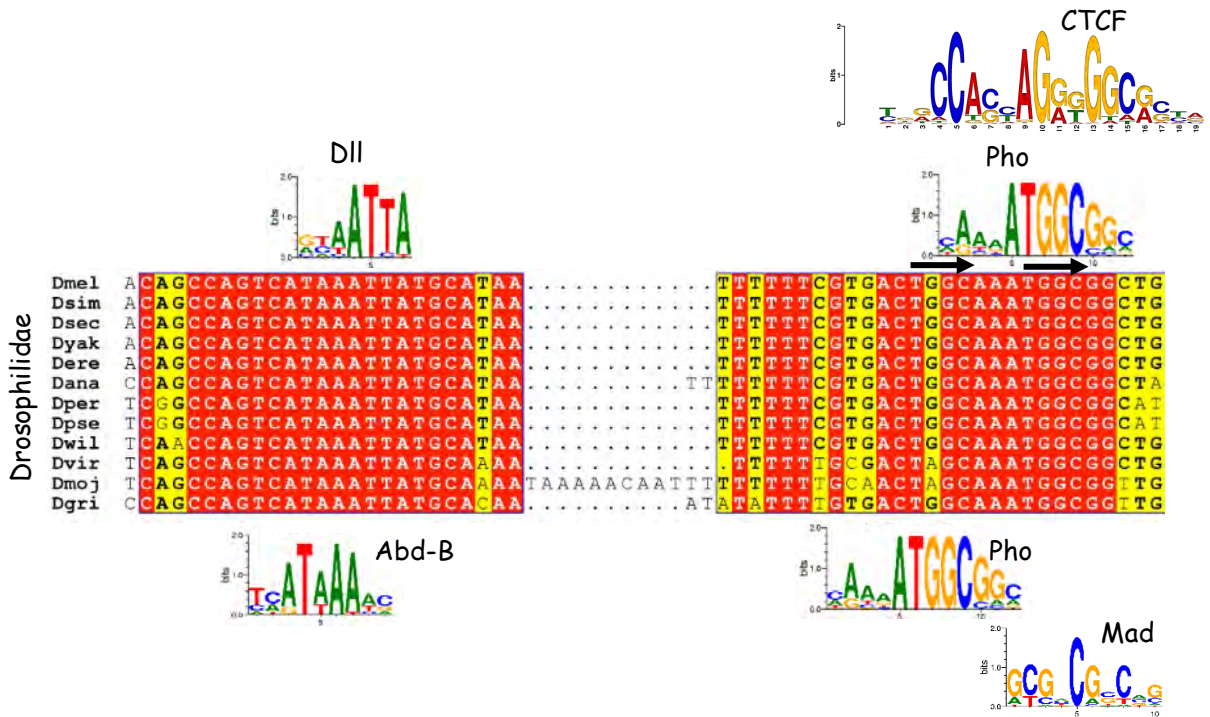


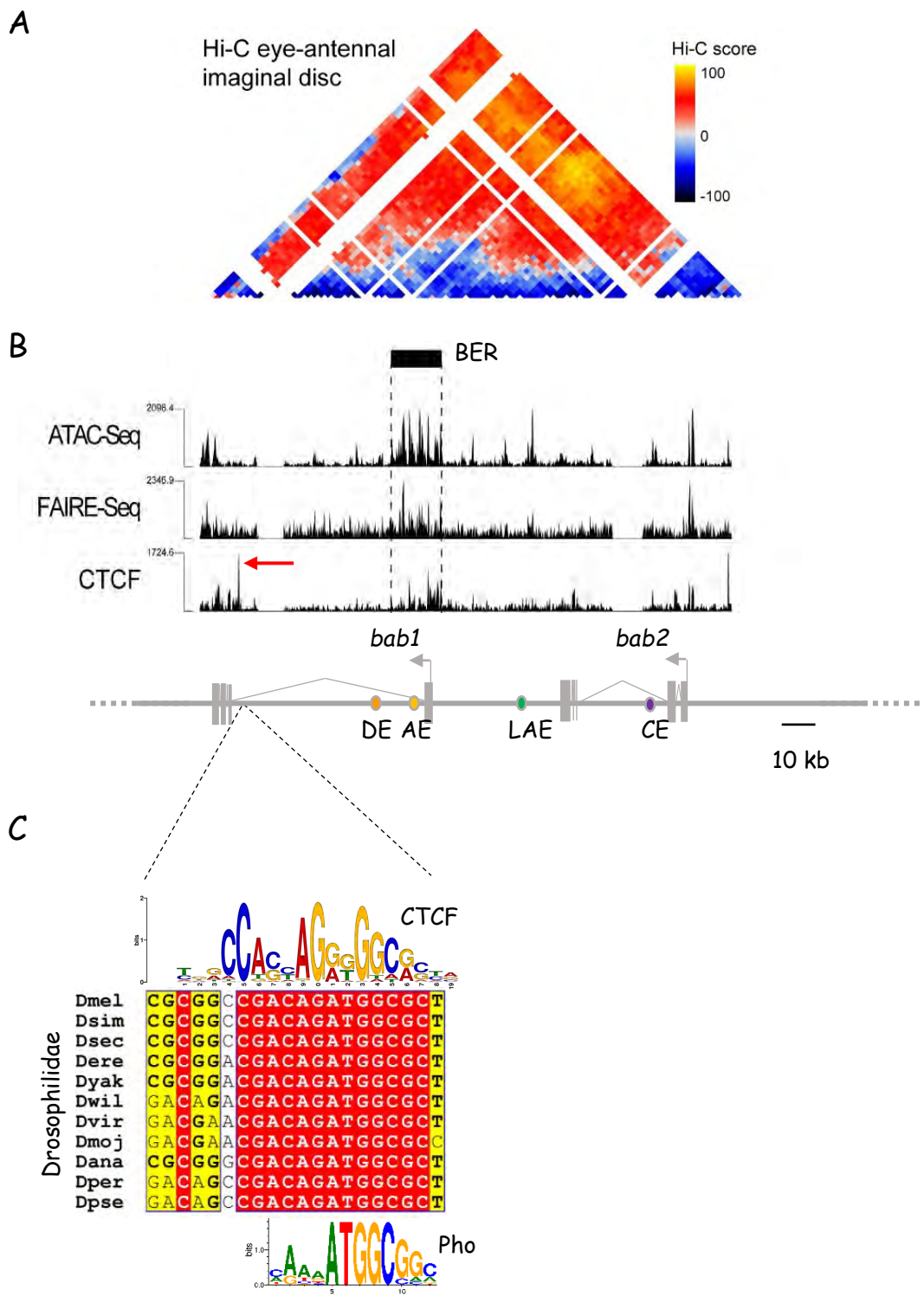
B

Leg/Eye-antenna/Wing/Haltere CS1 within OCS10



Leg/Eye-antenna/Wing/Haltere CS3 within OCS10





Supplementary data

Four letter abbreviations for investigated species	page 2
Predicted sequences for BTB-BabCD proteins	pages 3-20
Bab1 sequence conservation among muscomorphans	pages 21-22
Bab2 sequence conservation among muscomorphans	pages 23-24
Sequence conservation between Bab1/2 paralogs	pages 25-29
Sequence conservation between paralogous Bab1/2 proteins among muscomorphans. The four-letter species abbreviations are as listed below (page 2). Strictly conserved amino-acid residues are indicated by white characters on a red background while partially conserved ones are in black characters on a yellow background. Locations of the strongly-conserved BTB and BabCD domains are indicated along the right side (see black lines).	
BPE ^{OCS1} sequence conservation among Drosophilidae	pages 30-31
BPE ^{OCS2} sequence conservation among Drosophilidae	page 32
BPE ^{OCS3} sequence conservation among Drosophilidae	pages 33-34
BPE ^{OCS4} sequence conservation among Drosophilidae	page 35
BPE ^{OCS5} sequence conservation among Drosophilidae	page 36
BPE ^{OCS6} sequence conservation among Drosophilidae	pages 37-38
BPE ^{OCS7} sequence conservation among Drosophilidae	pages 39-40
BPE ^{OCS8} sequence conservation among Drosophilidae	pages 41-42
BPE ^{OCS9} sequence conservation among Drosophilidae	pages 43-45
BPE ^{OCS10} sequence conservation among Drosophilidae	pages 46-47
Enhancer sequence conservation among Drosophilidae	pages 48-57

Conservation among twelve reference drosophilids of *D. melanogaster* BER OCS1-10 as well as LAE, CE, AE and DE sequences. The four-letter Drosophilidae species abbreviations are as listed below (page 2). Locations of conserved sequence elements (CS) are indicated by underneath black bars. Sequence LOGOs of predicted binding sites for the Dll, Bowl, C15, Sp1, Rn, Sal/Salr, BabCD, GAF, Pho, Eip74EF, Dsx, Abd-B, Sd, Chinmo, Pan, Mad, GATA factors, Twist-Da and Lbe transcription factors are depicted above or below the alignments.

Four letter abbreviations for investigated species

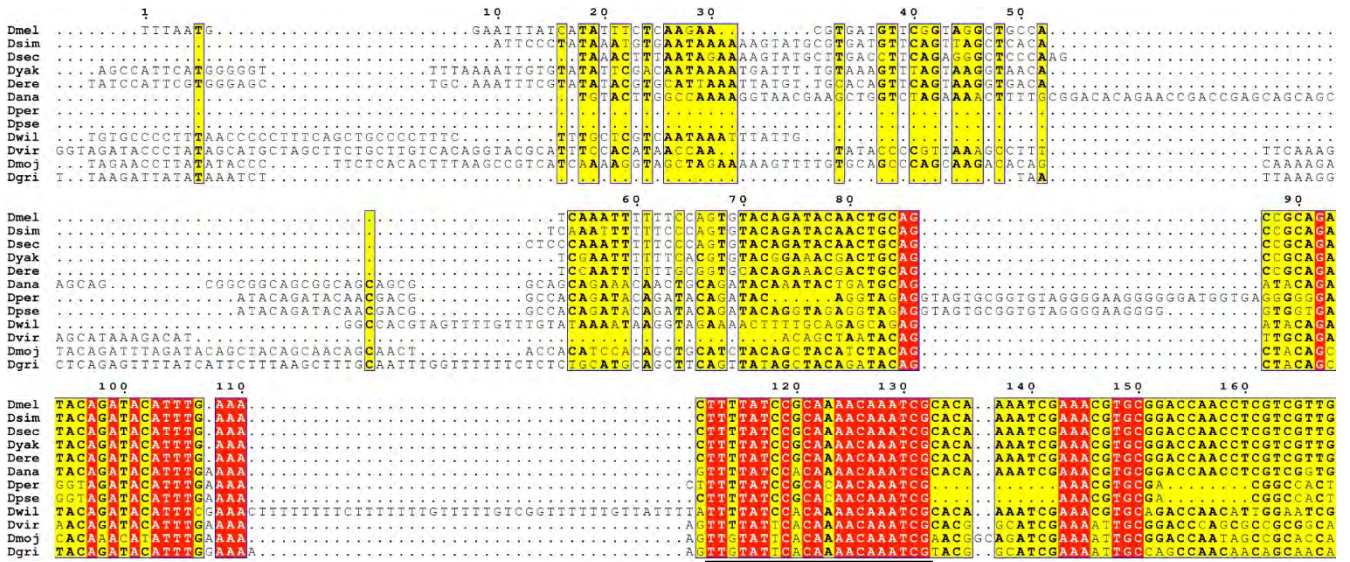
<i>Dmel: Drosophila melanogaster</i>	Drosophilidae	Acalyptratae	Schizophora	Muscomorpha/Cyclorrhapha	Brachycera
<i>Dsim: Drosophila simulans</i>					
<i>Dsec: Drosophila sechellia</i>					
<i>Dyak: Drosophila yakuba</i>					
<i>Dere: Drosophila erecta</i>					
<i>Dsuz: Drosophila suzukii</i>					
<i>Drho: Drosophila rhopaloa</i>					
<i>Dele: Drosophila elegans</i>					
<i>Dbip: Drosophila bipectinata</i>					
<i>Dana: Drosophila ananassae</i>					
<i>Dper: Drosophila persimilis</i>					
<i>Dpse: Drosophila pseudoobscura</i>					
<i>Dwil: Drosophila willistoni</i>					
<i>Dvir: Drosophila virilis</i>					
<i>Dmoj: Drosophila mojavensis</i>					
<i>Dgri: Drosophila grimshawi</i>	Calyptratae	Aschiza	Orthorrhapha		
<i>Tmin: Themira minor</i>					
<i>Tdal: Teleopsis dalmanni</i>					
<i>Blat: Bactrocera latifrons</i>					
<i>Ccap : Ceratitis capitata</i>					
<i>Mdom: Musca domestica</i>					
<i>Scal: Stomoxys calcitrans</i>					
<i>Lcup: Lucilia cuprina</i>					
<i>Preg: Phormia regina</i>					
<i>Chom: Cochliomyia hominivorax</i>					
<i>Pmac: Paykullia maculata</i>					
<i>Gbre: Glossina brevipalpis</i>					
<i>Gmor: Glossina morsitans</i>					
<i>Edim: Eristalis dimidiata</i>					
<i>Mabd: Megaselia abdita</i>				Nematocera	
<i>Cpat: Condylostylus patibulatus</i>					
<i>Cmol: Chrysotimus molliculus</i>					
<i>Pcoq: Proctacanthus coquilletti</i>					
<i>Ddia: Dasyopogon diadema</i>					
<i>Hfus: Holcocephala fusca</i>					
<i>Hill: Hermetia illucens</i>					
<i>Bval: Beris vallata</i>					
<i>Mdes: Mayetiola destructor</i>					
<i>Cfus: Coboldia fuscipes</i>					
<i>Ppat: Phlebotomus papatasi</i>					
<i>Llon: Lutzomyia longipalpis</i>					
<i>Agam: Anopheles gambiae</i>					
<i>Aaeg: Aedes aegypti</i>					
<i>Dpul: Daphnia pulex</i>	Crustacea				

