

Kulkarni et al.

1 ***oskar* acts with the transcription factor Creb to regulate long-term memory in crickets**

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24 **Abstract**

25
26 Novel genes have the potential to drive the evolution of new biological mechanisms, or to integrate into
27 pre-existing regulatory circuits and contribute to the regulation of older, conserved biological functions.
28 One such gene, the novel insect-specific gene *oskar*, was first identified based on its role in establishing
29 the *Drosophila melanogaster* germ line. We previously showed that this gene likely arose through an
30 unusual domain transfer event involving bacterial endosymbionts, and played a somatic role before
31 evolving its well-known germ line function. Here, we provide empirical support for this hypothesis in the
32 form of evidence for a novel neural role for *oskar*. We show that *oskar* is expressed in the adult neural
33 stem cells of a basally branching insect, the cricket *Gryllus bimaculatus*. In these stem cells, called
34 neuroblasts, *oskar* is required together with the ancient animal transcription factor *Creb* to regulate long-
35 term (but not short-term) olfactory memory. We provide evidence that *oskar* positively regulates *Creb*,
36 which plays a conserved role in long-term memory across animals, and that *oskar* in turn may be a direct
37 target of *Creb*. Together with previous reports of a role for *oskar* in nervous system development and
38 function in crickets and flies, our results are consistent with the hypothesis that *oskar*'s original somatic
39 role may have been in the insect nervous system. Moreover, its co-localization and functional cooperation
40 with the conserved pluripotency gene *piwi* in the nervous system may have facilitated *oskar*'s later co-
41 option to the germ line in higher insects.
42

43 Keywords: *oskar*, memory, crickets, *Creb*, co-option, neuroblast, mushroom body, Kenyon cells
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Kulkarni et al.

45 **Introduction**

46 *Oskar* (*osk*) is an insect-specific gene first discovered in *Drosophila melanogaster*, where it plays a
47 critical role in germline specification¹. *Oskar* mRNA is localized to the posterior of the developing *D.*
48 *melanogaster* oocyte^{2,3}. Local translation and anchoring of *Oskar* (*Osk*) protein leads to the posterior
49 accumulation of the mRNA and protein products of several genes with conserved expression and function
50 in animal germ lines, including *vasa* and *piwi*^{2,4,5}. Collectively called germ plasm, these cytoplasmic
51 contents act as necessary and sufficient determinants to specify embryonic germ cells^{2,3}. The current
52 model of *Osk* function in *D. melanogaster* germ plasm assembly is that it serves as a scaffolding protein,
53 facilitating the assembly of the ribonucleoprotein complexes that contain germ plasm components^{2,6,7}.
54
55 Interestingly, *osk* and several other genes originally identified as *D. melanogaster* germ line genes,
56 including *vasa*, *pumilio*, *staufer*, *orb*, and *piwi*-related genes including *aubergine* and *argonaute 3*, have
57 since been shown to have a variety of roles in animal nervous systems⁸⁻¹⁴. For example, in *D.*
58 *melanogaster*, *osk* RNAi in larval dendritic arborization neurons disrupts *nanos* mRNA localization,
59 ultimately leading to a defect in dendrite morphogenesis and an associated defect in motor response to
60 mechanical stimulation¹². Furthermore, *osk* plays a role in the embryonic nervous system, but not in the
61 germ line, in a basally branching hemimetabolous insect, the cricket *Gryllus bimaculatus*, where it is
62 important for proper neuroblast divisions and subsequent axonal patterning¹⁵. Our recent analysis of
63 hundreds of previously unidentified *osk* orthologs across insects showed that *osk* is expressed in at least a
64 dozen somatic tissues in species across the insect tree¹⁶. This suggests that a somatic function of *osk* may
65 be ancestral. However, the precise roles of *osk* in any somatic tissue, including the nervous system,
66 remain largely unknown.

67

68 Here, we demonstrate a role for *osk* in the adult brain of the cricket *Gryllus bimaculatus*, in a population
69 of neural stem cells in the mushroom body that persist throughout adult life. We show that *osk*, as well as
70 *Piwi* and *Vasa*, are enriched in a population of adult neuroblasts in the mushroom body, and that RNA

Kulkarni et al.

71 interference (RNAi) targeting *osk* or *piwi* in adult crickets impairs long-term, but not short-term memory
72 formation in an olfactory associative learning assay¹⁷. We also provide evidence that *osk* and *piwi*
73 function in a regulatory feedback loop with the cyclic AMP response element binding protein (Creb), a
74 transcription factor with well described conserved roles in long-term memory across metazoans¹⁸. Our
75 data demonstrate a novel somatic role for *osk*, and shed light on how a novel gene may acquire critical
76 roles by integrating with pre-existing gene regulatory systems comprising older, conserved genes.

77

78 **Results and Discussion**

79

80 ***osk* is expressed in adult neuroblasts of the mushroom body**

81 We previously showed that neuroblasts in the cricket embryo express *osk*, *vasa* and *piwi*, and that *osk* is
82 required for correct neuroblast division and embryonic nervous system morphology¹⁵. Interestingly, in
83 many insects, including crickets, a subset of embryonic neuroblasts persist throughout adulthood and
84 continuously give rise to new neurons called Kenyon cells that comprise the mushroom body^{19–21}. This
85 contrasts with flies like *D. melanogaster*, in which neuroblasts die prior to adulthood²², and in which adult
86 brains are thus essentially devoid of neurogenesis²³ (although there are reports of potential stem cells in
87 adult *D. melanogaster* brains^{23,24} which may be damage-dependent rather than homeostatic in function²⁵,
88 and which remain controversial²⁶.)

89

90 Given the role of *osk* in embryonic neuroblasts of crickets, we asked whether *osk* also plays a role in the
91 adult mushroom body neuroblasts. We used *in situ* hybridization to examine *osk* expression in the adult
92 brain, and found expression in a cluster of cells with the large, round nuclei and diffuse chromatin
93 characteristic of stem cells, at the apex of each of the two lobes of the mushroom body, consistent with
94 descriptions of adult neuroblasts in Orthopterans (Fig. 1A). EdU co-localization (Fig. 1B) confirmed the
95 identity of these cells as neuroblasts, the only proliferative cells in the adult brain²⁷. We also found that
96 mushroom body neuroblasts express high levels of Vasa and Piwi proteins (Fig. 1E).

Kulkarni et al.

97

98 Previous research has shown that mushroom body neuroblasts play an important role in long-term
99 olfactory memory formation in Orthoptera²⁸. Scotto-Lomassesse²⁸ found that ablation of mushroom body
100 neuroblasts using irradiation led to a dramatic reduction in olfactory, but not visual, learning after 24- and
101 48- hours, suggesting that newborn mushroom body neurons produced by those neuroblasts play a role in
102 forming new olfactory memories. We therefore sought to test whether *osk*, expressed specifically in
103 mushroom body neuroblasts, functions in these cells in the context of long-term memory formation.

104

105 We first tested whether Osk regulates the proliferation or survival of adult mushroom body neuroblasts.
106 Using an established technique for systemic RNAi in the adult cricket brain²⁹, we injected double
107 stranded *osk* RNA (dsRNA) into the head capsule, and confirmed efficiency of *osk* knockdown via qPCR
108 and small RNA profiling of *osk*^{RNAi} brains (Fig. 2A, 2D, Suppl. Tables S1, S2, Suppl. Fig. S1, S2). *osk*^{RNAi}
109 adult brains showed no gross anatomical defects relative to controls (data not shown). Moreover, neither
110 the total number of neuroblasts (p<0.05), nor the number of neuroblasts undergoing mitosis as revealed
111 by Edu labeling (p<0.05), were statistically significantly different between *osk*^{RNAi} adult brains and
112 controls (Fig. 1C, 1D). We stained *osk*^{RNAi} and control brains with Cleaved Caspase-3, a marker for
113 apoptosis, and did not observe any evidence of cell death (Suppl. Fig. S3A). We noted that one described
114 role for PIWI in the *Drosophila* germ line is to prevent DNA damage caused by transposon
115 mobilization³⁰. However, we observed no detectable increase in γ H2A staining, a marker for DNA
116 damage, in *osk*^{RNAi} brains (Suppl. Fig. S3B). These data suggest that *osk* is not required for the
117 proliferation, survival or genomic integrity of adult neuroblasts. However, the specific expression of *osk*,
118 Piwi, and Vasa in the mushroom body neuroblasts suggested that some or all of these genes could play a
119 role related to memory or learning.

120

121 ***oskar* RNAi impairs long-term but not short-term memory**

Kulkarni et al.

122 The mushroom body is the anatomical substrate for olfactory memory and learning in insects^{27,31,32}, and
123 ablation of the mushroom body or of the adult mushroom body neuroblasts impairs these processes^{28,33,34}.
124 Based on previous observations that mushroom body neuroblasts play a role in long-term olfactory
125 memory formation in crickets²⁸, we hypothesized that *osk* might play a role in this process. To test this
126 hypothesis, we assessed memory of *osk*^{RNAi} adult male crickets at one hour (“short-term memory”) and
127 one day (“long-term memory”) post-training using well-established cricket olfactory behavior assays¹⁷. In
128 control crickets (injected with dsRNA targeting DsRed³⁵), four training sessions led to a significant
129 ($p < 0.05$) short-term preference for the rewarded odor (peppermint) at one hour after training (short-term).
130 Trained control crickets retained this learned preference ($p < 0.01$) even at one day after training (Fig. 2A,
131 “DsRed” panel), demonstrating that long-term memory formation is intact in these controls. However,
132 although *osk*^{RNAi} crickets formed and retained memory for the rewarded odor by one hour after training
133 (short-term; Fig. 2A *osk* dsRNA #1, $p < 0.001$) this memory was lost by one day after training (long-term;
134 Fig. 2A *osk* dsRNA #1, $p > 0.05$), indicating a specific impairment of long-term memory formation. These
135 results were reproducible in a second experiment using a non-overlapping fragment of *osk* dsRNA (Fig.
136 2A, *osk* dsRNA #2; $p < 0.05$ for short-term, $p > 0.05$ for long-term), suggesting the impact was specific to
137 *osk* knockdown. Efficacy of the knockdown was confirmed via qPCR and small RNA sequencing (Suppl.
138 Fig. S1, S2, Suppl. Tables S1, S2), indicating that *osk* is required for cricket long-term memory.
139
140 Since both *piwi* and *vasa* were co-expressed with *osk* in cricket mushroom body neuroblasts¹⁵ (Fig. 1E),
141 we also assessed the role of these two genes in olfactory memory. We found that *piwi* (Fig. 2B, $p < 0.001$)
142 but not *vasa* (Suppl. Fig. S4A, $p > 0.05$; Suppl. Fig. S4B) was also required for cricket long-term memory.
143 qPCR analyses showed that *osk* RNAi led to a significant decrease in *piwi* transcript levels (Fig. 2D),
144 suggesting that *osk* positively regulates *piwi* in the cricket brain. However, *osk* transcript levels remained
145 unaffected in *piwi*^{RNAi} animals (Fig. 2E). Consistent with the phenotype of the single gene knockdowns,
146 *osk*^{RNAi}/*piwi*^{RNAi} double knockdown adults also showed a long-term memory impairment phenotype (Fig.

Kulkarni et al.

147 2C, $p < 0.01$). Thus, *osk* and *piwi* do not globally disrupt olfaction, learning or short-term memory
148 formation, but are required for consolidation of long-term memory in this species.

149

150 ***osk* and *piwi* positively regulate the nuclear transcription factor *CrebA***

151 To understand how a novel gene like *osk* might have gained a role in an ancient animal function like long-
152 term memory consolidation, we investigated the hypothesis that it might interact with conserved
153 regulators of animal memory. To test this hypothesis, we asked whether we could detect a functional or
154 regulatory interaction between *osk* and a highly conserved transcription factor with well-documented
155 roles in long-term memory formation across animals, cyclic AMP response element binding protein
156 (*Creb*)¹⁸. We first identified putative *Creb* orthologs in the *G. bimaculatus* genome³⁶ using a combination
157 of BLAST searches and phylogenetic analyses (Fig. 3A, Suppl. Table S3). These analyses yielded two
158 high-confidence *Creb* orthologs, which we called *CrebA* and *CrebB* based on their closest *D.*
159 *melanogaster* *Creb* gene relative (Fig. 3A). Analysis of previously generated transcriptomes³⁷ showed
160 that both genes are expressed in adult cricket brains (Suppl. Table S4). We performed *CrebA* and *CrebB*
161 RNAi experiments and discovered that *CrebA* was required for long-term memory in crickets (Fig. 3B;
162 $p < 0.003$ and $p < 0.001$ for dsRNA#1 and dsRNA#2 respectively; Suppl. Fig. S4C). Using quantitative PCR
163 (qPCR), we then asked whether transcript levels of this memory regulator were altered in *osk* or *piwi*
164 knockdown conditions, and found consistent downregulation of *CrebA* transcript levels in both single and
165 double RNAi backgrounds (Fig. 2D-F). In contrast, and consistent with the observation that *vasa*^{RNAi} had
166 no long-term memory impact (Suppl. Fig. 1B), qPCR revealed no reduction of *CrebA* transcripts in
167 *vasa*^{RNAi} conditions (Suppl. Fig. 1 C). This suggests that the long-term memory defects observed in *osk*^{RNAi}
168 and *piwi*^{RNAi} conditions (Fig. 2D-F) are due to a downregulation of *CrebA* in these animals.

169

170 ***osk* and *piwi* are regulated by *CrebA***

171 *Creb* proteins are transcription factors that bind cyclic AMP response element (CRE) binding sites within
172 the regulatory regions of target genes to initiate transcription¹⁸ (Fig. 4A). Since target gene transcription

Kulkarni et al.

173 and new protein synthesis is crucial for long-term memory formation, and given the similarity in long-
174 term memory phenotypes of *osk*^{RNAi}, *piwi*^{RNAi} and *CrebA*^{RNAi} animals, we asked whether *osk* and *piwi*
175 might also be *Creb* target genes in this cricket. qPCR revealed that transcript levels of both *osk* and *piwi*
176 are significantly decreased in *CrebA*^{RNAi} conditions (Fig. 4B), suggesting that *osk/piwi* and CrebA may
177 interact in a positive feedback loop to promote each other's transcription. To evaluate the possibility that
178 *osk* or *piwi* might be direct transcriptional targets of CrebA, we examined the genomic sequences flanking
179 both loci and found two bioinformatically predicted CRE binding sites within the 6kb upstream of the
180 transcription start sites for *osk* (Fig. 4C) and *piwi* (Fig. 4D). These predicted CRE binding were found
181 twice as frequently as we would expect to find such sequences in a randomly generated sequence of this
182 length (see Methods). Electrophoretic mobility shift assays showed that protein(s) within the adult cricket
183 brain bind specifically to the predicted CRE sites of both of *osk* and *piwi* (Fig. 4C, D). Given the current
184 lack of species-specific CrebA reagents for this cricket species, we cannot rule out the interpretation that a
185 protein(s) other than CrebA present in the adult cricket brain is causing the observed mobility shift by
186 binding the predicted CRE sites of *osk* and *piwi*. However, given our functional data indicating that RNAi
187 against *osk*, *piwi* and *CrebA* all yield long-term memory defects (Figs. 2, 3B), that *osk* and *piwi* transcript
188 levels are reduced in *CrebA*^{RNAi} brains (Fig. 2B), and that *osk* and *piwi* genomic loci contain predicted
189 CRE binding sites (Fig. 4C, D), the results of our gel shift assays are consistent with the hypothesis that
190 cricket *osk* and *piwi* are direct transcriptional targets of CrebA.

191
192 We have discovered a new role for *oskar* in the adult cricket brain (Fig.1). We have shown that *osk*, Piwi,
193 and Vasa are co-expressed in mushroom body neuroblasts (Fig. 1A, B), a population of stem neural cells
194 required for long-term olfactory memory formation²⁸, and that knockdown of *osk* and *piwi* disrupt
195 olfactory long-term memory formation. The precise role that the mushroom body neuroblasts play in
196 memory formation remains unknown, as does the molecular role of Osk in these cells. In *D.*
197 *melanogaster*, where there are no adult neural stem cells in the mushroom body, olfactory long-term
198 memory requires the ~2,500 differentiated Kenyon cell neurons of the mushroom body³⁸, which respond

Kulkarni et al.

199 with high selectivity to a small number of stimuli, allowing the mushroom body to house an explicit
200 representation of a large number of olfactory cues^{38,39}. Specific olfactory stimuli are associated with
201 learned behavioral responses via specific sets of neurons connecting the mushroom body to other brain
202 regions in a protein synthesis-dependent fashion, to form long-term memories^{9,40}. Thus, one possibility is
203 that adult-born Kenyon cells in *G. bimaculatus* (and other insects that display adult neurogenesis in the
204 mushroom body) are recruited into an existing circuit and allow for a constantly increasing repertoire of
205 olfactory associations. Our results suggest that *osk* could play a role in this process, as *osk* RNAi disrupts
206 long-term memory. We note that of the two mammalian brain regions known to undergo adult
207 neurogenesis, one (the subventricular zone) contributes to the olfactory bulb, and neurogenesis in this
208 region is involved in olfactory memory⁴¹.

209

210 Given that adult *D. melanogaster* lacks the mushroom body neuroblasts seen in *G. bimaculatus*⁴², a
211 straightforward test for a directly comparable *osk* function in this fruit fly is not possible. However,
212 although *D. melanogaster* mushroom body stem cells are absent in adults, analogous mushroom body
213 neuroblasts remain mitotically active late into pupal development⁴³. Thus, it will be interesting to test
214 whether *osk* functions in these neuroblasts during larval and/or pupal stages. We note that an insertion of
215 an enhancer trap transposable element over 3 kb upstream of the *osk* transcription start site in *D.*
216 *melanogaster* was recovered in an insertional mutagenesis screen for long term memory in this fruit fly⁸.
217 However, this insertion has not been confirmed as compromising the sequence or function of the *osk*
218 locus, nor has *osk* been tested directly to confirm a potential role in *D. melanogaster* learning or memory.

219

220 Although *D. melanogaster* lacks adult mushroom body neuroblasts, it is possible that *osk* could function
221 in fruit fly olfactory long-term memory in a neuroblast-independent manner. A recent study of the
222 mushroom body output neurons has suggested that long-term memory involves the activity-dependent de-
223 repression of mRNAs localized to granules containing Pum, Staufen, and Orb proteins⁹. Given that Osk
224 nucleates similar granules containing these proteins in the *Drosophila* oocyte^{44,45}, and Osk's ability to

Kulkarni et al.

225 nucleate phase-transitioned granules in *D. melanogaster* cells⁴⁶, it would be interesting to test whether
226 Osk is involved in the formation and/or activity of these granules in the brain. Given our recent
227 observations of highly similar molecular interactions of conserved molecules and animal germ lines and
228 neural cells¹⁴, future studies could test whether additional, traditionally known “germ line” genes other
229 than *vasa* and *piwi* also function in *G. bimaculatus* adult neuroblasts, which would suggest that *osk* acts
230 with conserved molecular partners in different cellular contexts.

231
232 Both germ cells and neuroblasts are stem cells that give rise to highly specialized, postmitotic daughter
233 cells, while remaining proliferative for long periods of time. Thus, the original role of *osk* in both cell
234 types could conceivably be related to stem cell maintenance and/or asymmetric division. Indeed, a variety
235 of highly conserved “germ line genes” including *vasa*, *nanos*, and *piwi* are found in a variety of
236 multipotent cells in diverse animals^{14,47}, raising the possibility that such genes were involved in
237 establishing multipotency rather than specifying germ cell fate *per se*. A broader understanding of the
238 function(s) of *osk* thus requires additional studies of phylogenetically diverse insects, as well as further
239 detailed biochemical analysis in the context of *Drosophila* germ cells and neurons.

240
241 Our results provide an example of how newly arisen genes may find stable homes in pre-existing genetic
242 regulatory circuits. In the case of *osk*, we hypothesize that by evolving binding sites responsive to the
243 conserved transcription factor Creb, *osk* may have gained expression in the brain, opening the door for
244 potential participation in neural roles. Future studies will be needed to elucidate the molecular
245 mechanisms of *osk* gene products in the cricket brain, and specifically in learning and memory. We
246 speculate that the biophysical properties of Osk protein that make it effective at sequestering RNAs and
247 participating in translational control in the germ line⁴⁸⁻⁵⁰, may have been advantageous in promoting the
248 rapid translation needed the synaptic plasticity that underlies learning and memory. These include Osk’s
249 ability to form phase transitioned condensates^{46,51}, its regions of high predicted disorder^{16,46,51}, and its
250 ability to achieve and maintain asymmetric subcellular localization, all of which are well known in the

Kulkarni et al.

251 germ line, and may have provided a selective advantage to *Osk* in the context of promoting neuronal
252 function.

253

254 **Materials and Methods**

255

256 ***G. bimaculatus* husbandry**

257 For behavior experiments, *G. bimaculatus* crickets were maintained in the Mizunami laboratory at 27°C
258 on a 12:12 light cycle, with a diet of insect food pellets, as previously described⁵². For gene expression
259 analysis, quantitative PCR, and cell proliferation experiments, crickets were maintained in the Extavour
260 laboratory at 28°C and 35% relative humidity on a 12:12 light cycle, with a diet of grain and cat food, as
261 previously described⁵³.

262

263 ***In situ* hybridization**

264 For in situ hybridization, brains were dissected and de-sheathed in ice-cold 1x Phosphate Buffered Saline
265 (1X PBS) as previously described^{29,54}. Brains were fixed one hour in 4% paraformaldehyde in 1X PBS,
266 followed by an additional overnight fixation in the same solution at 4°C, or for an additional 3-4 hours at
267 room temperature. *osk* transcripts were detected using a 788 bp probe, following standard protocols⁵³ with
268 the following modifications to reduce background: 20-minute Proteinase K (Thermo Fisher Scientific,
269 Cat# EO0491) treatment followed by a 30-minute fixation in 0.8% glutaraldehyde in 1X PBS and 4%
270 paraformaldehyde in 1X PBS. The *osk* probe was used at 1.0 ng/μl concentration and hybridized at 69-
271 70°C. Brains were sectioned after in situ development was completed, by embedding in 4% low-melt
272 agarose in distilled water, and sectioning at 50-90μM using a Leica VT1000S vibratome.

273

274 **Immunostaining**

275 For immunostaining, primary antibodies used were as follows: rabbit anti-Gb-Vasa and anti-Gb-Piwi⁵⁵
276 were used at 1:300, mouse anti-RNA polymerase II pSer 6 Mab H5 (Covance MMS-129R) 1:100, FITC-

Kulkarni et al.

277 conjugated anti-alpha Tubulin (Sigma F2168) 1:100 and rabbit anti-*Drosophila* Vasa (kind gift of Paul
278 Lasko, McGill University) 1:500 following standard procedures as previously described⁵³. Goat anti-
279 rabbit secondary antibodies conjugated to Alexa 488, Alexa 555 or Alexa 568 (Invitrogen) were used at
280 1:500 or 1:1000. Counterstains used were Hoechst 33342 (Sigma B2261) at 0.1 to 0.05 mg/ml and FITC-
281 conjugated phalloidin (Sigma P5282) at 1 U/ml. For antibody staining, brains were embedded in 4% low-
282 melt agarose in distilled water, and sectioned at 50-90 μ m using a Leica VT1000S vibratome, prior to
283 incubation with the primary antibody. For in situ hybridization, brains were sectioned after staining had
284 been completed. For analysis of apoptosis and DNA damage, brains were fixed 4h after EdU injection and
285 sectioned via vibratome, with EdU detection performed first (using Invitrogen's Click-iT protocol),
286 followed by antibody staining and confocal analysis.

287

288 ***RNA extractions, cDNA preparation and cloning of gene fragments for production of dsRNA***

289 Brains from unmated male adults were dissected in ice-cold 1X PBS, then immediately homogenized in
290 TRIzol (ThermoFisher Scientific, catalog number 15596026). Total RNA was extracted following the
291 manufacturer's instructions, including a 30-minute DNase treatment. 1 μ g of total RNA was used as
292 template for cDNA synthesis using SuperScript III (ThermoFisher Scientific, catalog number 18-080-044)
293 with oligo-dT primers. cDNA was diluted 1:10 prior to PCR with gene specific primers, and 2 μ L of
294 template was used per 25 μ L PCR reaction. PCR products were run on a 1% agarose gel and desired
295 bands were gel eluted following IBI Scientific's PCR purification and gel elution kit (catalog number
296 IB47030). Then, products were cloned into Zero blunt TOPO PCR cloning kit (ThermoFisher Scientific,
297 catalog number 450245) using electro-competent *DH5 α -E* cells (ThermoFisher Scientific, catalog number
298 11319019).

299

300 ***Identification and annotation of Piwi Proteins in G. bimaculatus***

301 We previously used a *G. bimaculatus* transcriptome⁵⁶ to identify two RNA fragments corresponding to
302 two Piwi family proteins (*piwi*: JQ434103 and *piwi-2*: KC242806.1^{15,55}). All previous published analyses

Kulkarni et al.

303 of *piwi* in *G. bimaculatus* were performed with “*piwi*” (JQ434103), as only this gene showed enriched
304 expression in embryonic germ cells⁵⁵. Since the time of our initial studies on *piwi*, we assembled and
305 annotated an updated *G. bimaculatus* genome³⁶. For the present study, we therefore performed new
306 BLAST searches to clarify the status of *piwi* orthologs in this cricket (Suppl. Fig. S5). We found both
307 previously identified fragmented *piwi* RNA sequences within the new gene annotations³⁶ with gene IDs
308 GBI_17641 (containing “*piwi*” fragment JQ434103) and GBI_07509 (containing “*piwi-2*” fragment
309 KC242806.1) respectively. We also identified two additional putative novel *piwi*-like genes annotated in
310 the *G. bimaculatus* genome, with gene IDs GBI_09750 and GBI_09796³⁶.

311
312 Using InterProScan, we confirmed that the amino acid sequences of the proteins encoded by these four
313 putative *piwi* genes contained the typical characteristics of the Argonaute/Piwi proteins⁵⁷ namely a Paz
314 domain followed by a C-terminal Piwi domain. Additionally, we inferred the gene tree of the
315 Argonaute/Piwi protein family using the putative *G. bimaculatus* Piwi and Argonaute protein amino acid
316 sequences obtained from the genome, together with sequences of publicly available Piwi and Argonaute
317 proteins from other insects (*Drosophila melanogaster*, *Apis mellifera*, *Bombyx mori*, *Tribolium*
318 *castaneum*, *Blattella germanica*, *Zootermopsis nevadensis*, *Acyrtosiphon pisum*, and *Locusta*
319 *migratoria*). Protein sequence alignments were performed with MUSCLE^{58,59} in Geneious (v3.8.425;
320 www.geneious.com), and the gene tree was inferred with RAxML v8.2.11⁶⁰ with 100 bootstrap iterations
321 to obtain the support values of each node. The tree was then visualized with ggtree^{61,62}. The resulting tree
322 differentiated four groups of sequences with bootstrap values above 90%, each of which contained
323 different Piwi/Argonaute subfamilies as follows: Argonaute 1 proteins (AGO1; included GBI_02015),
324 Argonaute 2 (AGO2; included GBI_13717), Argonaute 3 (AGO3; included GBI_01357), and Piwi
325 proteins and their paralogs (Aub and Siwi, the Piwi paralogs in *D. melanogaster* and *B. mori* respectively;
326 included GBI_17641) (Suppl. Fig. S5). This indicated that our previous analyses⁵⁵ had indeed targeted the
327 true *piwi* ortholog in *G. bimaculatus*. Accordingly, all gene expression and function analyses in the
328 present study were also performed on this true *piwi* ortholog (GBI_17641).