Bab2 sequence conservation among muscomorphans (Part1)

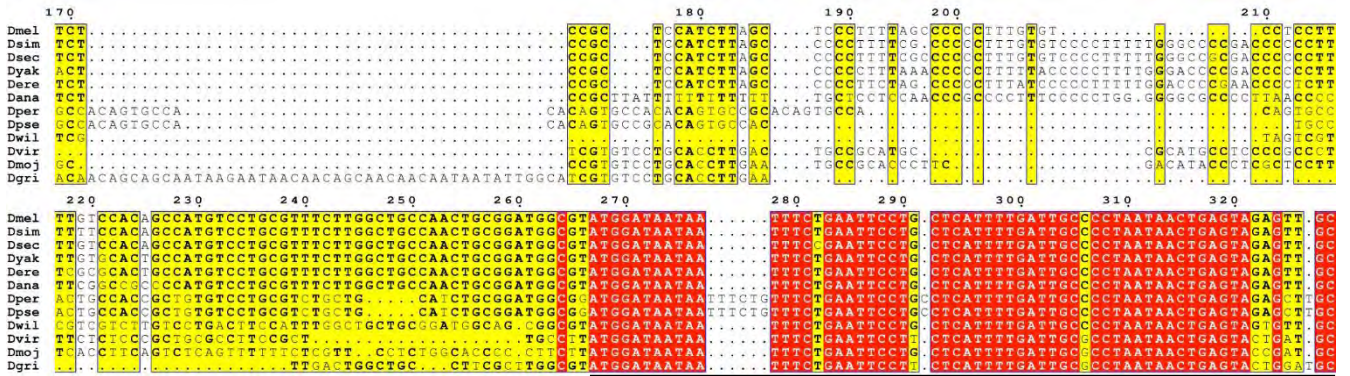
Dmel12	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Dspe2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Dvir12	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Bla12	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Ccap2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Tmin2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Tdal12	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Mdom2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Scal12	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Lcup12	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Preg2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Chom2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Fmac2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Gbre2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Gmo2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Mequ2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Edim2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Mabd2	1	MDMTKQIVDFEIK..SE.....LIGE.....IDQFEASDYTMAPP.....EELPKMVESEPOLGHLSDNRRKYSFEREVEPTLQDPSEVV..DMQ.....
Dmel12	77	KDIESVGEVGSFPEKVDVETELVKSKAS.....PMNDQALFPPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Dspe2	66	ATGPATEKAAADLDEADEEAEKPKON.....LTKDPLPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Dvir12	64	SAEQOQOARAELEVKLEKQEKPTFS.....VLRHEPLPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Bla12	76	NTAHAQAQLEQHKSTQANWHEPFAH..ETANEM.SVDRKSTETAINRATVQ..AIMSGDLSYVIVT..ASFPFAQPTOFFEADIDGMTSFPNSCLGSGPSTPN
Ccap2	89	GAEOCQOQTRPLETQOADAAYOQPRE..ELNDTVAVEDEIKAIASQPPKIVGAAVVSGDLPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Tmin2	108	TLCKADNNTSLALAQTSPK.....PFOQOAVDVPDGLDLECA.....KYVGDMMMPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Tdal12	67	TGGAANNTSVALENTAF.VRPEKSLPTDLMKTFGN.....ITAGLDLPPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Gmo2	72	ALGSENEHNAEFAEKMPAQIQKPTTFLQIENHTAN.VSIIK.....PAMGRDIPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Scal12	55	MOQEKNFNEIPLIKMPLVEQKPTTFLQIENHTAN.VSIIK.....PAMGRDIPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Lcup12	51	ALGSENEHNAEFAEKMPAQIQKPTTFLQIENHTAN.VSIIK.....PAMGRDIPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Preg2	56	ATNTIQEHSNRIEMKIPPERKQKPTTFLQIENHTAN.VSIIK.....PAMGRDIPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Chom2	59	ATNTIQEHSNRIEMKIPPERKQKPTTFLQIENHTAN.VSIIK.....PAMGRDIPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Fmac2	54	ITETAYNVEKVNKISAQIQKPTTFLQIENHTAN.VSIIK.....PAMGRDIPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Gbre2	47	FVAPAGVLSRKTTHWEKVRNPFELLENMDK..SIIK.....AANTCDLPPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Gmo2	47	FVAPAGVLSRKTTHWEKVRNPFELLENMDK..SIIK.....AANTCDLPPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Mequ2	8FSESEIFPL.KSLLS.....FASIGDLPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Edim2	8FSESEIFPL.KSLLS.....FASIGDLPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Mabd2	7FSESEIFPL.KSLLS.....FASIGDLPPLPRPLTSSSEVVGIRDP..EHLRRLRMC..LEAKKRSRSPVSGPQPFIN.....
Dmel12	154HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Dspe2	143HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Dvir12	141HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Bla12	185HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Ccap2	200HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Tmin2	201HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Tdal12	205HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Mdom2	167HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Lcup12	145HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Preg2	150HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Chom2	152HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Fmac2	148HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Gbre2	140HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Gmo2	140HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Mequ2	64HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Edim2	64HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Mabd2	44HLA.....GSALEFFGQRSS.....FVE.....TKIKTNKTPPR.....RKIV.....FVSG.....ECQOFCRLRNN
Dmel12	205	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GG
Dspe2	204	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...DC
Dvir12	263	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GM
Bla12	203	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GM
Ccap2	290	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GM
Tmin2	285	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Tdal12	214	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Mdom2	230	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Scal12	227	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Lcup12	199	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Lcup2	204	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Chom2	206	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Fmac2	202	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Gbre2	193	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Gmo2	128	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Mequ2	126	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Edim2	128	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Mabd2	82	YQNLNINVFDDLLQNSSEVVDVTAACGGH...IKAKRMVLSACSPYPQAFDNDPCQHPH...IMRDVRS...DKALVEMFYKGEINVCDDQ...NPLKVAET...KIRGLAEVSAAGRGE...GT
Dmel12	318	AS.....ALP.....MSAFDDEDEEELASATAIQOD..GDAD.....PDEEMKARPLRLP.....EGV.....LDLN.....QRKRKRSDGYSATPSPSPL.....R
Dspe2	317	AS.....ALP.....MSAFDDEDEEELASATAIQOD..GDAD.....PDEEMKARPLRLP.....EGV.....LDLN.....QRKRKRSDGYSATPSPSPL.....R
Dvir12	313	AS.....ALP.....MSAFDDEDEEELASATAIQOD..GDAD.....PDEEMKARPLRLP.....EGV.....LDLN.....QRKRKRSDGYSATPSPSPL.....R
Bla12	376	AA.....PMMQCRMTIFDEEDVSDDD.....TESA.....EDGCGHPRKRLAKT.....RAGDSA.....LDLN.....QRKRKRSDGYSATPSPSPL.....R
Ccap2	408	AA.....PMMQCRMTIFDEEDVSDDD.....TESA.....EDGCGHPRKRLAKT.....RAGDSA.....LDLN.....QRKRKRSDGYSATPSPSPL.....R
Tmin2	394	GT.....GHTLVFPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Tdal12	325	VA.....AHMPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Mdom2	341	VA.....AHMPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Scal12	342	VA.....AHMPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Lcup12	310	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Lcup2	315	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Preg2	310	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Chom2	317	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Fmac2	313	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Gbre2	304	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Gmo2	241	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Mequ2	204	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Edim2	239	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Mabd2	195	MA.....AQLPLPERMTIVYDDEPESSEDDA.....DNDDIGLMDGFGHFKPRLYERNFVIAAAA.AAA.AAAVRSANRKRKRSDGYSATPSPSPL.....R
Dmel12	390	QGRS.....EISERGSSGT.....PQSQSOSQ...LAMI...TSIVRN...FASFPFO.....TLEGR.....
Dspe2	394	QGRS.....EISERGSSGT.....PQSQSOSQ...LAMI...TSIVRN...FASFPFO.....TLEGR.....
Dvir12	390	QGRS.....EISERGSSGT.....PQSQSOSQ...LAMI...TSIVRN...FASFPFO.....TLEGR.....
Bla12	459	GANASNO..PTIESASPKNQTTNAITDEISGADVSGAQPQVAVIT...TSIVRN...FASFPFO.....TLEGR.....
Ccap2	485	GIFTEH..TAGONATMSQVATAT..NGTADTASQPPVAVIT...TSIVRN...FASFPFO.....TLEGR.....
Tmin2	490	YGNRA..YSPASPERAAE..ASDNMPEPPLALASTIVRN...FASFPFO.....TLEGR.....
Tdal12	417	SRSH..PLPRTTEKQHNAAAEE..NAPANDAASNTNAMI...TSIVRN...FASFPFO.....TLEGR.....
Mdom2	433	NTRNLGLDLSGQLKDKESC.....GFSSPAPNGGII...TSIVRN...FASFPFO.....TLEGR.....
Scal12	436	SRRS.....RSPQRDKFAE.....AASINPVMAT...TSIVRN...FASFPFO.....TLEGR.....
Lcup12	395	SRRS.....RSPQRDKFAE.....AASINPVMAT...TSIVRN...FASFPFO.....TLEGR.....
Preg2	402	SRRS.....RSPQRDKFAE.....AASINPVMAT...TSIVRN...FASFPFO.....TLEGR.....
Chom2	404	SRRS.....RSPQRDKFAE.....AASINPVMAT...TSIVRN...FASFPFO.....TLEGR.....
Fmac2	403	SRRS.....RSPQRDKFAE.....AASINPVMAT...TSIVRN...FASFPFO.....TLEGR.....
Gbre2	393	DYRS.....ATSMFAEKNI...STTPFIVM...TSIVRN...FASFPFO.....TLEGR.....
Gmo2	393	DYRS.....ATSMFAEKNI...STTPFIVM...TSIVRN...FASFPFO.....TLEGR.....
Mequ2	312	QOQQOQH.QOQQOQQOQQOQHLYOQR..QSLPQCP..TFPMSSSVRN...FASFPFO.....TLEGR.....
Edim2	310	QOQQOQH.QOQQOQQOQQOQHLYOQR..QSLPQCP..TFPMSSSVRN...FASFPFO.....TLEGR.....
Mabd2	245	FMRLT.LGSSHSPIEN.....YQDR.....QQLPQCP..TFPMSSSVRN...FASFPFO.....TLEGR.....
Dmel12	437NSAMNAVQRKS.....PAP.....TATGSHNGN.....SGAAHSDP...GGVA.....VQALPFPHM.AAIV.....
Dspe2	466SSSAAS..SSAGATYRT.AARCSPPPHQHQHGHHPGGSGNS.....AGALHSDP...GGVGS.....GGQSSLPFPHM.AAIVAA.....
Dvir12	470SSSMGSSGSAAYRS.CSAPFPFPP.....PAHISNG.....SRAALHSDP...GGVGS.....GGQSSLPFPHM.AAIVAA.....
Bla12	548SARLSTATASVRS.PM.....ESANS.....LKGAAHSDP...GGVGS.....GGQSSLPFPHM.AAIVAA.....
Ccap2	571	ITPHGSSATPTDPTISHATAHSYRF.....PDM.....ESANS.....LKGAAHSDP...GGVGS.....GGQSSLPFPHM.AAIVAA.....
Tmin2	587	SAV..SMGFSSTASNTPSASGSIYR..QVCCPEL.....TAAEITVAAPPEPFAQSQQPMIPSAVLSLGGSSPDSNNS.....AGHLSVHSH.....
Tdal12	501SAAYRF..TVCSEBFL.....FS.AHG.....NSAQ...MDNGIT.....NSANHSVHSHAAAAAA.....
Mdom2	518SARLSTATASVRS.PM.....ESANS.....LKGAAHSDP...GGVGS.....GGQSSLPFPHM.AAIVAA.....
Scal12	509AAAAASPNVVALPYRS.LERSCSPPM.....MTPPCA.R.....AQSSVSN.....TSPSLASPNSSSAGP.....SGTSPISHSH.AAIVAA.....
Lcup12	470FAAASYRA..RELSLSPSL..MT.AHFGT.....TSLASPNSSSAGP.....SGTSPISHSH.AAIVAA.....
Preg2	477SAAYTYRF..RELSLSPSL..MT.SHTGT.....GHLLE...TSPST.....PQISHPFH.AAIVAA.....
Chom2	479SAAYTYRF..RELSLSPSL..MT.SHTGT.....GHLLE...TSPST.....PQISHPFH.AAIVAA.....
Fmac2	478SAAYTYRF..RELSLSPSL..MT.SHTGT.....GHLLE...TSPST.....PQISHPFH.AAIVAA.....
Gbre2	464SVTLFPRS.MTRCSLSPSL..AAAAHTR.....EVLAV...TSPST.....VHS.RSAGS.....
Gmo2	463SVTLFPRS.MTRCSLSPSL..AAAAHTR.....EVLAV...TSPST.....VHS.RSAGS.....
Mequ2	396SVTLFPRS.MTRCSLSPSL..AAAAHTR.....EVLAV...TSPST.....VHS.RSAGS.....
Edim2	365AGGGGACGSSGSGS.....VRLNLPSP.....ITGGP...TSPST.....VHS.RSAGS.....
Mabd2	311AGGGGACGSSGSGS.....VRLNLPSP.....ITGGP...TSPST.....VHS.RSAGS.....

BTB

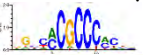
BER^{OCS1} sequence conservation among Drosophilidae (Part1)



CS1

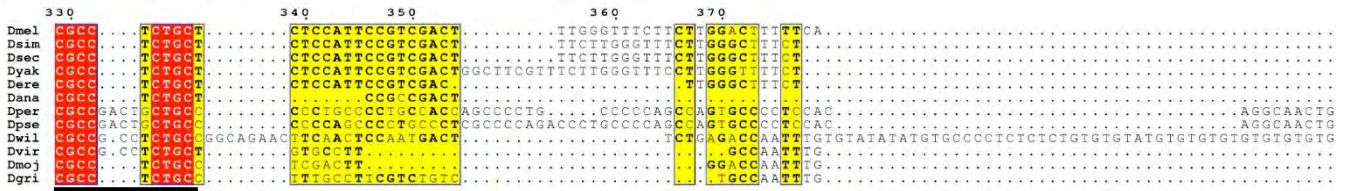
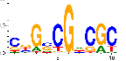


Spl



CS2

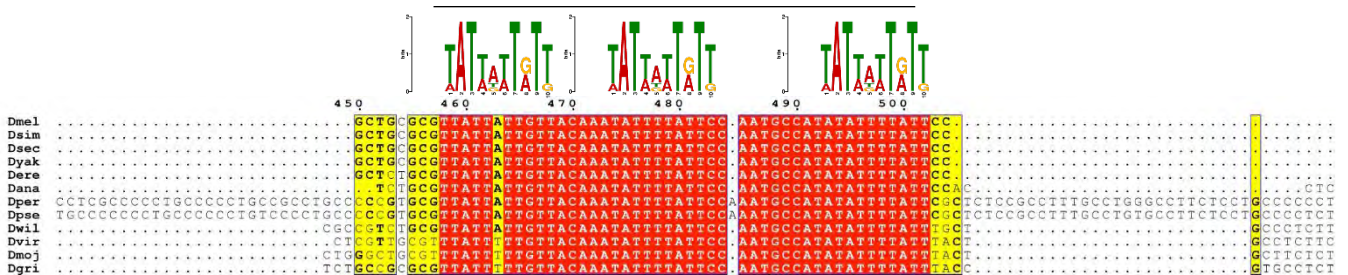
Mad



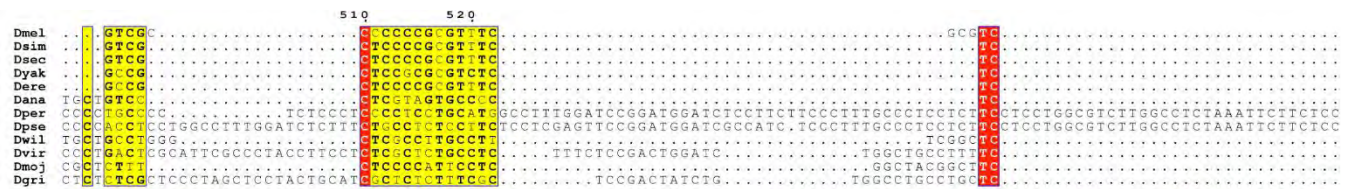
BER^{OCS1} sequence conservation among Drosophilidae (Part2)



BabCD



Leg-specific CS3



Leg-specific CS4



BER^{OCS2} sequence conservation among Drosophilidae

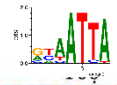
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Dmel	CGGGAGCAATATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dsim	GGGAGCAGATATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dsec	GGGAGCAGATATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dyak	GGGAGCAGATATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dere	GGGAGCAGATATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dana	GTGGGAACAGATAACATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dper	CACACAAAGCAGC	CGCAATAAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dspe	CACACAAAGCAGC	CGCAATAAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dwil	TACATACATATATAAGTATGG	GGCAGATATAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dvir	CACACTCCGACT	CGCAATAAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dmoj	AACAT	CGCAATAAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	
Dgri	CACACACACACT	CGCAATAAA	GGCAATAAA	TACAAAAATTATCCATTCA	CGTC	

	60	70	80	90	100
Dmel	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dsim	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dsec	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dyak	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dere	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dana	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dper	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dspe	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dwil	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dvir	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dmoj	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				
Dgri	ATAAACTTCAATTTTCGAAAAATCATTTCCTTAGCAGCTGCGCATTAAAAAAA				

CS1

	110	120	130	140
Dmel	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dsim	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dsec	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dyak	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dere	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dana	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dper	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dspe	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dwil	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dvir	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dmoj	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA
Dgri	AAACACAGAAAGCAGAAAAATAAAGA		ACGCCAACATAAAAA	TGNA

DII



	150	170	180	190	200	210	220
Dmel	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dsim	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dsec	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dyak	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dere	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dana	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dper	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dspe	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dwil	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dvir	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dmoj	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA
Dgri	TAAATGATTTAAGGCTCGACAGTGGGCA	TGATTTGCCCAACTGCCAGCAAT				C	GAGATATCGCAATCAATA

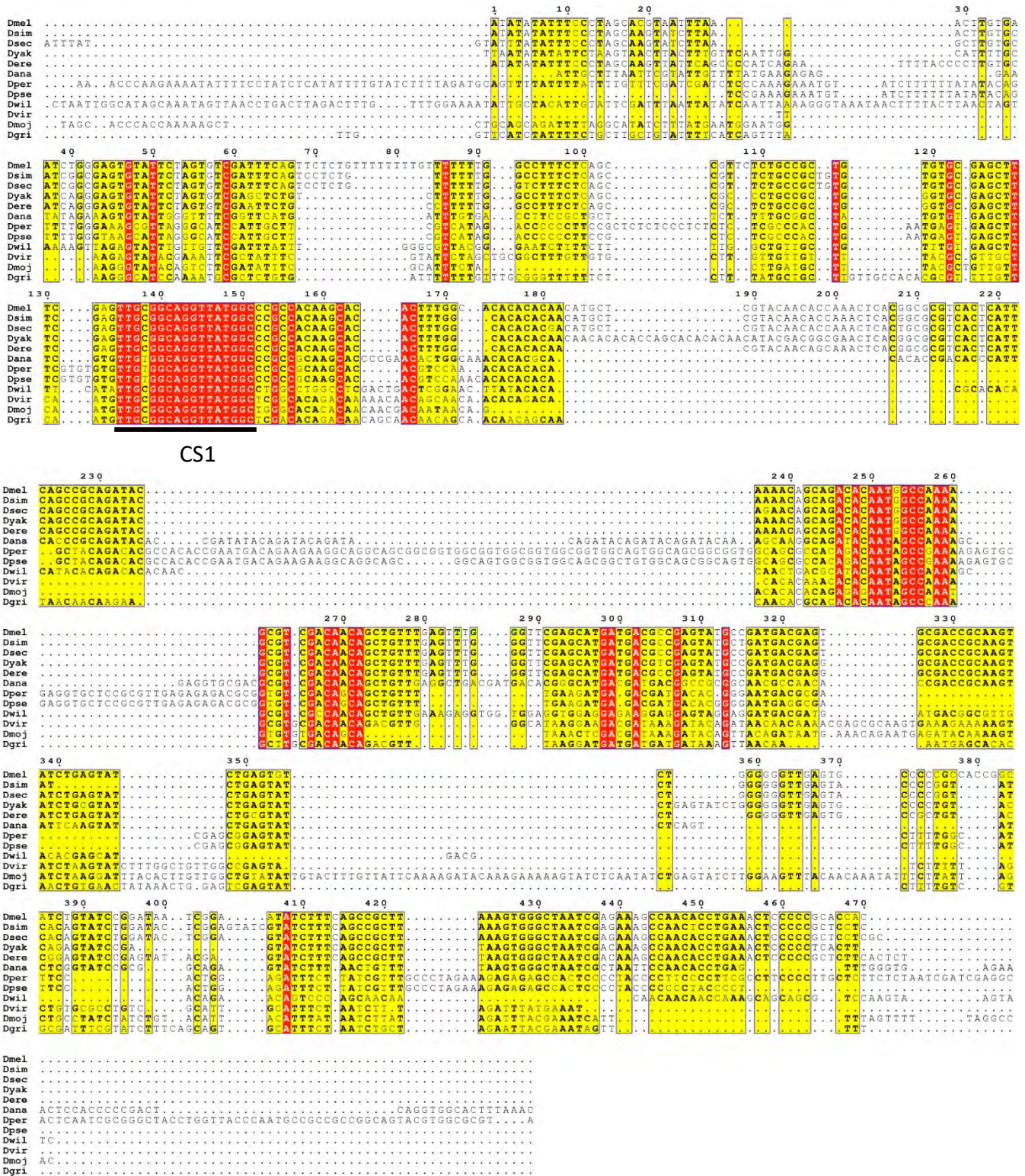
CS2

	230	240	250	260
Dmel	TACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dsim	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dsec	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dyak	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dere	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dana	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dper	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dspe	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dwil	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dvir	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dmoj	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		
Dgri	CACCT	CACCTGCCAAAGTCATTTGTCGTGAGTAG		