Kulkarni et al.

329
330 Analysis of the small RNA species present in *piwi*^{RNAi} animals indicated that our *piwi* RNAi experiments
331 specifically targeted the true *piwi* (GBI_17641), and did not affect or impact expression of the other 3
332 *piwi* subfamily genes (Suppl. Table S2). The quality control of the eight small RNA-seq samples (3
333 *piwi*^{RNAi}, 2 *osk*^{RNAi}, 2 *DsRed*^{RNAi}, and 1 untreated or wild-type, WT) was performed with FastQC v0.11.8⁶³,
334 and the adapters were trimmed with Cutadapt v1.8.1⁶⁴. Clean reads were mapped to the *G. bimaculatus*
335 genome assembly³⁶ with Bowtie 2 v2.3.4.1⁶⁵ using parameters “-L 18, -N 0”. The numbers of sequenced
336 reads, clean reads, and mapped reads are shown in Suppl. Table S1. The mapped reads were retrieved
337 using samtools v1.9⁶⁶ for obtaining the read length distributions (Suppl. Fig. S2). The proportion of
338 miRNAs and piRNAs in each sample was extrapolated as the percentage of reads of 22-23 nts and 28-29
339 nts respectively (Suppl. Fig. S2). The FeatureCounts function from the R package Rsubread v2.0.0⁶⁷ was
340 used to count the number of reads mapped to all annotated genes and build a table of counts. The counts
341 of *osk* (GBI_0140) and the four *piwi* genes (GBI_09750, GBI_09796, GBI_07509, and GBI_17641) were
342 retrieved (Suppl. Table S2). The small RNA-seq reads mapping to the target genes were assumed to be
343 reads of the siRNAs produced from the dsRNA⁶⁸. The thousands of such reads mapping to our targeted
344 *piwi* (GBI_17641) and the absence of such siRNA reads mapping to other *piwi* genes, suggest that no off-
345 target effects impaired the expression of other *piwi* genes.

346

347 ***RNA interference***

348 Unmated adult male crickets within one week of their final molt were injected with 2 μ L of double-
349 stranded RNA (dsRNA) through a hole pierced in the median ocellus⁶⁹ using a 10 μ L syringe fitted with
350 26S gauge tip (WPI, Tokyo, Japan; Hamilton Inc., Nevada, USA). Behavioral tests were repeated using
351 two non-overlapping fragments of *oskar* (742bp and 503bp), a 646bp fragment of *piwi*, a 541bp fragment
352 of *vasa*, two non-overlapping fragments of *CrebA* (both 387bp fragments), a 384bp fragment of *CrebB*,
353 and a 678bp fragment of *DsRed* as a negative control¹⁵. Double-stranded RNA concentrations used were

Kulkarni et al.

354 10 μ M for *oskar*; 3.38 μ g/ μ L for *piwi*; 2.71 μ g/ μ L for *vasa*; 2.97 μ g/ μ L for *DsRed*; 6 μ g/ μ L for *oskar/piwi*
355 double knock down; 7 μ g/ μ L for *CrebA*; 7 μ g/ μ L for *CrebB*; and 7 μ g/ μ L for *DsRed* (Suppl. Table S5).

356

357 ***Construction, sequencing and analysis of small RNA libraries from G. bimaculatus adult brains***

358 Unmated adult male crickets within one week of their final molt to adulthood were injected with dsRNA
359 as described above (see *RNA interference*). At 48h post RNAi injections, brains (Suppl. Table S5) were
360 dissected in ice-cold 1x PBS, and transferred into Trizol, following which total RNA was extracted from
361 them following manufacturers protocols. Next, RNA was size selected for 18-30nt size range after
362 denaturing PAGE. A 2S rRNA specific oligo was used for 2S rRNA depletion. The small RNAs were
363 ligated at the 3' and 5' ends by the respective adapters and purified by denaturing PAGE after each
364 ligation. PCR was performed after reverse transcription. The PCR product was gel purified from an
365 agarose gel to obtain the final library. The libraries were sequenced using Illumina NextSeq500 1x75bp.
366 The resulting data were uploaded onto NCBI SRA database and are publicly available under the
367 BioProject ID PRJNA837371.

368

369 ***Gene expression from mRNA-seq data***

370 To check the expression of *G. bimaculatus* genes in nervous systems in wild-type animals, previously
371 generated mRNA-seq libraries were used³⁷ and the complete CDS for genes of interest were obtained
372 from the recently published genome³³. Reads were trimmed with Cutadapt v3.4⁶⁴, and mapped to the full
373 *G. bimaculatus* CDS using Geneious Read Mapper⁷⁰. DESeq2 normalized counts in fragments per
374 kilobase per exon per million mapped fragments (FPKMs) were then obtained for all genes of interest⁷³.
375 The expression of *osk* and *piwi* genes (Suppl. Table S4) shows that the depleted *piwi* (GBI_17641) is one
376 of the two *piwi* genes expressed in wild-type brains of males and females. In the same way, we obtained
377 the expression in FPKMs of *vasa* (GBI_17344), *CrebA* (GBI_04244), and *CrebB* (GBI_02305) (Suppl.
378 Table S4).

Kulkarni et al.

379

380 *Olfactory learning behavioral memory assays*

381 Adult male crickets at eight days after the final molt were used in all experiments. Three days before
382 conditioning, individual crickets were separated into 100-mL beakers and deprived of drinking water to
383 enhance their motivation to search for water. Two days before conditioning (ten days after the imaginal
384 molt), each cricket was injected with dsRNA as described above. Two days after dsRNA injection, each
385 cricket was subjected to an odor preference test, in which the animal was allowed to freely visit
386 peppermint and vanilla odors⁷¹. The time spent at each of the peppermint and vanilla odor sources was
387 measured cumulatively to evaluate relative odor preference⁷¹. Crickets were subjected to 4-trial
388 conditioning, in which an odor was paired with water reward, with an inter-trial interval of five
389 minutes^{29,72}. For conditioning, a small filter paper was attached to the needle of a hypodermic syringe.
390 The syringe was filled with water reward (unconditioned stimulus), and the filter paper was soaked with
391 peppermint essence (conditioned stimulus). At one hour and one day after the end of the conditioning,
392 each cricket was subjected to an odor preference test. The relative odor preference of each conditioned
393 and control animal was measured using the preference index (PI) for rewarded odor (peppermint), defined
394 as $tP/(tP+tV) * 100$ (%), where tP is the time spent exploring the peppermint source and tV is the time
395 spent exploring the vanilla (unrewarded) odor. Wilcoxon's test was used to compare odor preferences
396 before and after training. For multiple comparisons, Holm's method was used to adjust the significance
397 level.

398

399 *Quantitative PCR (qPCR)*

400 Two days after dsRNA injection, brains were dissected from unmated male adults (within a week of final
401 molt into adulthood) in ice-cold 1x PBS, then immediately homogenized in TRIzol (ThermoFisher
402 Scientific, catalog number 15596026). Total RNA was extracted from a total of six brains per treatment,
403 following the manufacturer's instructions, including a 30-minute DNase treatment. 1 µg of total RNA
404 was used as template for cDNA synthesis using SuperScript III (ThermoFisher Scientific, catalog number

Kulkarni et al.

405 18-080-044) with oligo-dT primers. cDNA was diluted 1:10 prior to qPCR, and 6 μ L of template was
406 used per 25 μ L qPCR reaction. (PerfeCta SYBR Green SuperMix, Low ROX, Quanta Biosciences,
407 catalog number 101414-158). qPCR reactions were conducted in triplicate, and fold change was
408 calculated using the delta-delta Ct method⁷³, with standard deviation propagated following standard
409 methods. *Beta-tubulin* was used as a reference gene⁵³. Primers amplifying a 130bp fragment of *Gb-*
410 *CrebA*, a 140bp fragment of *Gb-CrebB*, a 234bp fragment of *Gb-oskar*, a 166 bp fragment of *Beta-*
411 *tubulin*⁵³, a 129bp fragment of *Gb-piwi*, a 150bp fragment of *Gb-vasa* and a 120bp fragment of *Gb-FGFR*
412 (Fibroblast Growth Factor Receptor) were used (Suppl. Table S6).

413

414 ***EdU Assay***

415 Cell proliferation was assayed using the Click-iT EdU Alexa 488 kit (Life Technologies, Cat# C10637).
416 Crickets were injected with 10-15 μ l of EdU either into the abdomen or into the head capsule through the
417 median ocellus (both methods successfully labeled dividing neuroblasts), and brains were dissected to
418 visualize EdU incorporation four hours post-injection. Brains were dissected and de-sheathed in ice-cold
419 1x PBS. Calyces were removed with a microscalpel and incubated in 0.1M citric acid for 15-30 minutes
420 on a poly-lysinated slide (Sigma Aldrich, Cat. No. P8920-100ML). Calyces were then spread into a
421 monolayer by adding a Sigma cote-covered coverslip, and the entire slide was flash-frozen in liquid
422 nitrogen. The coverslip was removed, leaving the mushroom body monolayer on the slide. Slides were
423 air-dried and were then fixed for 15 minutes in 4% PFA. EdU detection was then carried out following
424 manufacturer's instructions. EdU-positive cells were photographed under epifluorescence on a Zeiss
425 AxioImager Z.1 compound microscope using Zen, and manually quantified in ImageJ. For any mushroom
426 body where the EdU-positive cluster of cells was damaged or destroyed during preparation, that sample
427 was discarded and not included in the analysis. For tissue double stained to visualize transcripts and EdU
428 incorporation simultaneously, the in situ hybridization was conducted before the visualization of
429 incorporated EdU. AxioImager Z.1, LSM 780 or LSM 880 confocal microscopes (Zeiss) were used for
430 microscopy, driven by AxioVision or Zen (Zeiss).

Kulkarni et al.

431

432 ***Identification of G. bimaculatus Creb genes and construction of Creb phylogenetic tree***

433 Putative orthologs of *Creb/ATF* family members from several animal species were initially identified by
434 BLAST searches and then downloaded from NCBI. These sequences were then used to search for
435 putative *G. bimaculatus CrebA* orthologs in the *G. bimaculatus* genome³⁶. All identified sequences were
436 then aligned with MAFFT (v 7.510)⁷⁴. A maximum likelihood tree was created in RAxML using the
437 PROTGAMEWAG model⁶⁰, and plotted with the FigTree package v1.4.4
438 (<http://tree.bio.ed.ac.uk/software/figtree>) (Fig.3B, Suppl. Table S3).

439

440 ***Bioinformatic prediction of CRE sites in osk and piwi upstream regulatory regions***

441 A position frequency matrix (PFM) for the full cyclic AMP response element (CRE) octameric
442 palindrome was obtained from the JASPAR database (an open source database for transcription factor
443 binding sites⁷⁵ (Suppl. File 1). In addition to CRE, PFMs for the TATA box were also obtained from the
444 same database. We included TATA box proximity among our search criteria for putative CRE sites, since
445 TATA boxes are often a feature of functional promoters, and functional promoter-proximal CRE sites are
446 reported as often occupied by Creb. These raw PFM data (Suppl. File 1) were then used as an input in
447 FIMO (Find Individual Motif Occurrence) in the MEME suite (a motif-based sequence analysis tool⁷⁶,
448 and up to 10Kb of the gene sequences upstream of the predicted transcription start site for each of *G.*
449 *bimaculatus oskar*, *piwi*, and *vasa* was scanned for the presence of the CRE and TATA motifs using the
450 annotated *G. bimaculatus* genome³⁶, and using $p < 0.0001$ as the stringency criteria. For comparison, *G.*
451 *bimaculatus beta actin*, *alpha tubulin* and *FGFR* loci were subjected to the same analyses (also with
452 $p < 0.0001$ as stringency criteria) to assess the possibility that any *G. bimaculatus* gene would be predicted
453 to have CRE sites in the 10Kb region upstream of their transcription start site using this method (Suppl.
454 File 2). We found that for the latter three genes, there were no CRE predictions in their upstream regions
455 (up to 10Kb from the transcription start site). Further, we bioinformatically generated one thousand 10Kb
456 long DNA fragments of random sequence using the “random DNA sequence” tool in the Sequence

Kulkarni et al.

457 Manipulation Suite⁷⁷ and then tested them for CRE prediction. Our results indicate that a CRE site is
458 expected to occur in a randomly generated sequence at a frequency of ~1.6 CRE sites for every 10Kb
459 tested (Suppl. Files 1,2).

460

461 ***PCR amplification, sequence confirmation and cloning of CRE sites***

462 Based on bioinformatic predictions of putative CRE sites, primers were designed in the upstream
463 regulatory regions of *osk* (Suppl. Table S7 #1 and #2) and *piwi* (Suppl. Table S7 #3 and #4). Once all
464 four CRE sites were sequence confirmed by Sanger sequencing (2 CRE sites/gene), the ~30bp fragments
465 containing each CRE site were synthetically generated as duplexes (with 3'A overhangs) for use as
466 EMSA pre-probes (CRE site in bold) (Suppl. Table S7). The 3'A overhangs were then used to clone all
467 EMSA pre-probes into a pGEM-T easy vector following manufacturer's instructions (Promega, catalog
468 number A1360) using One-Shot chemically competent TOP10 *E. coli* cells (Thermo-Fischer, catalog
469 number C4040-06).