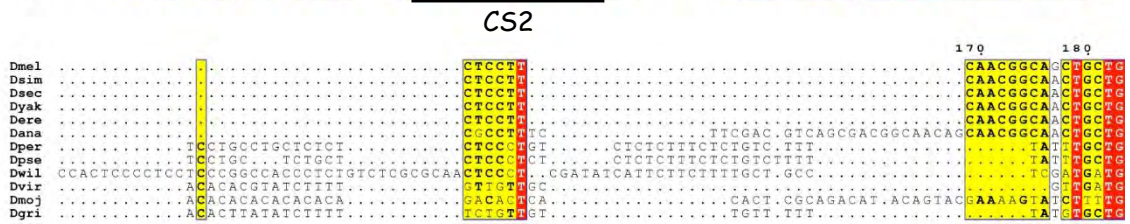
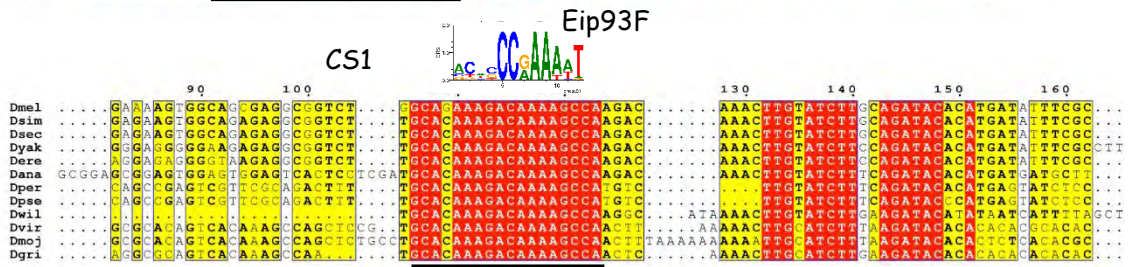
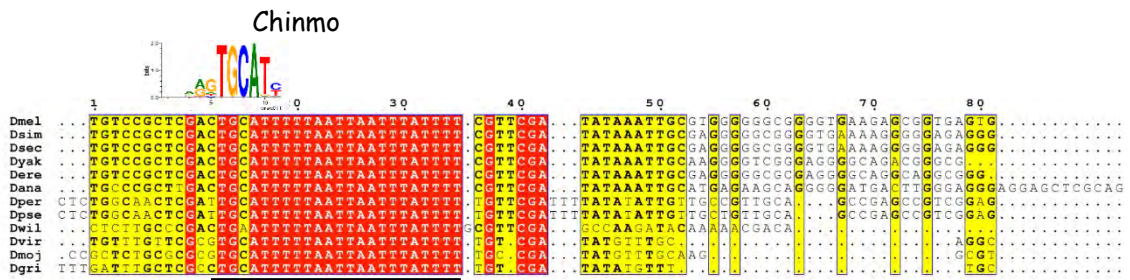
	270	280	290	300	310	320
Dmel	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dsim	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dsec	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dyak	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dere	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dana	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dper	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dspe	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dwil	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dvir	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dmoj	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		
Dgri	GCTGCTGATTGATGTCGATGTC	AAGCAAAGCGAAGTTGGCAA	ATA	GTCCGAGCTGCTAG		

	330	340	350	360
Dmel	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dsim	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dsec	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dyak	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dere	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dana	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dper	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dspe	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dwil	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dvir	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dmoj	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG
Dgri	TC	GA	AAATGGGGGCAAA	CAAATGTTGTCAGTAG

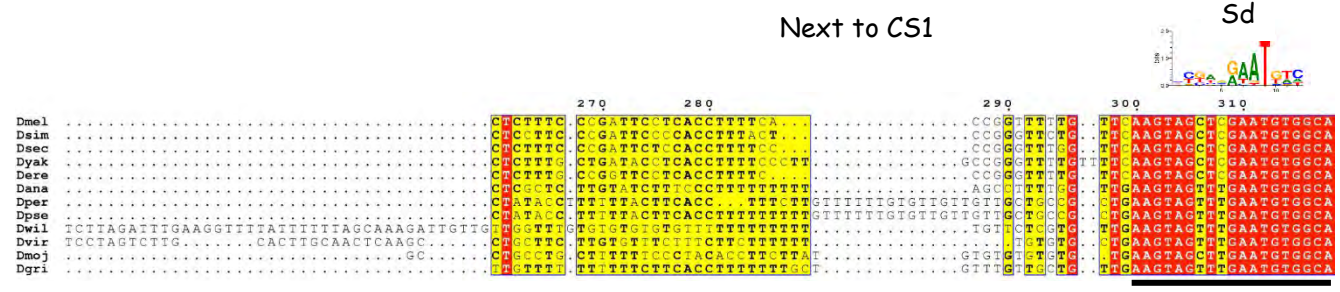
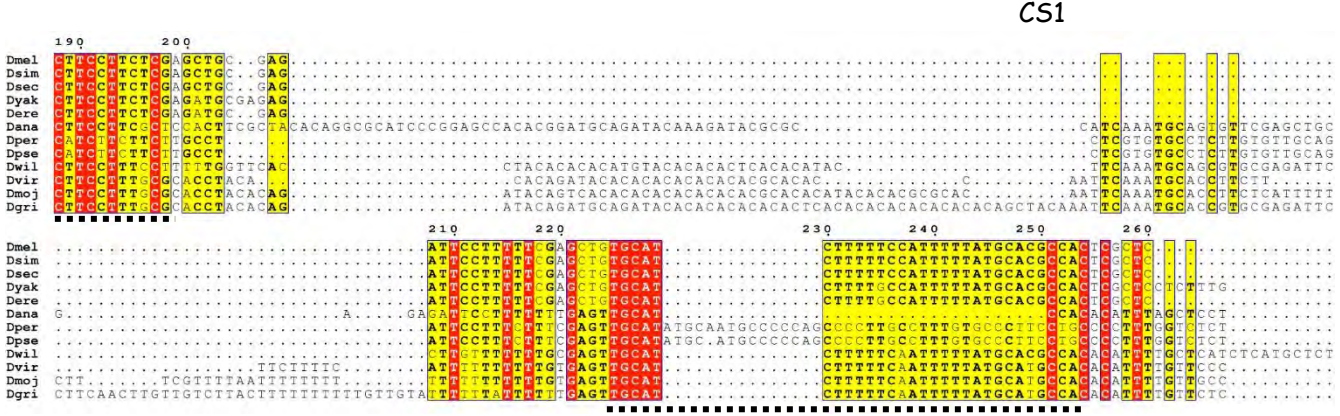
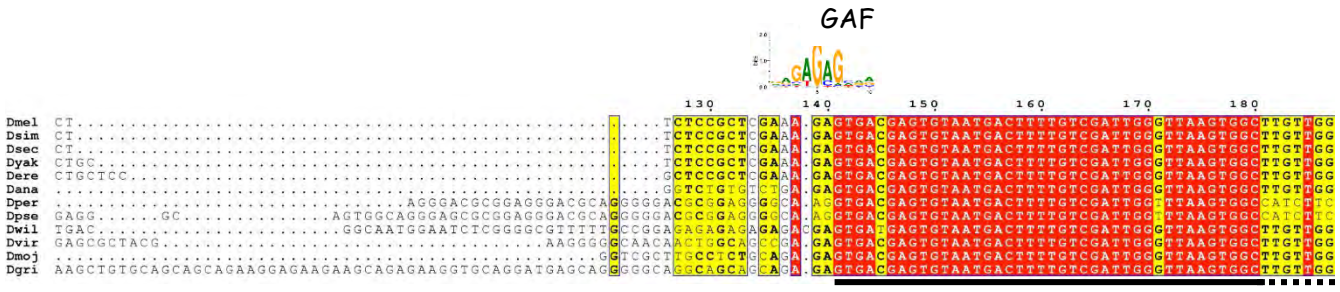
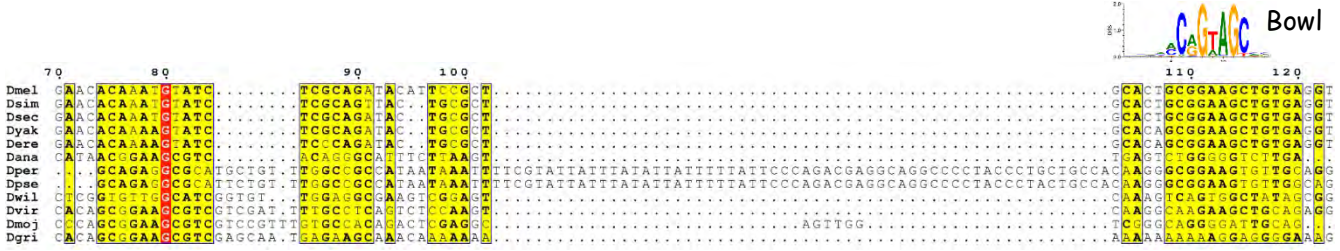
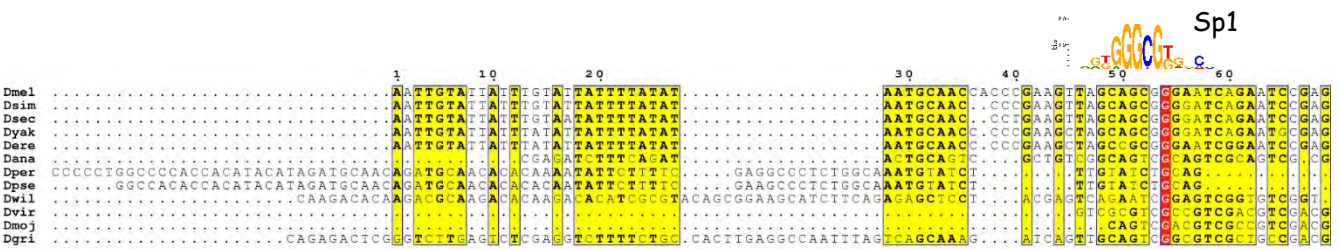
BER^{OC54} sequence conservation among Drosophilidae



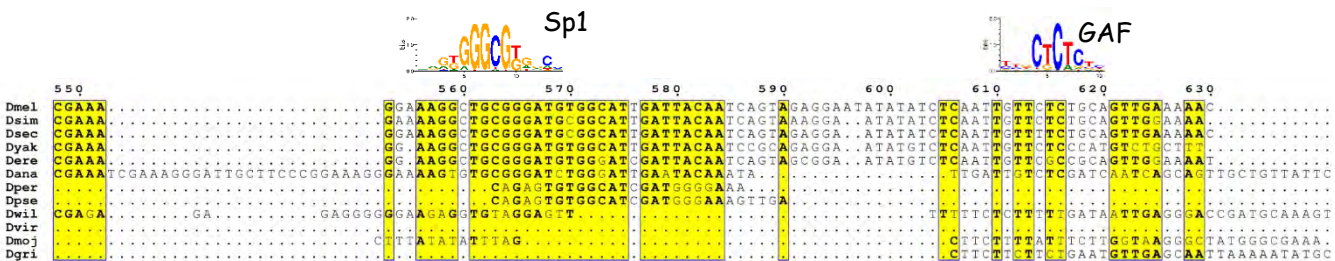
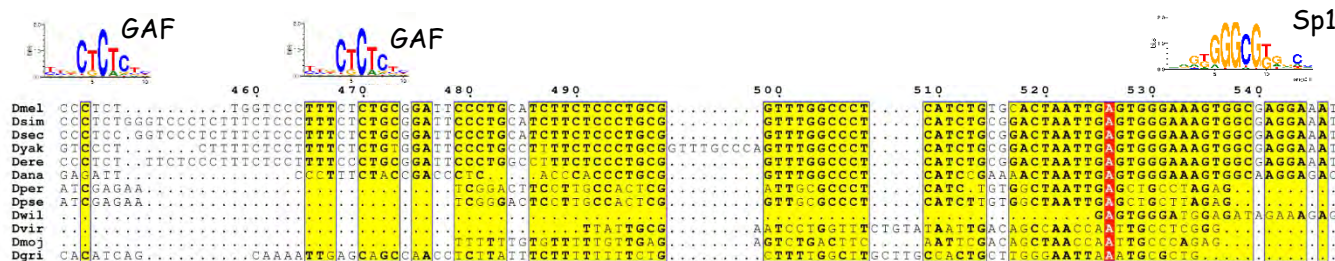
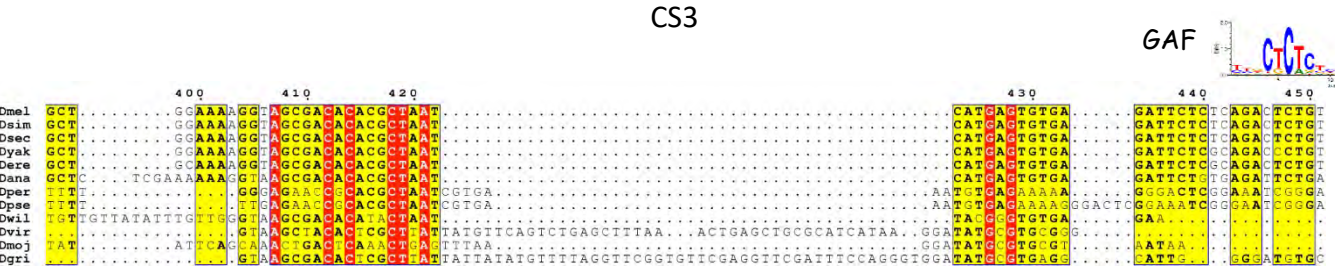
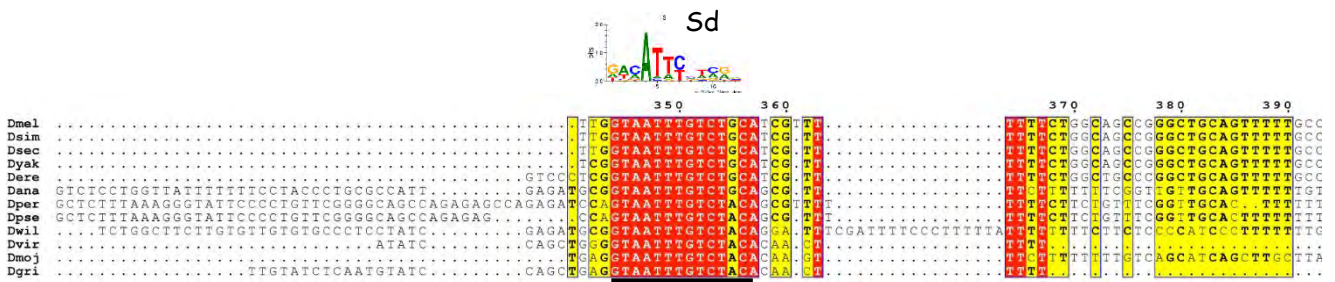
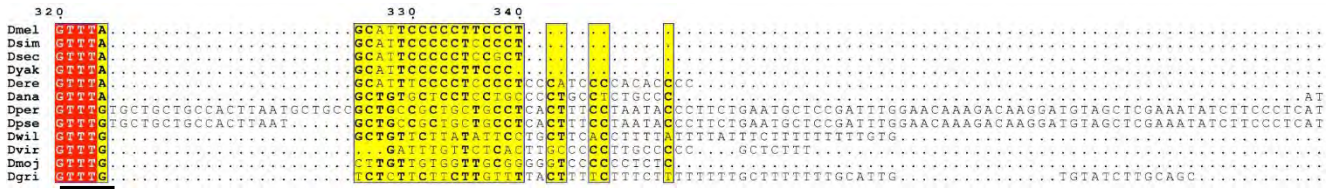
BER^{OC55} sequence conservation among Drosophilidae



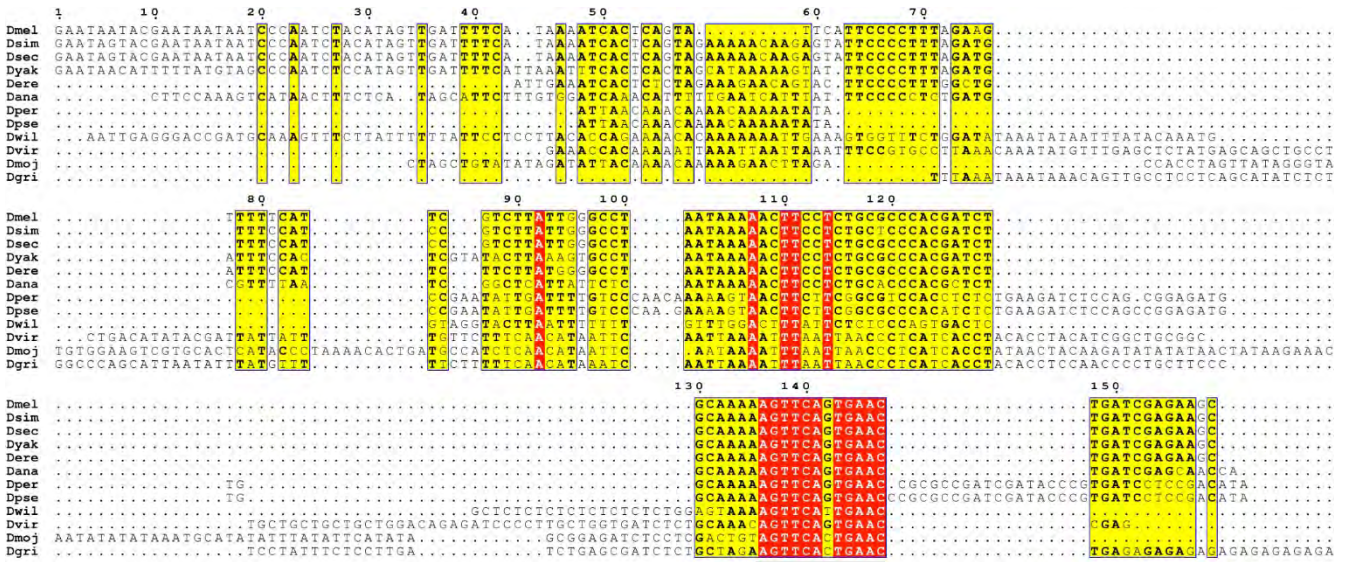
BER^{OCS6} sequence conservation among Drosophilidae (Part1)



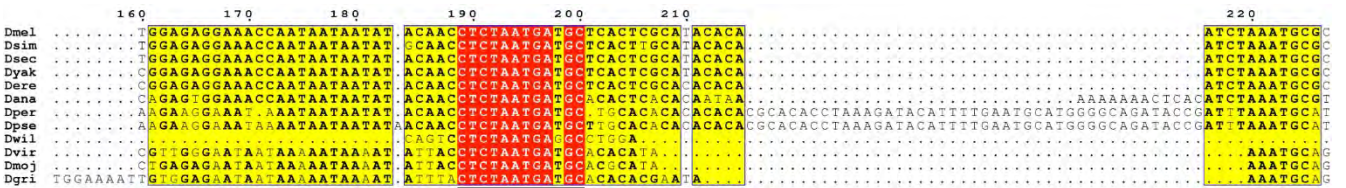
BER^{OC56} sequence conservation among Drosophilidae (Part2)



BER^{OCST} sequence conservation among Drosophilidae (Part1)

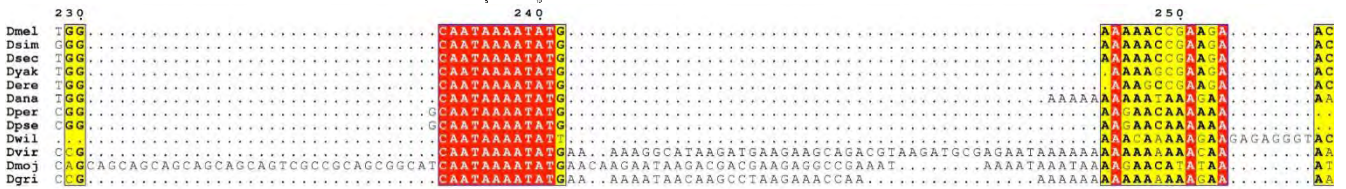
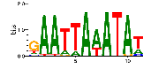