470

471 ***Generation of 5'Cy5 labelled Electrophoretic Mobility Shift Assay (EMSA) Probes and EMSA***

472 Once cloned, pGEM-T easy specific duplex forward primer (5'Cy5-ACGTCGCATGCTCCCGGCCATG,
473 reverse complement 5'Cy5-CATGGCCGGGAGCATGCGACGT), and reverse primer (5'Cy5-
474 GTCGACCTGCAGGCGGCCGCGAATT, reverse complement 5-Cy5-
475 AATTCGCGGCCGCCTGCAGGTCGAC) were designed with 5'Cy5 modifications to amplify inserts
476 and generate fluorescently labeled double stranded EMSA probes, using a two-step PCR program with
477 the following conditions: (98°C for 60 seconds (x1cycle); 98°C for 15 seconds followed by 72°C for 30
478 seconds (x30 cycles); 72°C for 5 minutes (x1cycle) (Suppl. Table S7). The PCR product was loaded onto
479 a 1% Agarose Gel and the desired bands were gel eluted following IBI Scientific's PCR purification and
480 gel elution kit (catalog number IB47030) in 30µl water. A second round of PCR amplification following
481 the conditions described above was performed using the eluted DNA from previous steps to increase
482 probe yield. All steps starting with the first round of PCR were done in the dark to protect fluorescently

Kulkarni et al.

483 labeled probes. Probe concentrations were measured using Nanodrop, and diluted to a final concentration
484 of 40 fmol/probe for use in EMSA⁷⁸. 20% native PAGE gels were used to study gel shifts. Gels were
485 imaged using an Azure Sapphire Biomolecular Imager (VWR).

486

487 ***Nuclear Protein extracts from unmated adult male *G. bimaculatus* brains***

488 Brains were dissected from unmated *G. bimaculatus* males within one week of their final molt that were
489 anesthetized briefly on ice prior to dissection in 1x PBS. Nuclear protein extracts were prepared from
490 dissected brains following manufacturer's instructions (Abcam Nuclear Extraction Kit, catalog number
491 ab113474).

492

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500

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506

507 **Author Contributions**

Kulkarni et al.

508 AK performed all qPCRs, bioinformatic analysis of CRE sites, gel shift assays, cricket brain, nerve cord
509 and other tissue transcriptomes referenced in this study, and prepared small RNA libraries with the
510 assistance of SP in consultation with WET; BEC performed whole mount gene expression and EdU
511 assays; KT, YM, YL, TW performed olfactory learning assays, GY performed small RNA library analysis
512 and the cricket Piwi/AGO identification and phylogenetic analysis, JK provided technical support for gel
513 shift assays; MM and CGE conceived of the project and experimental design; AK and CGE wrote the
514 manuscript with input from all co-authors; WET, MM and CGE obtained funding for the project.

Kulkarni et al.

515 Figure Legends

516

517 **Figure 1. *oskar*, *Piwi* and *Vasa* are expressed in *G. bimaculatus* adult mushroom body neuroblasts.**

518 (A) In situ hybridization on adult *G. bimaculatus* brains detects *osk* transcripts in the cells of the
519 mushroom body (arrows). Inset in top left panel shows the overall structure of the adult brain; shaded box
520 indicates a single mushroom body lobe, corresponding to the region shown in micrographs in top row.
521 Bottom row: dorsal views of both mushroom body lobes, indicating *oskar* expression in neuroblast
522 clusters (arrows). White asterisks indicate non-specific binding of probe to tracheal remnants in the brain.
523 (B) Edu labelling (green) of the adult brain shows that *osk*-expressing cells (grey) are mitotically active,
524 consistent with their identity as neuroblasts. (C) Quantification of EdU-positive cells shows no significant
525 difference between *osk^{RNAi}* and control brains ($p < 0.05$). (D) Quantification of total number of mitotically
526 active cells shows no significant difference between *osk^{RNAi}* and control brains ($p < 0.05$). Numbers within
527 bars indicate sample sizes in (C) and (D). (E) Detection of *Vasa* & *Piwi* proteins in adult mushroom body
528 neuroblasts (MBNBs). Scale bars = 50 μ m in top panels of (A) and (B) and in (E), and 200 μ M in bottom
529 panels of (A).

530

531 **Figure 2. *oskar^{RNAi}* and *piwi^{RNAi}* impairs cricket long-term memory.**

532 (A) Results of olfactory memory
533 assay in *oskar^{RNAi}* animals. (B) Results of olfactory memory assay in *piwi^{RNAi}* animals. (C) Results of
534 olfactory memory assay in *osk^{RNAi}/piwi^{RNAi}* double knockdown animals. For each assay, relative preference
535 between the rewarded odor (peppermint) and control odor (vanilla) was tested before training, one hour
536 post-training, and one day post training for *DsRed* controls and for *osk* (using two different non-
537 overlapping *osk* fragments #1 and #2), *piwi* and *osk/piwi* double RNAi. Boxes represent the 1st and 3rd
538 quartiles surrounding the median (middle line). Whiskers extend to extreme values within 1.5x of
539 interquartile range. Wilcoxon's test was used for comparison of preference before and after conditioning.
540 For multiple comparisons, the Holm method was used to adjust the significance level (* $p < 0.05$, **
541 $p < 0.01$, *** $p < 0.001$, n.s. = not significantly different). (D-F). Quantitative qPCR results showing the
542 extent of downregulation of different *G. bimaculatus* genes in *osk*, *piwi* and *osk/piwi* double RNAi
543 backgrounds. Effectiveness of RNAi per background is also shown in each case. Data is plotted as mRNA
544 fold change (+/- standard deviation) based on the $\Delta\Delta$ Ct method (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,
545 **** $p < 0.0001$, n.s. = not statistically significant).

546

547 **Figure 3. Cricket *CrebA* is required for cricket long-term memory.**

548 (A) GenBank IDs of *Creb/ATF*
549 family member orthologs in mouse and insects (Suppl. Table S3) were used to construct a *Creb*
550 phylogenetic tree to infer the evolutionary relationships between mammalian *Creb* proteins and their
551 insect counterparts. *G. bimaculatus* *CrebA* and *CrebB* are indicated in black in the tree (B) *CrebA^{RNAi}*
552 impairs long-term memory formation in crickets. Relative preference between the rewarded odor
553 (peppermint) and control odor (vanilla) was tested before training, one hour post-training, and one day
554 post training for *DsRed* controls and *CrebA^{RNAi}* (using two different non-overlapping *CrebA* fragments #1
555 and #2 for independent confirmation). Boxes represent the 1st and 3rd quartiles surrounding the median
556 (middle line). Whiskers extend to extreme values within 1.5x of interquartile range. Wilcoxon's test was
557 used for comparison of preference before and after conditioning. For multiple comparisons, the Holm
558 method was used to adjust the significance level. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not
559 significantly different). $n=9$ for *CrebA* and $n=10$ for *DsRed*.

560

561 **Figure 4. Cricket *CrebA* regulates *oskar*.**

562 (A) Schematic diagram of the transcription factor cAMP
563 response element binding protein (Creb) protein (top) displaying only the two domains relevant to this
564 study, the Kinase-Inducible Domain (KID) that can facilitate kinase-inducible transcription activation and
565 the basic leucine zipper (bZIP) domain that is important for dimerization and DNA binding. Creb proteins
566 bind the cyclic AMP response element (CRE), a sequence present in the promoter regions of many
567 cellular genes to increase (middle schematic) or decrease (bottom schematic) transcription of target gene.
568 (B) Quantitative RT-PCR results showing the expression levels of *G. bimaculatus* *osk*, *piwi*, and *vasa* in

Kulkarni et al.

566 *CrebA* RNAi knockdown conditions. The extent of *CrebA* downregulation is also shown to assess
567 efficiency of RNAi knockdown. Data is plotted as mRNA fold change (+/- standard deviation) based on
568 the $\Delta\Delta\text{Ct}$ method (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, * $p < 0.0001$ n.s. = not statistically significant).
569 (C) Schematic of *G. bimaculatus oskar* gene (top) showing exons (depicted as rectangular grey boxes)
570 along with its presumptive regulatory region containing two predicted CRE sites, which we call CRE I
571 (~3.6Kb upstream of predicted transcription start site marked by fMet) and CRE II (~5.3Kb upstream of
572 predicted fMet). (Bottom) Electrophoretic Mobility Shift Assay (EMSA) to detect possible Creb binding
573 to *oskar*'s CRE I (top, in green) and CRE II (bottom, in blue). “*oskar* CRE+ probe” indicates predicted CRE
574 site-containing probe “*oskar* CRE- probe” indicates probe without predicted CRE sites; “cricket brain
575 protein” indicates *G. bimaculatus* brain protein extract; “BSA” indicates 1% BSA as a non-specific
576 protein control. The complete sequences of the EMSA probes used for CRE I and II experiments are
577 indicated in green and blue text underneath the gel image, with black representing the predicted CRE
578 sequence (D) Schematic of *G. bimaculatus piwi* gene (top) showing its exons and presumptive regulatory
579 region containing two predicted CRE sites, CRE I (~3.2Kb upstream of predicted fMet) and II (~5.7Kb
580 upstream of predicted fMet). (Bottom) EMSA results showing a mobility shift (marked with pink
581 asterisk) for *piwi* CREs. Lane labels are as indicated in (C). The complete sequence of the EMSA probes
582 are indicated in green (*piwi* CRE I) and blue (*piwi* CRE II) text underneath the gel image, with the CRE
583 sequence in black.
584

Kulkarni et al.

585 Supplementary Figure Legends

586

587 **Figure S1. Read Length Distribution:** Percentage of mapped reads of each length from 18 to 29
588 nucleotides in each sequenced small RNA library colored by RNAi treatment. The two peaks at ~22 and
589 ~28 mainly correspond to miRNAs and piRNAs.

590

591 **Figure S2. miRNAs vs piRNA:** Estimated proportion of reads corresponding to microRNAs and piRNAs
592 in each sample (colored by condition) based on the percentage of reads of 22-23 nucleotides in length and
593 28-29 nucleotides respectively.

594

595 **Figure S3.** Apoptosis marker Cleaved caspase 3 (**A**) and DNA damage marker gamma H2AX
596 immunostaining (**B**) in adult mushroom bodies, including neuroblasts of control and *osk*^{RNAi} brains.

597

598 **Figure S4.** (**A**) *vasa* RNAi fails to recapitulate the long-term olfactory memory phenotype seen in *osk*
599 and *piwi* RNAi. (**B**) qPCR on *vasa*^{RNAi} brains shows significant up-regulation of *CrebA*. (**C**) *CrebB* RNAi
600 does not recapitulate the long-term memory phenotype shown by *CrebA*^{RNAi}. N=9 for *CrebB*, and N=10
601 for *DsRed*.

602

603 **Figure S5.** *G. bimaculatus piwi* ortholog identification and phylogenetic analysis. Argonaute family gene
604 tree generated with the PIWI, AUB, AGO1, AGO2, and AGO3 protein sequences from *Drosophila*
605 *melanogaster*, *Apis mellifera*, *Bombyx mori*, *Tribolium castaneum*, *Blattella germanica*, *Zootermopsis*
606 *nevadensis*, *Acyrtosiphon pisum*, and *Locusta migratoria*. Values at nodes represent bootstrap support,
607 in boxes color-coded from dark (lowest) to light (highest) blue. *G. bimaculatus piwi* and *argonaut* genes
608 indicated by red and green asterisks respectively.

609

610

Kulkarni et al.

611 **Supplementary Table & File Legends**

612

613 **Table S1.** Number of raw sequenced small RNA reads, number and percentage of clean reads, and
614 number and percentage of reads mapped to the *G. bimaculatus* genome.

615

616 **Table S2.** Number of small RNA-seq reads mapped to *osk* and *piwi* genes to show specificity of RNAi
617 knockdowns. These small RNA-seq reads come from the siRNA detected following injections of dsRNA
618 for these respective genes. The targeted sequence in each library is highly enriched by small RNA-seq
619 reads. In the *piwi*^{RNAi} experiments, only the targeted *piwi* (GBI_17641) shows a high number of mapped
620 reads in the libraries generated from animals injected with the dsRNA against *piwi*, indicating that the
621 other three *piwi* orthologs present in the *G. bimaculatus* genome were unlikely to be targeted by our
622 approach.

623

624 **Table S3.** GenBank IDs of *Creb/ATF* family member orthologs in mouse and other insects including the
625 cricket *G. bimaculatus*. This information was used to construct a *Creb* phylogenetic tree (Fig. 3B) to infer
626 the evolutionary relationships between mammalian *Creb* proteins and their insect counterparts.

627

628 **Table S4.** Gene expression levels for *osk*, *piwi*, *vasa*, and *CrebA/B* (in FPKM per tissue) from brain and
629 ventral cord transcriptomes of male and female adult *Gryllus bimaculatus*³⁷. Gene IDs as per the
630 annotated cricket genome³⁶.

631

632 **Table S5.** A total of 128 unmated, adult male cricket brains (16 brains from Control 1 "uninjected
633 controls", 37 brains from Control 2 "*DsRed* injected", 45 brains from "*piwi*" dsRNA injected, and 30
634 brains from "*osk*" dsRNA injected) were dissected 48h post dsRNA injection and processed for making
635 small RNA libraries.

636

637 **Table S6.** Primers used for quantitative PCR of all listed *G. bimaculatus* genes.

638

639 **Table S7.** Primers used for cloning and generation of EMSA probes for *G. bimaculatus oskar* and *piwi*.

640

641 **Supplementary File 1:** The Frequency Matrices (PFM) from JASPAR database used to predict CRE
642 sites and TATA boxes in the presumptive regulatory regions of *G. bimaculatus* genes.

643

644 **Supplementary File 2:** FASTA file containing the simulated one thousand 10-Kb long DNA fragments
645 generated to test the frequency of occurrence of CRE sites in randomly generated sequences.

Kulkarni et al.