Eye/Antennal CS1

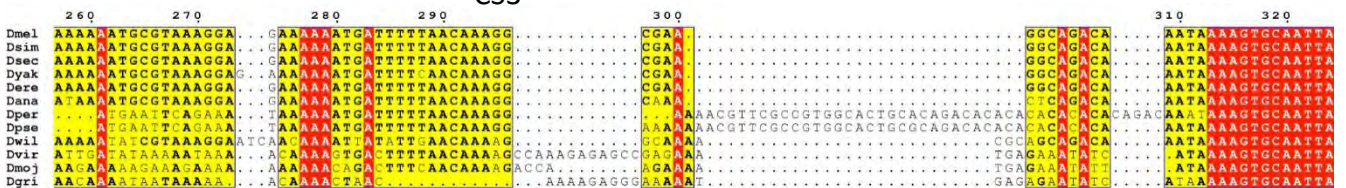


Eye/Antennal CS2

BabCD

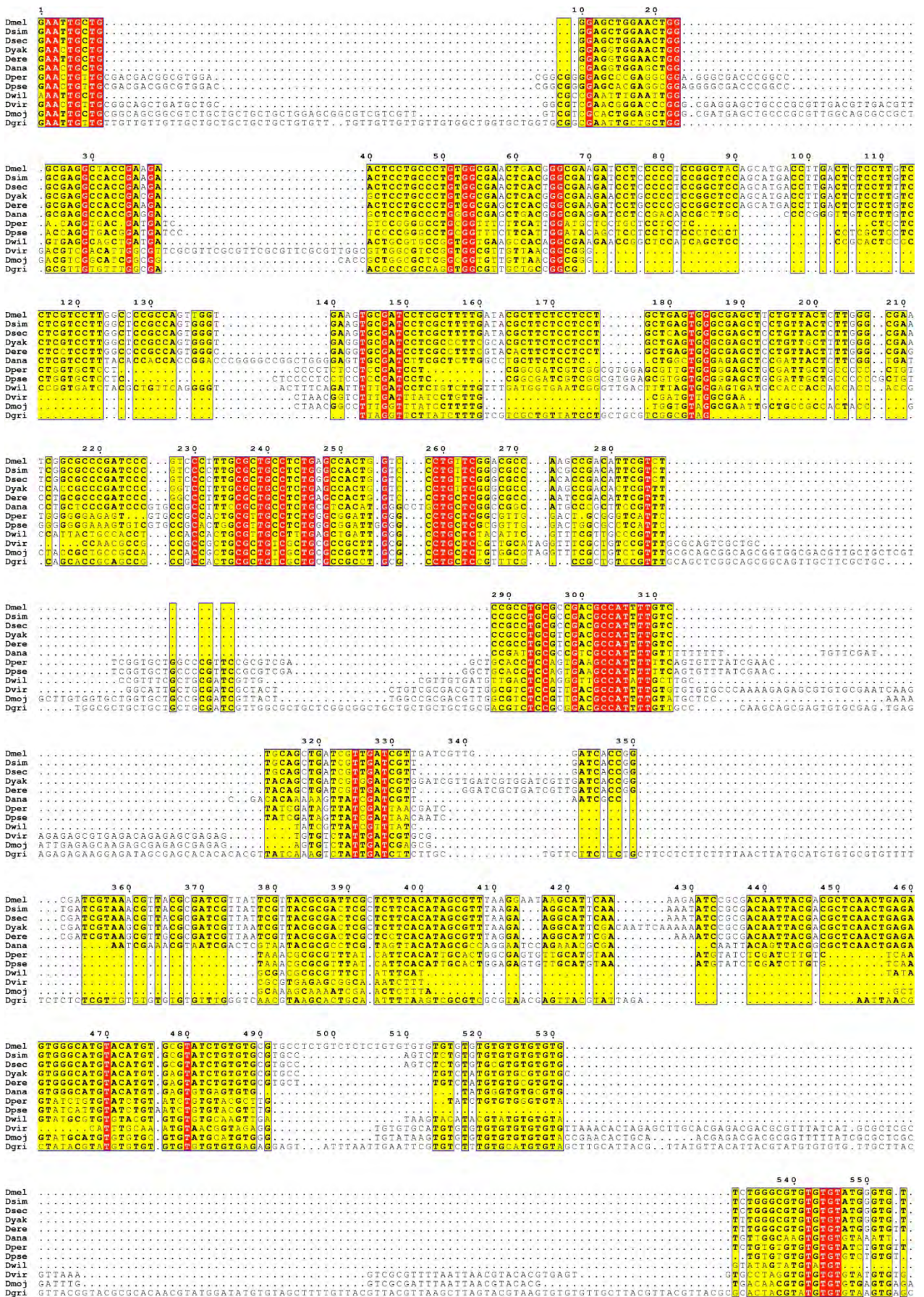


CS3



CS4

BER^{OC}S⁹ sequence conservation among Drosophilidae (Part1)



BER^{OCs9} sequence conservation among Drosophilidae (Part2)

Sequence alignment for positions 560-570. Species: Dmel, Dsim, Dsec, Dyak, Dere, Dana, Dper, Dpse, Dwil, Dvir, Dmoj, Dgri. Conserved regions are highlighted in yellow and red.

Sequence alignment for positions 580-620. Conserved regions are highlighted in yellow and red.

Sequence alignment for positions 630-700. Conserved regions are highlighted in yellow and red.

Sequence alignment for positions 710-810. Conserved regions are highlighted in yellow and red.

Sequence alignment for positions 820-870. Conserved regions are highlighted in yellow and red.

Sequence alignment for positions 880-950. Conserved regions are highlighted in yellow and red.

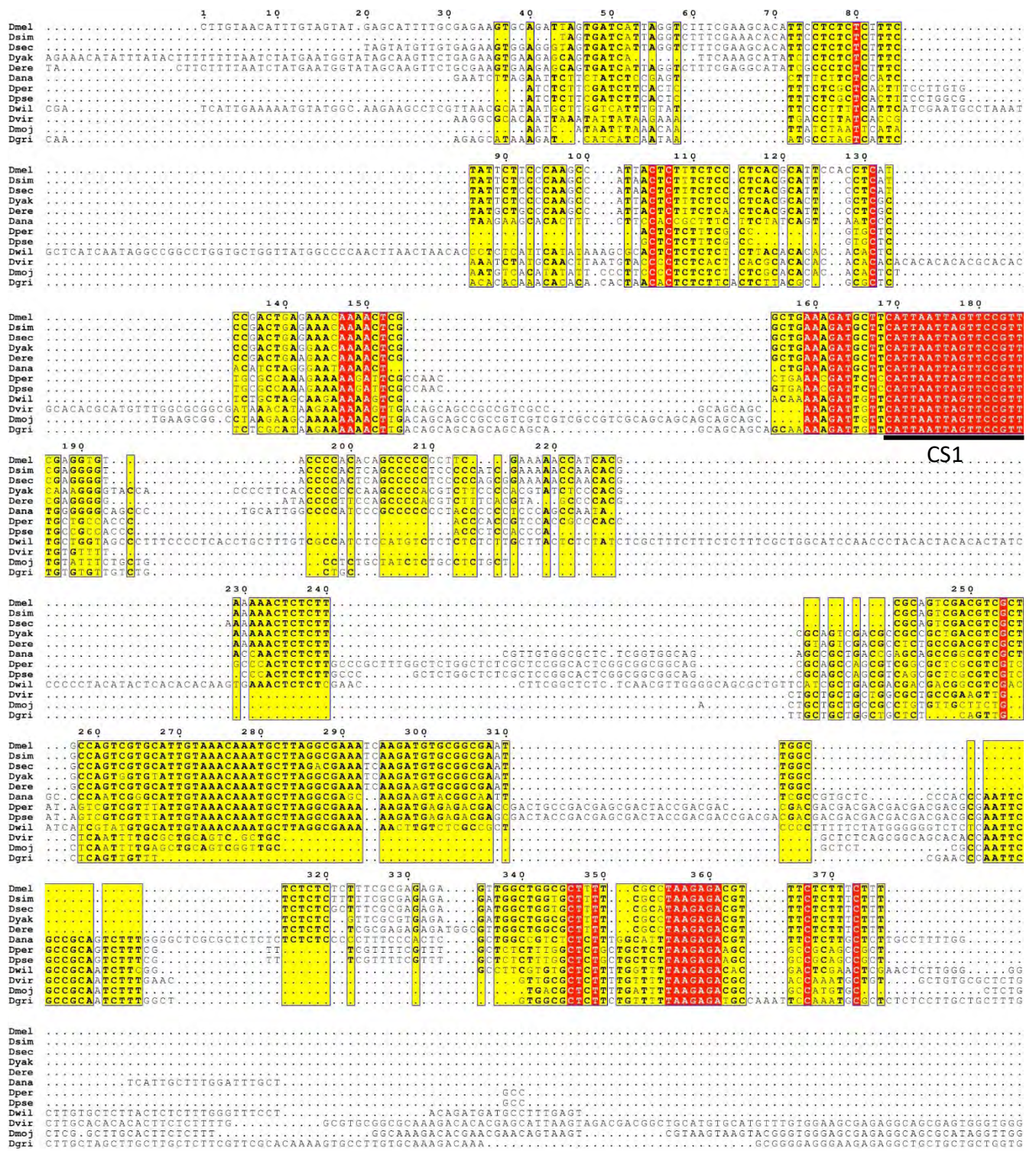
Sequence alignment for positions 960-1030. Conserved regions are highlighted in yellow and red.

Sequence alignment for positions 1040-1070. Conserved regions are highlighted in yellow and red.

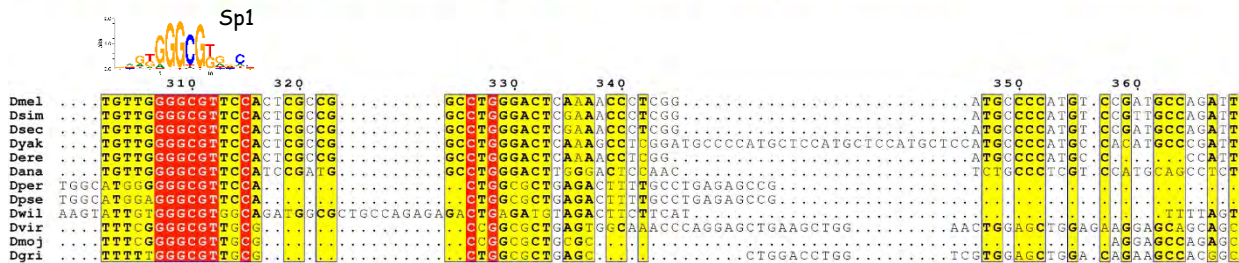
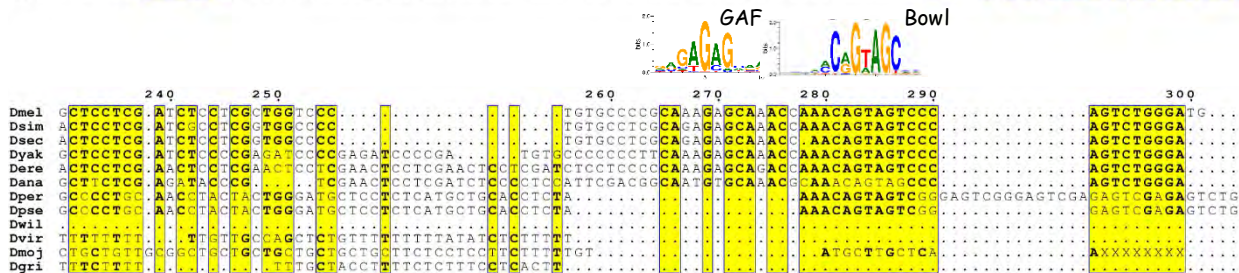
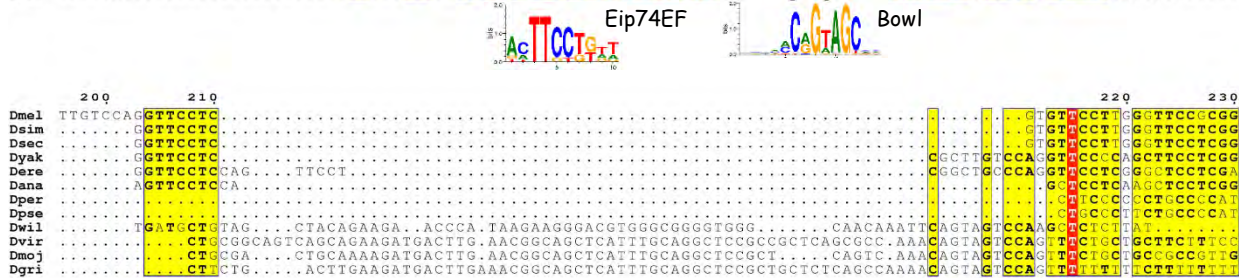
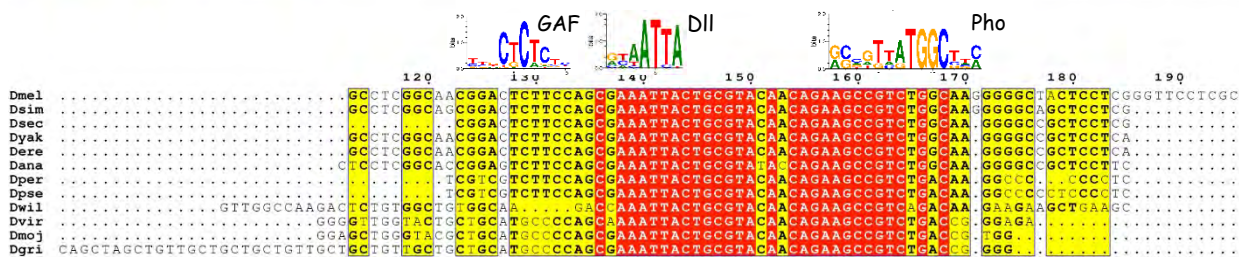
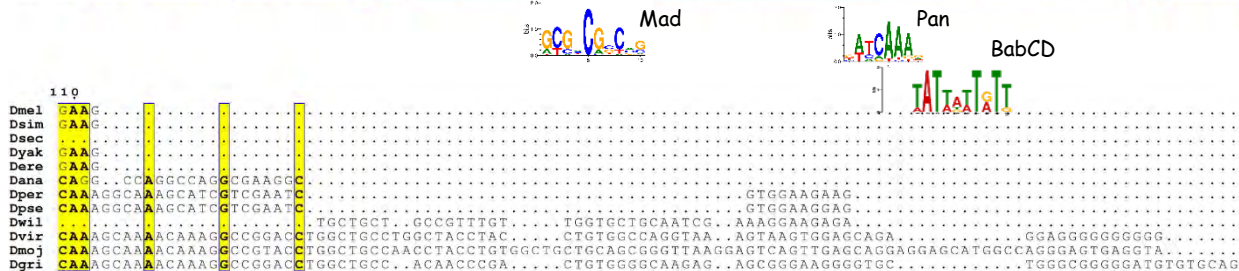
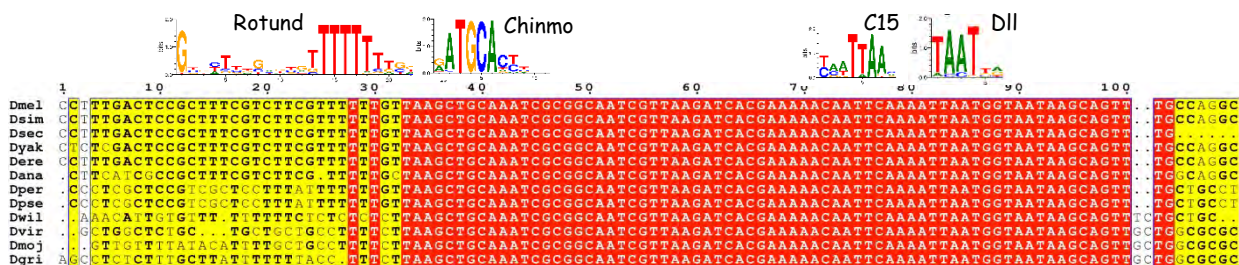
Sequence alignment for positions 1080-1090. Conserved regions are highlighted in yellow and red.