646 **References**
647

- 648 1. Lehmann, R., and Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and
649 embryonic polarity require the localized activity of oskar, a maternal gene in drosophila. *Cell* *47*, 141–
650 152. [10.1016/0092-8674\(86\)90375-2](https://doi.org/10.1016/0092-8674(86)90375-2).
- 651 2. Ephrussi, A., Dickinson, L.K., and Lehmann, R. (1991). oskar organizes the germ plasm and directs
652 localization of the posterior determinant nanos. *Cell* *66*, 37–50. [10.1016/0092-8674\(91\)90137-n](https://doi.org/10.1016/0092-8674(91)90137-n).
- 653 3. Kim-Ha, J., Smith, J.L., and Macdonald, P.M. (1991). oskar mRNA is localized to the posterior pole of
654 the *Drosophila* oocyte. *Cell* *66*, 23–35. [10.1016/0092-8674\(91\)90136-m](https://doi.org/10.1016/0092-8674(91)90136-m).
- 655 4. Hay, B., Jan, L.Y., and Jan, Y.N. (1990). Localization of vasa, a component of *Drosophila* polar
656 granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development* *109*, 425–
657 433.
- 658 5. Lasko, P.F., and Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not
659 sufficient for, pole cell development. *Gene Dev* *4*, 905–921. [10.1101/gad.4.6.905](https://doi.org/10.1101/gad.4.6.905).
- 660 6. Jones, J.R., and Macdonald, P.M. (2007). Oskar controls morphology of polar granules and nuclear
661 bodies in *Drosophila*. *Development* *134*, 233–236. [10.1242/dev.02729](https://doi.org/10.1242/dev.02729).
- 662 7. Lehmann, R. (2016). Germ Plasm Biogenesis - An Oskar-Centric Perspective. *Curr Top Dev Biol* *116*,
663 679–707. [10.1016/bs.ctdb.2015.11.024](https://doi.org/10.1016/bs.ctdb.2015.11.024).
- 664 8. Dubnau, J., Chiang, A.-S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F.,
665 Scott, R., Certa, U., et al. (2003). The staufen/pumilio Pathway Is Involved in *Drosophila* Long-Term
666 Memory. *Curr Biol* *13*, 286–296. [10.1016/s0960-9822\(03\)00064-2](https://doi.org/10.1016/s0960-9822(03)00064-2).
- 667 9. Pai, T.-P., Chen, C.-C., Lin, H.-H., Chin, A.-L., Lai, J.S.-Y., Lee, P.-T., Tully, T., and Chiang, A.-S.
668 (2013). *Drosophila* ORB protein in two mushroom body output neurons is necessary for long-term
669 memory formation. *Proc National Acad Sci* *110*, 7898–7903. [10.1073/pnas.1216336110](https://doi.org/10.1073/pnas.1216336110).
- 670 10. Perrat, P.N., DasGupta, S., Wang, J., Theurkauf, W., Weng, Z., Rosbash, M., and Waddell, S. (2013).
671 Transposition-Driven Genomic Heterogeneity in the *Drosophila* Brain. *Science* *340*, 91–95.
672 [10.1126/science.1231965](https://doi.org/10.1126/science.1231965).
- 673 11. Wharton, R.P., Sonoda, J., Lee, T., Patterson, M., and Murata, Y. (1998). The Pumilio RNA-Binding
674 Domain Is Also a Translational Regulator. *Mol Cell* *1*, 863–872. [10.1016/s1097-2765\(00\)80085-4](https://doi.org/10.1016/s1097-2765(00)80085-4).
- 675 12. Xu, X., Brechbiel, J.L., and Gavis, E.R. (2013). Dynein-Dependent Transport of nanos RNA in
676 *Drosophila* Sensory Neurons Requires Rumpelstiltskin and the Germ Plasm Organizer Oskar. *J Neurosci*
677 *33*, 14791–14800. [10.1523/jneurosci.5864-12.2013](https://doi.org/10.1523/jneurosci.5864-12.2013).
- 678 13. Ye, B., Petritsch, C., Clark, I.E., Gavis, E.R., Jan, L.Y., and Jan, Y.N. (2004). nanos and pumilio Are
679 Essential for Dendrite Morphogenesis in *Drosophila* Peripheral Neurons. *Curr Biol* *14*, 314–321.
680 [10.1016/j.cub.2004.01.052](https://doi.org/10.1016/j.cub.2004.01.052).

Kulkarni et al.

- 681 14. Kulkarni, A., Lopez, D.H., and Extavour, C.G. (2020). Shared Cell Biological Functions May
682 Underlie Pleiotropy of Molecular Interactions in the Germ Lines and Nervous Systems of Animals.
683 *Frontiers Ecol Evol* 8, 215. 10.3389/fevo.2020.00215.
- 684 15. Ewen-Campen, B., Srouji, J.R., Schwager, E.E., and Extavour, C.G. (2012). oskar Predates the
685 Evolution of Germ Plasm in Insects. *Curr Biol* 22, 2278–2283. 10.1016/j.cub.2012.10.019.
- 686 16. Blondel, L., Besse, S., Rivard, E.L., Ylla, G., and Extavour, C.G. (2021). Evolution of a Cytoplasmic
687 Determinant: Evidence for the Biochemical Basis of Functional Evolution of the Novel Germ Line
688 Regulator Oskar. *Mol Biol Evol* 38, 5491–5513. 10.1093/molbev/msab284.
- 689 17. Matsumoto, Y., and Mizunami, M. (2004). Context-Dependent Olfactory Learning in an Insect. *Learn*
690 *Memory* 11, 288–293. 10.1101/lm.72504.
- 691 18. Silva, A.J., Kogan, J.H., Frankland, P.W., and Kida, S. (1998). CREB and Memory. *Neuroscience* 21,
692 127–148. 10.1146/annurev.neuro.21.1.127.
- 693 19. Farris, S.M., and Sinakevitch, I. (2003). Development and evolution of the insect mushroom bodies:
694 towards the understanding of conserved developmental mechanisms in a higher brain center. *Arthropod*
695 *Struct Dev* 32, 79–101. 10.1016/s1467-8039(03)00009-4.
- 696 20. Malaterre, J., Strambi, C., Chiang, A., Aouane, A., Strambi, A., and Cayre, M. (2002). Development
697 of cricket mushroom bodies. *J Comp Neurol* 452, 215–227. 10.1002/cne.10319.
- 698 21. Urbach, R., and Technau, G.M. (2003). Early steps in building the insect brain: neuroblast formation
699 and segmental patterning in the developing brain of different insect species. *Arthropod Struct Dev* 32,
700 103–123. 10.1016/s1467-8039(03)00042-2.
- 701 22. Pinto-Teixeira, F., Konstantinides, N., and Desplan, C. (2016). Programmed cell death acts at
702 different stages of *Drosophila* neurodevelopment to shape the central nervous system. *Febs Lett* 590,
703 2435–2453. 10.1002/1873-3468.12298.
- 704 23. Simões, A.R., and Rhiner, C. (2017). A Cold-Blooded View on Adult Neurogenesis. *Front Neurosci-*
705 *switz* 11, 327. 10.3389/fnins.2017.00327.
- 706 24. Fernández-Hernández, I., Rhiner, C., and Moreno, E. (2013). Adult Neurogenesis in *Drosophila*. *Cell*
707 *Reports* 3, 1857–1865. 10.1016/j.celrep.2013.05.034.
- 708 25. Simões, A.R., Neto, M., Alves, C.S., Santos, M.B., Fernández-Hernández, I., Veiga-Fernandes, H.,
709 Brea, D., Durá, I., Encinas, J.M., and Rhiner, C. (2022). Damage-responsive neuro-glial clusters
710 coordinate the recruitment of dormant neural stem cells in *Drosophila*. *Dev Cell* 57, 1661-1675.e7.
711 10.1016/j.devcel.2022.05.015.
- 712 26. Li, G., and Hidalgo, A. (2020). Adult Neurogenesis in the *Drosophila* Brain: The Evidence and the
713 Void. *Int J Mol Sci* 21, 6653. 10.3390/ijms21186653.
- 714 27. Strausfeld, N.J. (2012). *Arthropod Brains: Evolution, Functional Elegance, and Historical*
715 *Significance* (Harvard University Press).

Kulkarni et al.

- 716 28. Scotto-Lomassese, S., Strambi, C., Strambi, A., Aouane, A., Augier, R., Rougon, G., and Cayre, M.
717 (2003). Suppression of Adult Neurogenesis Impairs Olfactory Learning and Memory in an Adult Insect. *J*
718 *Neurosci* 23, 9289–9296. 10.1523/jneurosci.23-28-09289.2003.
- 719 29. Takahashi, T., Hamada, A., Miyawaki, K., Matsumoto, Y., Mito, T., Noji, S., and Mizunami, M.
720 (2009). Systemic RNA interference for the study of learning and memory in an insect. *J Neurosci Meth*
721 179, 9–15. 10.1016/j.jneumeth.2009.01.002.
- 722 30. Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., Cook, H.A., and Theurkauf,
723 W.E. (2007). *Drosophila* rasiRNA Pathway Mutations Disrupt Embryonic Axis Specification through
724 Activation of an ATR/Chk2 DNA Damage Response. *Dev Cell* 12, 45–55. 10.1016/j.devcel.2006.12.001.
- 725 31. Belle, J. de, and Heisenberg, M. (1994). Associative odor learning in *Drosophila* abolished by
726 chemical ablation of mushroom bodies. *Science* 263, 692–695. 10.1126/science.8303280.
- 727 32. Heisenberg, M., Borst, A., Wagner, S., and Byers, D. (1985). *Drosophila* Mushroom Body Mutants
728 are Deficient in Olfactory Learning: Research Papers. *J Neurogenet* 2, 1–30.
729 10.3109/01677068509100140.
- 730 33. Mizunami, M., Weibrecht, J.M., and Strausfeld, N.J. (1998). Mushroom bodies of the cockroach:
731 Their participation in place memory. *J Comp Neurol* 402, 520–537. 10.1002/(sici)1096-
732 9861(19981228)402:4<520::aid-cne6>3.0.co;2-k.
- 733 34. Malun, D., Plath, N., Giurfa, M., Moseleit, A.D., and Müller, U. (2002). Hydroxyurea-induced partial
734 mushroom body ablation in the honeybee *Apis mellifera*: Volumetric analysis and quantitative protein
735 determination. *J Neurobiol* 50, 31–44. 10.1002/neu.10015.
- 736 35. Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien,
737 R.Y. (2002). A monomeric red fluorescent protein. *Proc National Acad Sci* 99, 7877–7882.
738 10.1073/pnas.082243699.
- 739 36. Ylla, G., Nakamura, T., Itoh, T., Kajitani, R., Toyoda, A., Tomonari, S., Bando, T., Ishimaru, Y.,
740 Watanabe, T., Fuketa, M., et al. (2021). Insights into the genomic evolution of insects from cricket
741 genomes. *Commun Biology* 4, 733. 10.1038/s42003-021-02197-9.
- 742 37. Whittle, C.A., Kulkarni, A., and Extavour, C.G. (2021). Evolutionary dynamics of sex-biased genes
743 expressed in cricket brains and gonads. *J Evolution Biol* 34, 1188–1211. 10.1111/jeb.13889.
- 744 38. Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat Rev Neurosci* 4, 266–
745 275. 10.1038/nrn1074.
- 746 39. Caron, S.J.C., Ruta, V., Abbott, L.F., and Axel, R. (2013). Random Convergence of Olfactory Inputs
747 in the *Drosophila* Mushroom Body. *Nature* 497, 113–117. 10.1038/nature12063.
- 748 40. Chen, C.-C., Wu, J.-K., Lin, H.-W., Pai, T.-P., Fu, T.-F., Wu, C.-L., Tully, T., and Chiang, A.-S.
749 (2012). Visualizing Long-Term Memory Formation in Two Neurons of the *Drosophila* Brain. *Science*
750 335, 678–685. 10.1126/science.1212735.

Kulkarni et al.

- 751 41. Lazarini, F., and Lledo, P.-M. (2011). Is adult neurogenesis essential for olfaction? *Trends Neurosci*
752 34, 20–30. 10.1016/j.tins.2010.09.006.
- 753 42. Cayre, M., Scotto-Lomassese, S., Malaterre, J., Strambi, C., and Strambi, A. (2007). Understanding
754 the Regulation and Function of Adult Neurogenesis: Contribution from an Insect Model, the House
755 Cricket. *Chem Senses* 32, 385–395. 10.1093/chemse/bjm010.
- 756 43. Ito, K., and Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of
757 *Drosophila melanogaster*. *Dev Biol* 149, 134–148. 10.1016/0012-1606(92)90270-q.
- 758 44. Breitwieser, W., Markussen, F.H., Horstmann, H., and Ephrussi, A. (1996). Oskar protein interaction
759 with Vasa represents an essential step in polar granule assembly. *Gene Dev* 10, 2179–2188.
760 10.1101/gad.10.17.2179.
- 761 45. Chang, J.S., Tan, L., and Schedl, P. (1999). The *Drosophila* CPEB Homolog, Orb, Is Required for
762 Oskar Protein Expression in Oocytes. *Dev Biol* 215, 91–106. 10.1006/dbio.1999.9444.
- 763 46. Kistler, K.E., Trcek, T., Hurd, T.R., Chen, R., Liang, F.-X., Sall, J., Kato, M., and Lehmann, R.
764 (2018). Phase transitioned nuclear Oskar promotes cell division of *Drosophila* primordial germ cells. *Elife*
765 7, e37949. 10.7554/elife.37949.
- 766 47. Juliano, C.E., Swartz, S.Z., and Wessel, G.M. (2010). A conserved germline multipotency program.
767 *Development* 137, 4113–4126. 10.1242/dev.047969.
- 768 48. Macdonald, P.M., Kanke, M., and Kenny, A. (2016). Community effects in regulation of translation.
769 *Elife* 5, e10965. 10.7554/elife.10965.
- 770 49. Rongo, C., Gavis, E.R., and Lehmann, R. (1995). Localization of oskar RNA regulates oskar
771 translation and requires Oskar protein. *Dev Camb Engl* 121, 2737–2746.
- 772 50. Kulkarni, A., and Extavour, C.G. (2017). Convergent evolution of germ granule nucleators: A
773 hypothesis. *Stem Cell Res* 24, 188–194. 10.1016/j.scr.2017.07.018.
- 774 51. Bose, M., Lampe, M., Mahamid, J., and Ephrussi, A. (2022). Liquid-to-solid phase transition of oskar
775 ribonucleoprotein granules is essential for their function in *Drosophila* embryonic development. *Cell* 185,
776 1308-1324.e23. 10.1016/j.cell.2022.02.022.
- 777 52. Matsumoto, Y., and Mizunami, M. (2000). Olfactory learning in the cricket *Gryllus bimaculatus*. *J*
778 *Exp Biology* 203, 2581–2588. 10.1242/jeb.203.17.2581.
- 779 53. Kainz, F., Ewen-Campen, B., Akam, M., and Extavour, C.G. (2011). Notch/Delta signalling is not
780 required for segment generation in the basally branching insect *Gryllus bimaculatus*. *Development* 138,
781 5015–5026. 10.1242/dev.073395.
- 782 54. Hamada, A., Miyawaki, K., Honda-Sumi, E., Tomioka, K., Mito, T., Ohuchi, H., and Noji, S. (2009).
783 Loss-of-function analyses of the fragile X-related and dopamine receptor genes by RNA interference in
784 the cricket *Gryllus bimaculatus*. *Dev Dynam* 238, 2025–2033. 10.1002/dvdy.22029.