CS1 (promoter region)

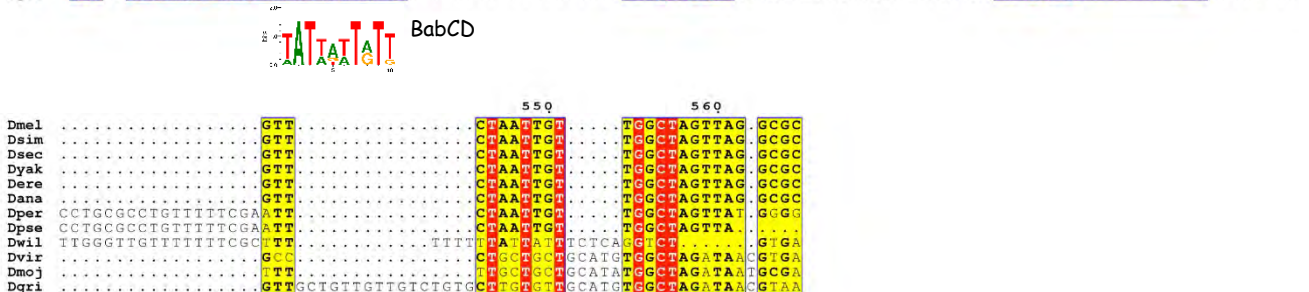
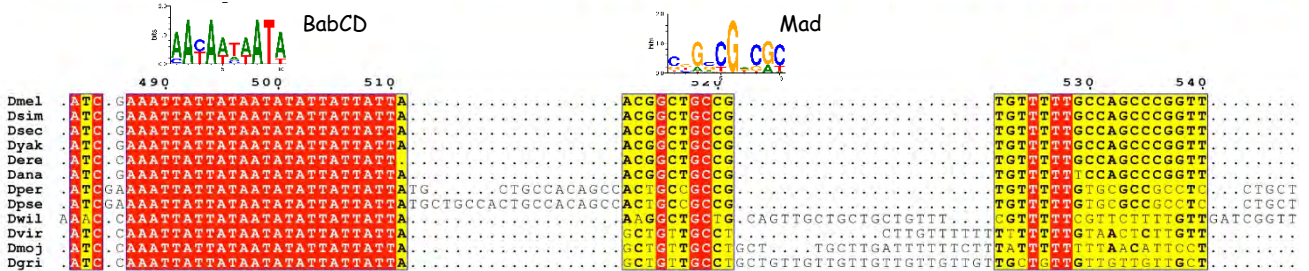
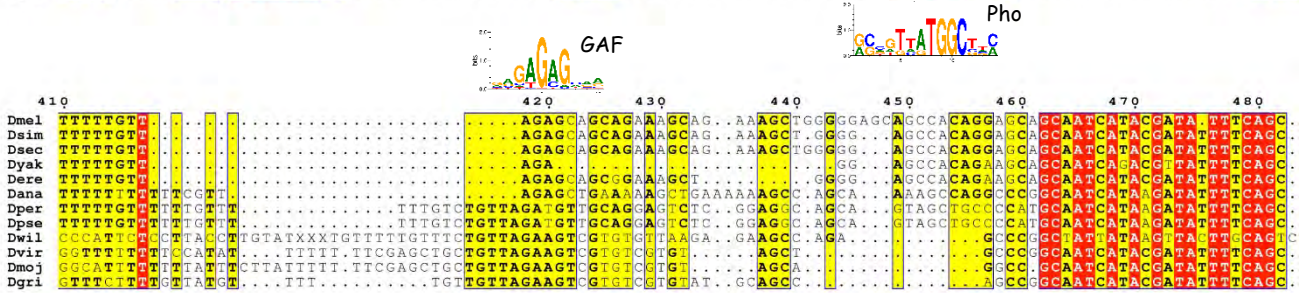
BER^{OC}S10 sequence conservation among Drosophilidae (Part1)



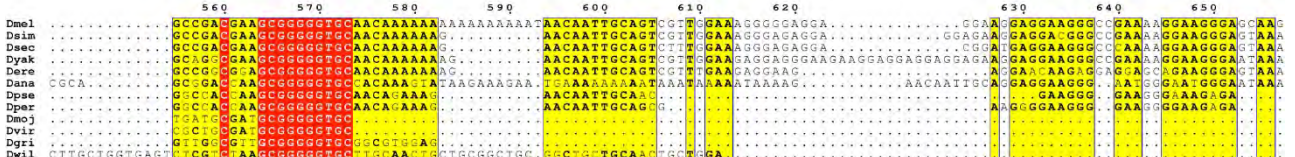
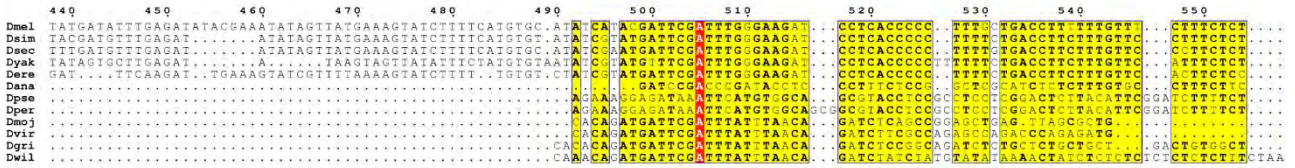
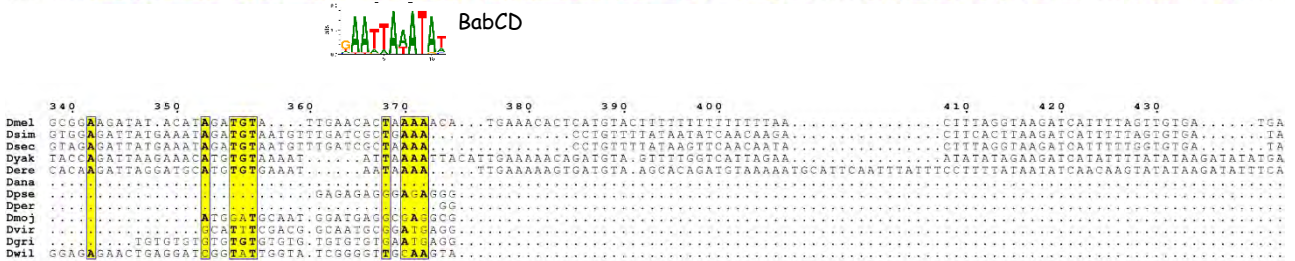
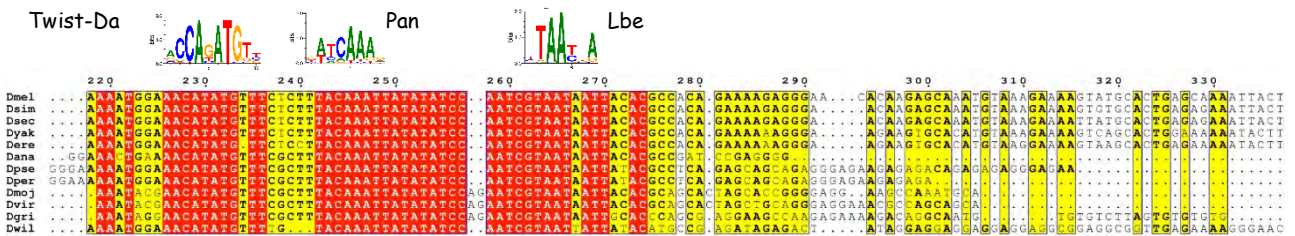
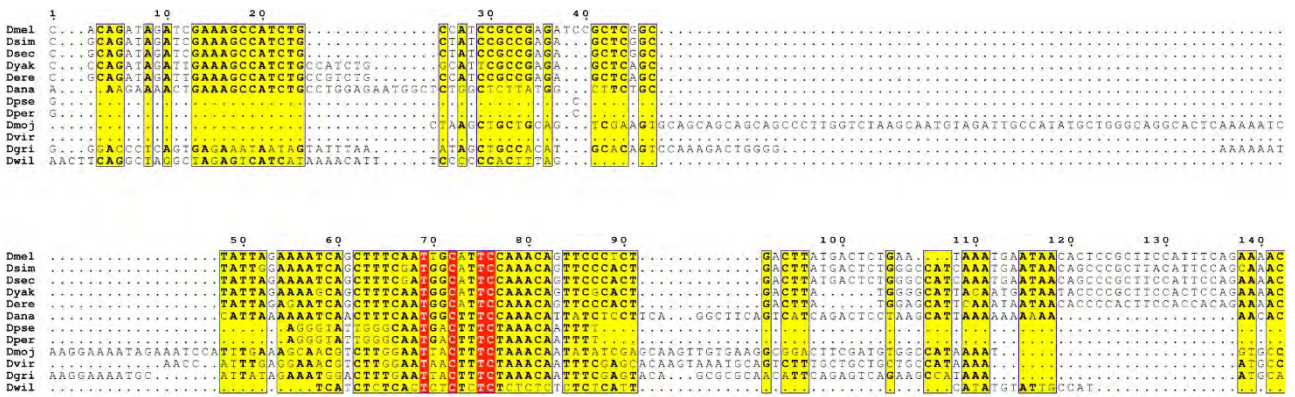
LAE sequence conservation among Drosophilidae (Part1)