Kulkarni et al.

- 785 55. Ewen-Campen, B., Donoughe, S., Clarke, D.N., and Extavour, C.G. (2013). Germ Cell Specification
786 Requires Zygotic Mechanisms Rather Than Germ Plasm in a Basally Branching Insect. *Curr Biol* 23,
787 835–842. 10.1016/j.cub.2013.03.063.
- 788 56. Zeng, V., Ewen-Campen, B., Horch, H.W., Roth, S., Mito, T., and Extavour, C.G. (2013).
789 Developmental Gene Discovery in a Hemimetabolous Insect: De Novo Assembly and Annotation of a
790 Transcriptome for the Cricket *Gryllus bimaculatus*. *Plos One* 8, e61479. 10.1371/journal.pone.0061479.
- 791 57. Zhou, X., Liao, Z., Jia, Q., Cheng, L., and Li, F. (2007). Identification and characterization of Piwi
792 subfamily in insects. *Biochem Bioph Res Co* 362, 126–131. 10.1016/j.bbrc.2007.07.179.
- 793 58. Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space
794 complexity. *Bmc Bioinformatics* 5, 113. 10.1186/1471-2105-5-113.
- 795 59. Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput.
796 *Nucleic Acids Res* 32, 1792–1797. 10.1093/nar/gkh340.
- 797 60. Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large
798 phylogenies. *Bioinformatics* 30, 1312–1313. 10.1093/bioinformatics/btu033.
- 799 61. Yu, G. (2020). Using ggtree to Visualize Data on Tree-Like Structures. *Curr Protoc Bioinform* 69,
800 e96. 10.1002/cpbi.96.
- 801 62. Yu, G., Smith, D.K., Zhu, H., Guan, Y., and Lam, T.T. (2017). ggtree: an r package for visualization
802 and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol Evol* 8,
803 28–36. 10.1111/2041-210x.12628.
- 804 63. Andrews, S. (2010). Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput
805 Sequence Data. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
- 806 64. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads.
807 *Embnet J* 17, 10–12. 10.14806/ej.17.1.200.
- 808 65. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9,
809 357–359. 10.1038/nmeth.1923.
- 810 66. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G.,
811 Durbin, R., and Subgroup, 1000 Genome Project Data Processing (2009). The Sequence Alignment/Map
812 format and SAMtools. *Bioinformatics* 25, 2078–2079. 10.1093/bioinformatics/btp352.
- 813 67. Liao, Y., Smyth, G.K., and Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and
814 better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res* 47, gkz114-
815 10.1093/nar/gkz114.
- 816 68. Montañés, J.C., Rojano, C., Ylla, G., Piulachs, M.D., and Maestro, J.L. (2021). siRNA enrichment in
817 Argonaute 2-depleted *Blattella germanica*. *Biochimica Et Biophysica Acta Bba - Gene Regul Mech* 1864,
818 194704. 10.1016/j.bbagr.2021.194704.

Kulkarni et al.

- 819 69. Matsumoto, Y., Noji, S., and Mizunami, M. (2003). Time Course of Protein Synthesis-Dependent
820 Phase of Olfactory Memory in the Cricket *Gryllus bimaculatus*. *Zool Sci* 20, 409–416.
821 10.2108/zsj.20.409.
- 822 70. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A.,
823 Markowitz, S., Duran, C., et al. (2012). Geneious Basic: An integrated and extendable desktop software
824 platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649.
825 10.1093/bioinformatics/bts199.
- 826 71. Matsumoto, Y., and Mizunami, M. (2002). Temporal determinants of long-term retention of olfactory
827 memory in the cricket *Gryllus bimaculatus*. *The Journal of Experimental Biology* 205, 1429–1437.
- 828 72. Matsumoto, Y., Hatano, A., Unoki, S., and Mizunami, M. (2009). Stimulation of the cAMP system by
829 the nitric oxide-cGMP system underlying the formation of long-term memory in an insect. *Neurosci Lett*
830 467, 81–85. 10.1016/j.neulet.2009.10.008.
- 831 73. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-
832 Time Quantitative PCR and the $2^{-\Delta\Delta C T}$ Method. *Methods* 25, 402–408. 10.1006/meth.2001.1262.
- 833 74. Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). MAFFT: a novel method for rapid multiple
834 sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 30, 3059–3066.
835 10.1093/nar/gkf436.
- 836 75. Castro-Mondragon, J.A., Riudavets-Puig, R., Rauluseviciute, I., Berhanu Lemma, R., Turchi, L.,
837 Blanc-Mathieu, R., Lucas, J., Boddie, P., Khan, A., Manosalva Pérez, N., et al. (2021). JASPAR 2022:
838 the 9th release of the open-access database of transcription factor binding profiles. *Nucleic Acids Res* 50,
839 gkab1113-. 10.1093/nar/gkab1113.
- 840 76. Bailey, T.L., Johnson, J., Grant, C.E., and Noble, W.S. (2015). The MEME Suite. *Nucleic Acids Res*
841 43, W39–W49. 10.1093/nar/gkv416.
- 842 77. Stothard, P. (2000). The Sequence Manipulation Suite: JavaScript Programs for Analyzing and
843 Formatting Protein and DNA Sequences. *Biotechniques* 28, 1102–1104. 10.2144/00286ir01.
- 844 78. Hsieh, Y.-W., Alqadah, A., and Chuang, C.-F. (2016). An Optimized Protocol for Electrophoretic
845 Mobility Shift Assay Using Infrared Fluorescent Dye-labeled Oligonucleotides. *J Vis Exp*.
846 10.3791/54863.
- 847