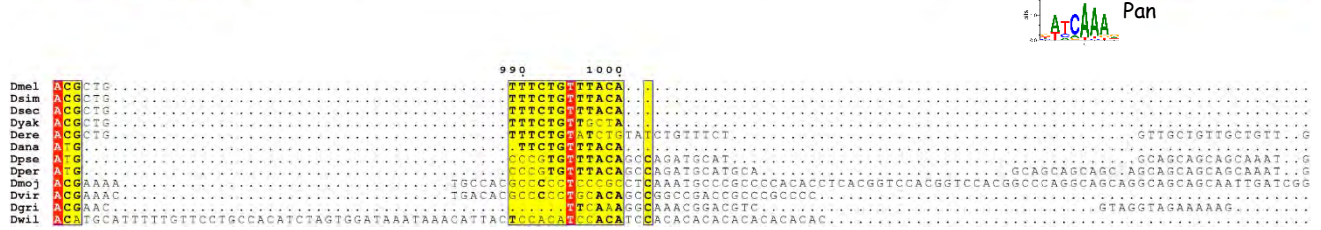
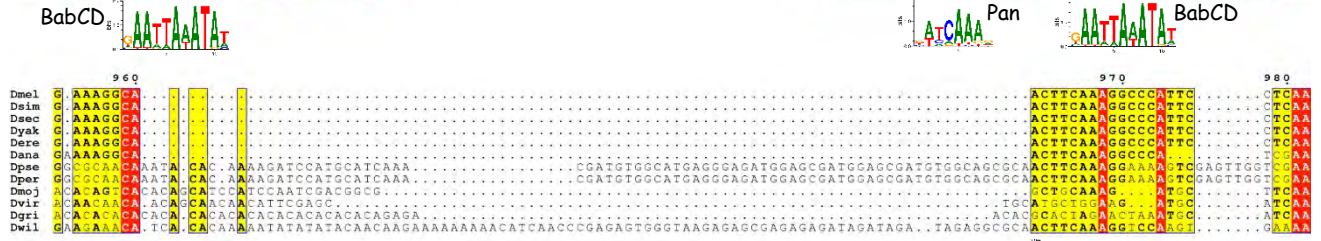
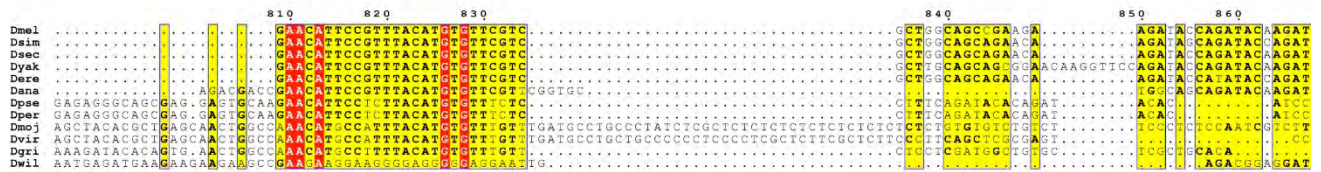
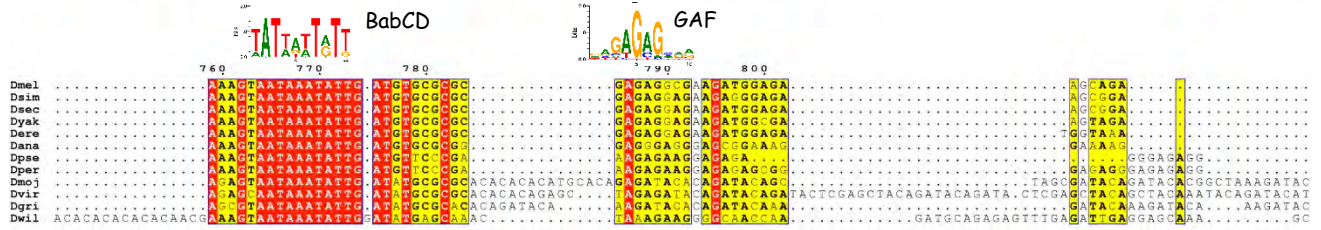
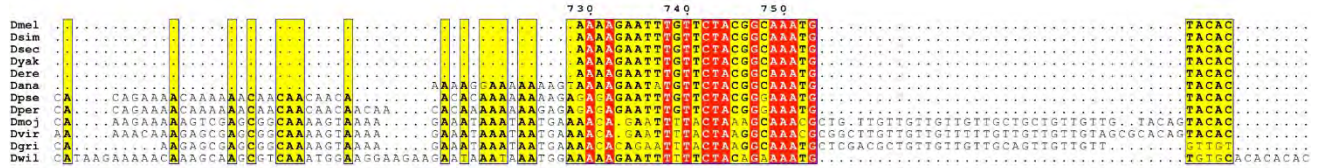
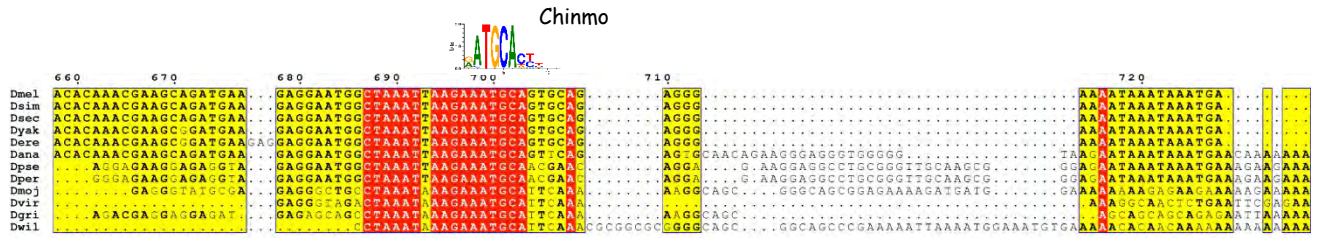
LAE sequence conservation among Drosophilidae (Part2)



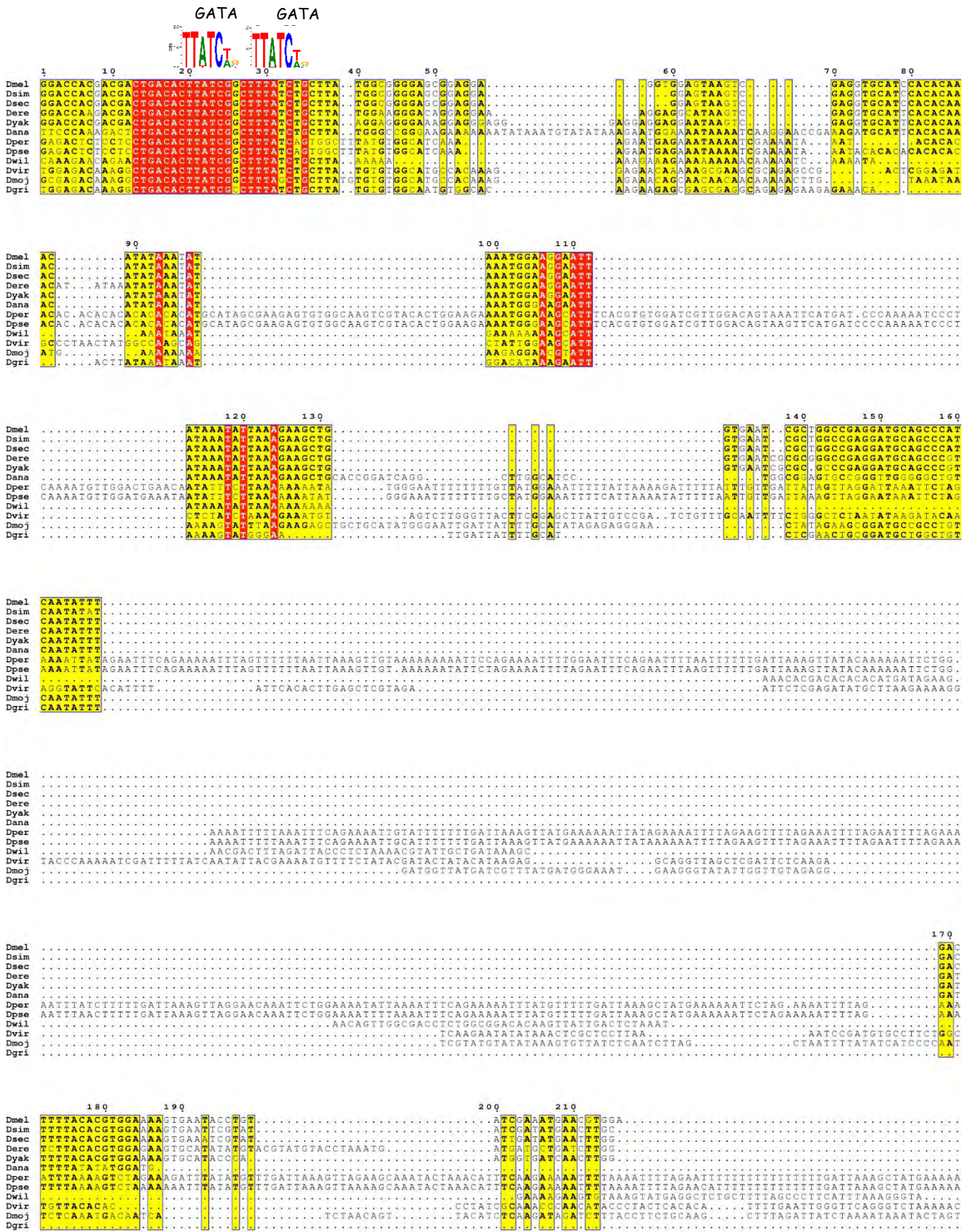
Cardiac CE sequence conservation among Drosophilidae (Part1)



Cardiac CE sequence conservation among Drosophilidae (Part2)



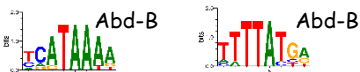
Abdominal AE sequence conservation among Drosophilidae (Part1)



Abdominal AE sequence conservation among Drosophilidae (Part4)

Sequence alignment showing conservation across species (Dmel, Dsdim, Dssec, Dderek, Ddyak, Ddana, Ddper, Ddpse, Ddwil, Ddvir, Ddmoj, Ddgr1) from position 930 to 970. Conserved regions are highlighted in yellow and red.

Sequence alignment showing conservation across species (Dmel, Dsdim, Dssec, Dderek, Ddyak, Ddana, Ddper, Ddpse, Ddwil, Ddvir, Ddmoj, Ddgr1) from position 980 to 1040. Conserved regions are highlighted in yellow and red.



Sequence alignment showing conservation across species (Dmel, Dsdim, Dssec, Dderek, Ddyak, Ddana, Ddper, Ddpse, Ddwil, Ddvir, Ddmoj, Ddgr1) from position 1050 to 1100. Conserved regions are highlighted in yellow and red.



Abdominal DE sequence conservation among Drosophilidae (Part1)