Figure 1

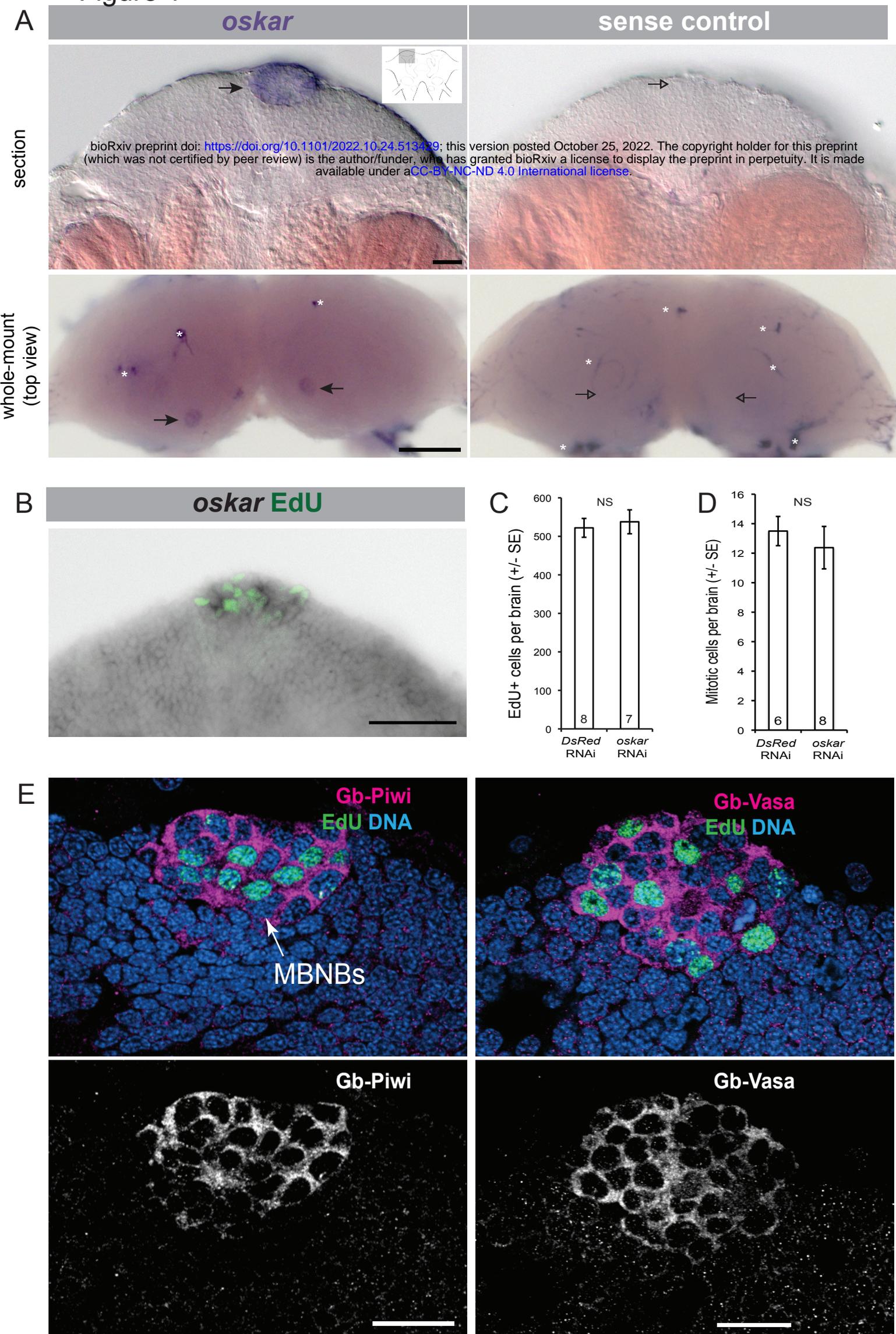


Figure 2

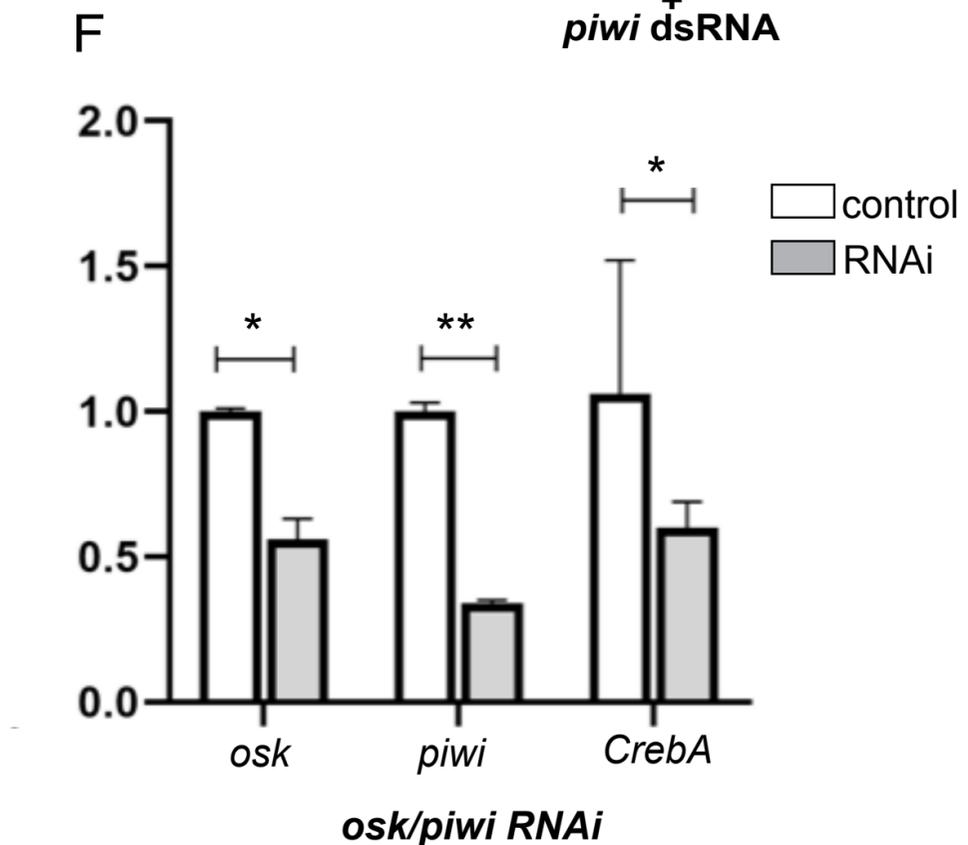
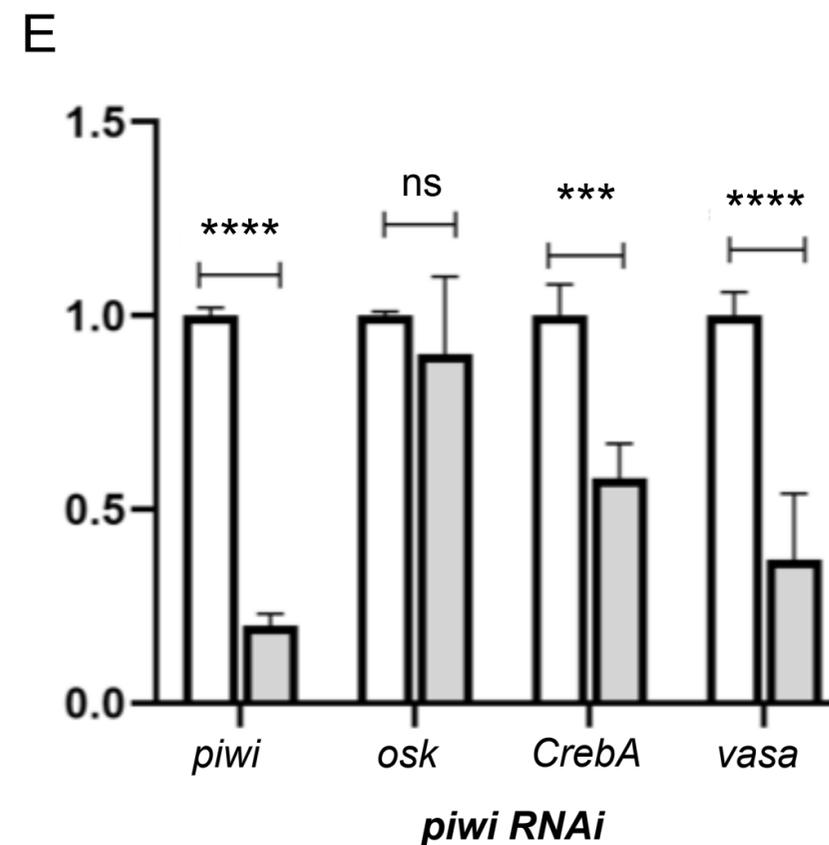
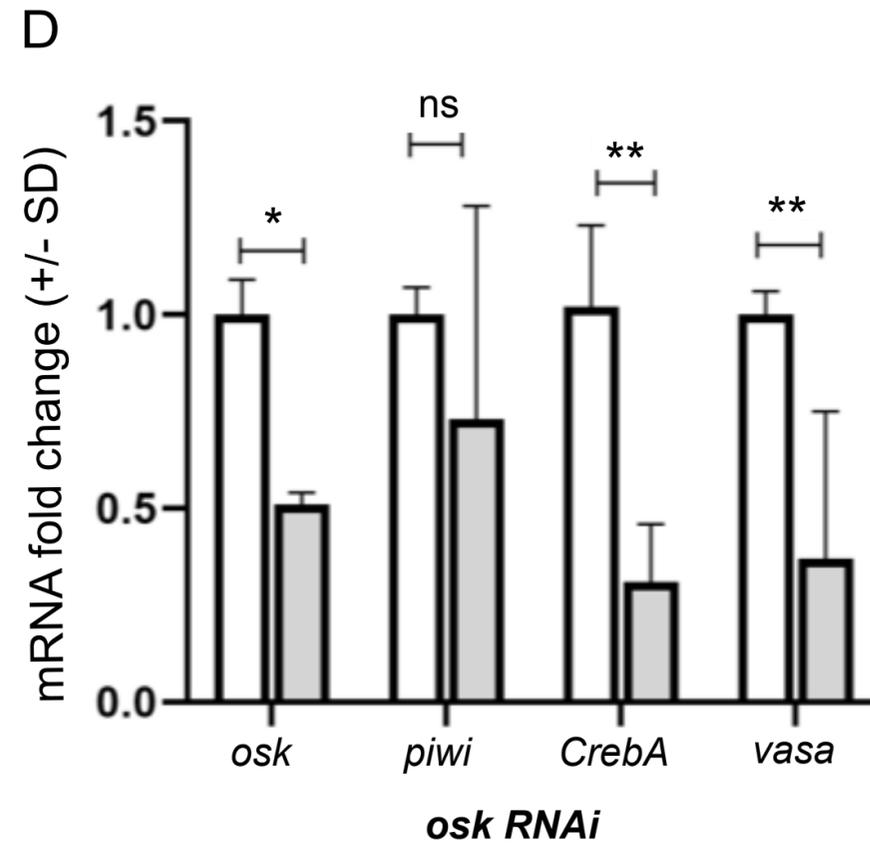
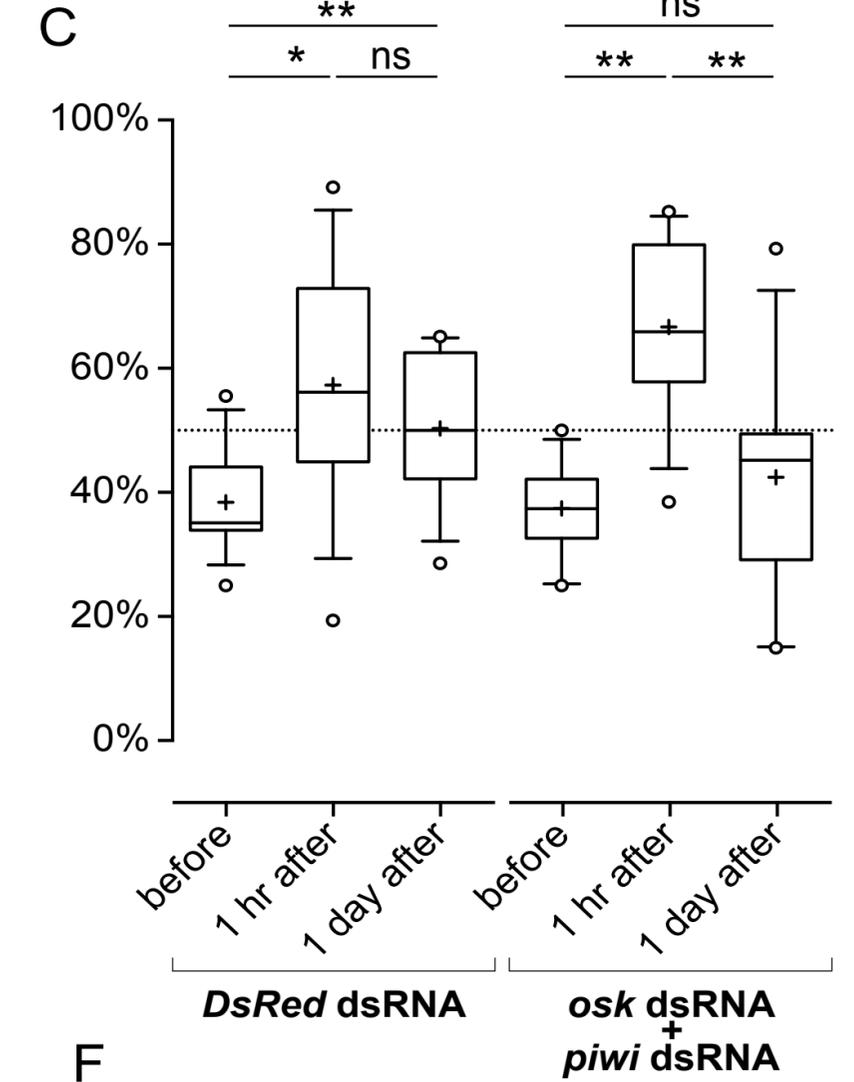
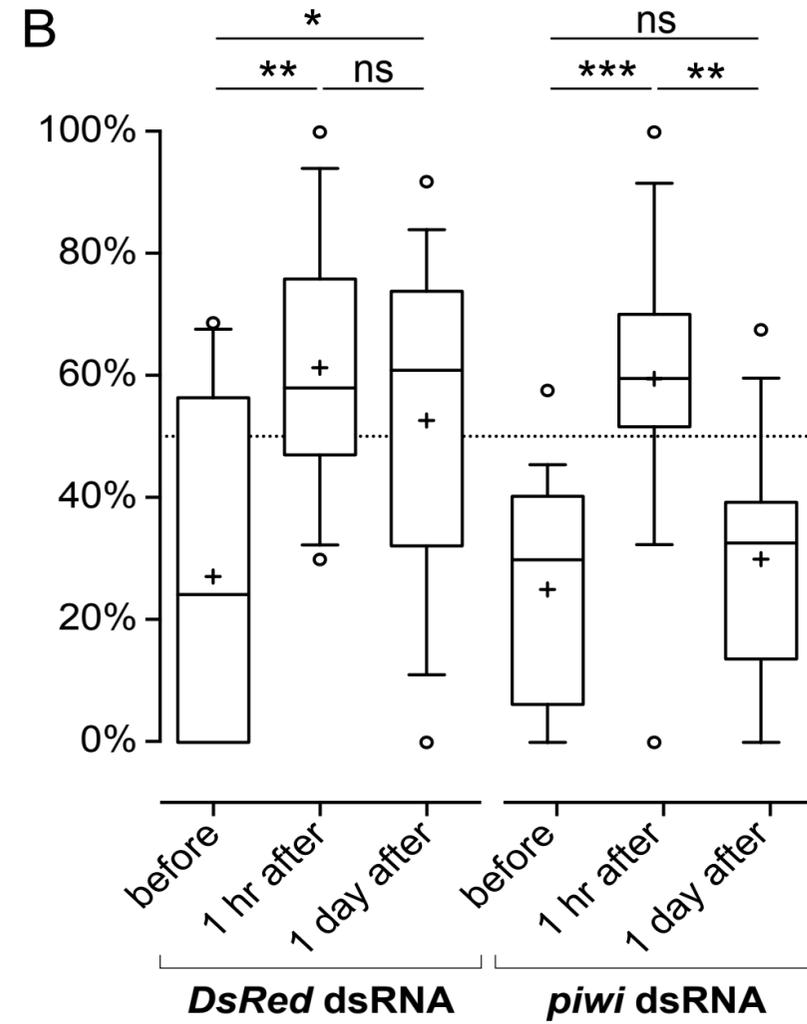
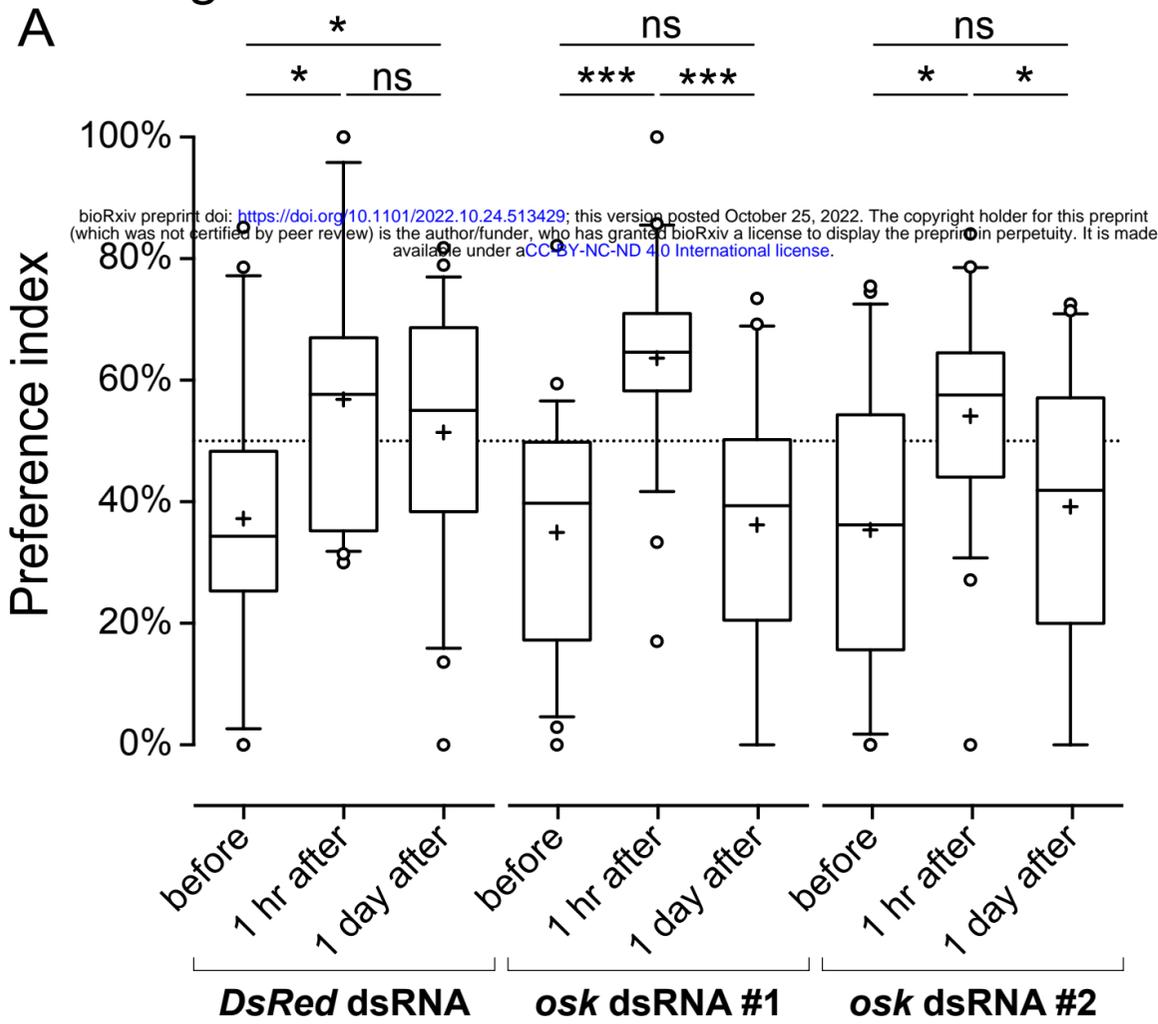


Figure 3

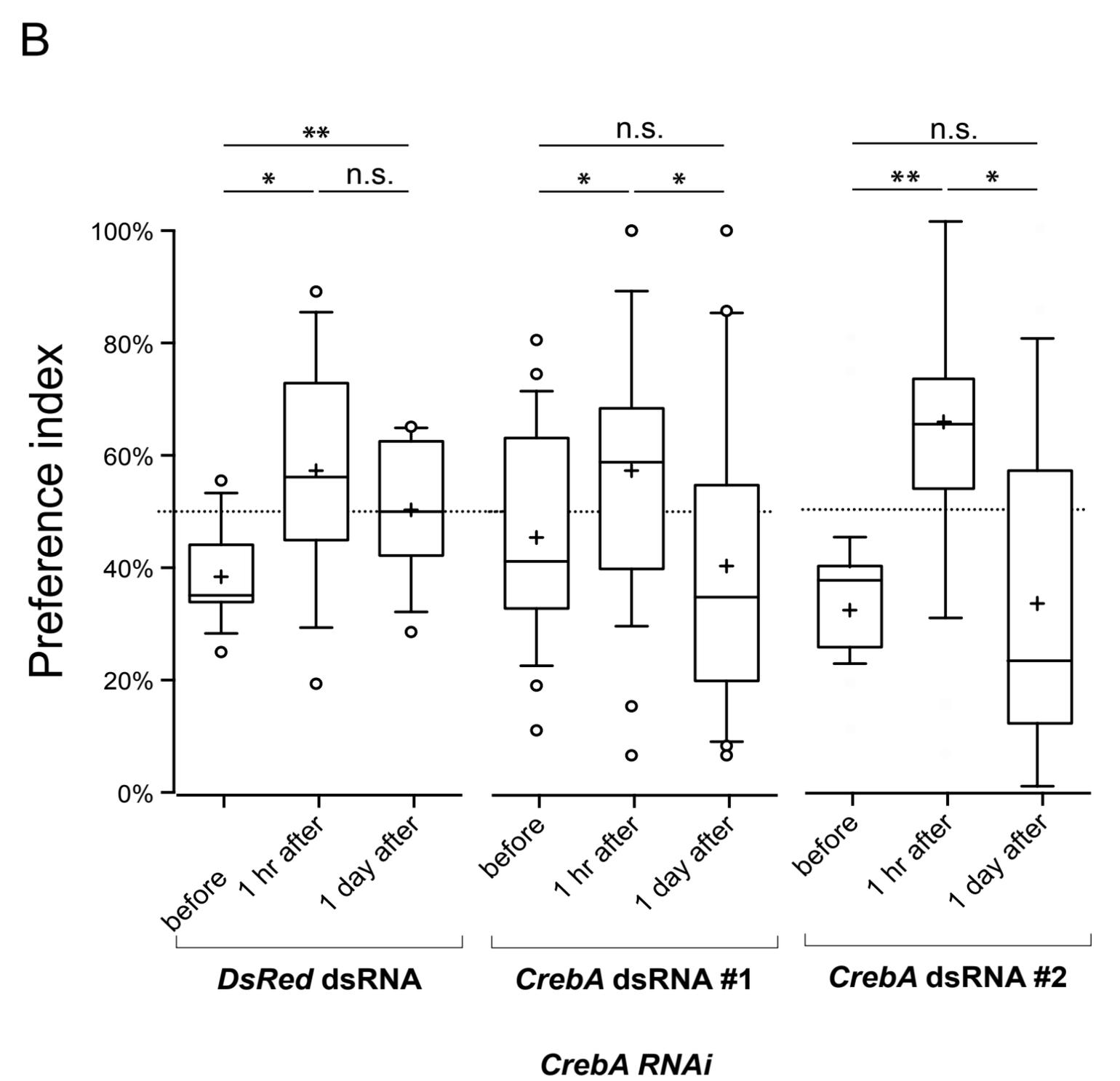
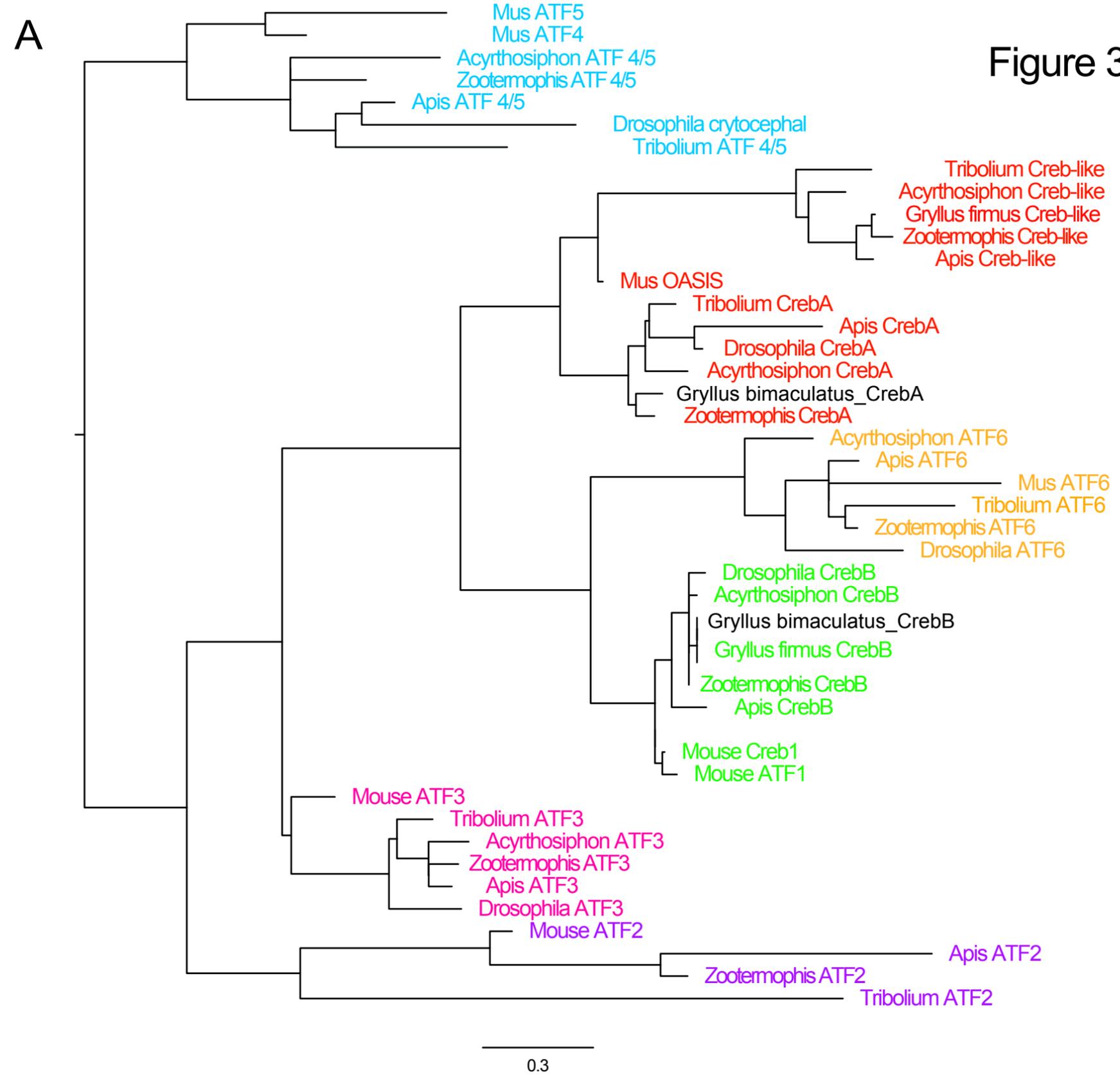
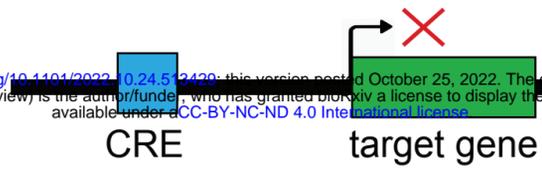
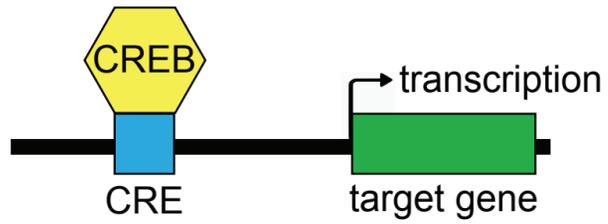
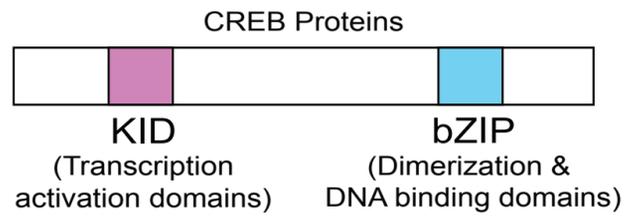


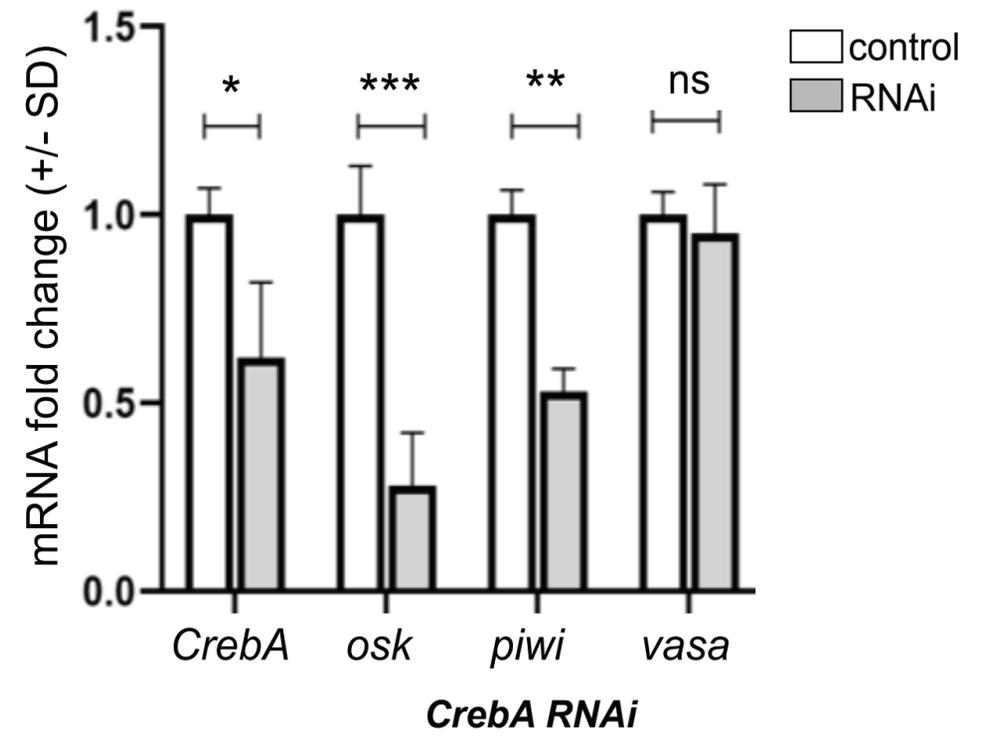
Figure 4

A

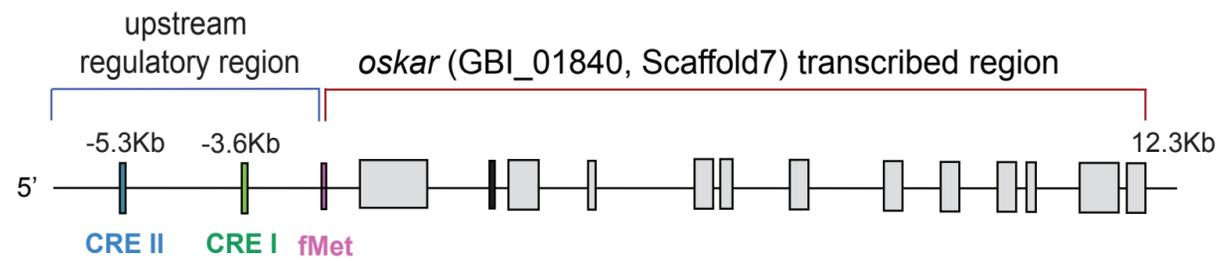


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B



C



oskar CRE+ probe

oskar CRE- probe

cricket brain protein

BSA

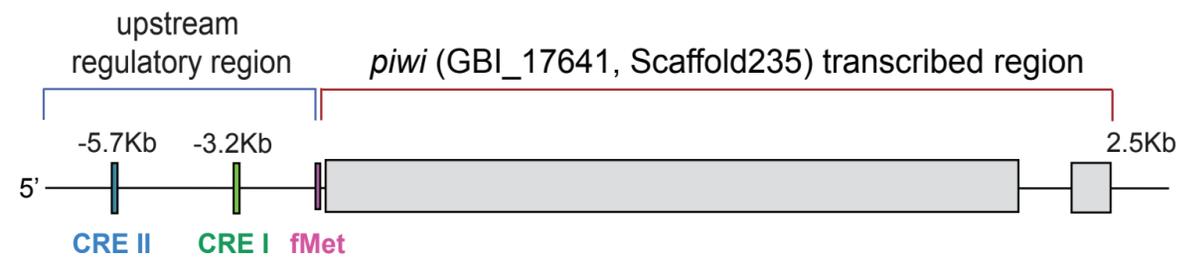
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oskar CRE- probe	+	-	-	-	-	-	-	-
cricket brain protein	+	-	-	-	+	+	+	+
BSA	-	+	+	-	-	-	-	-



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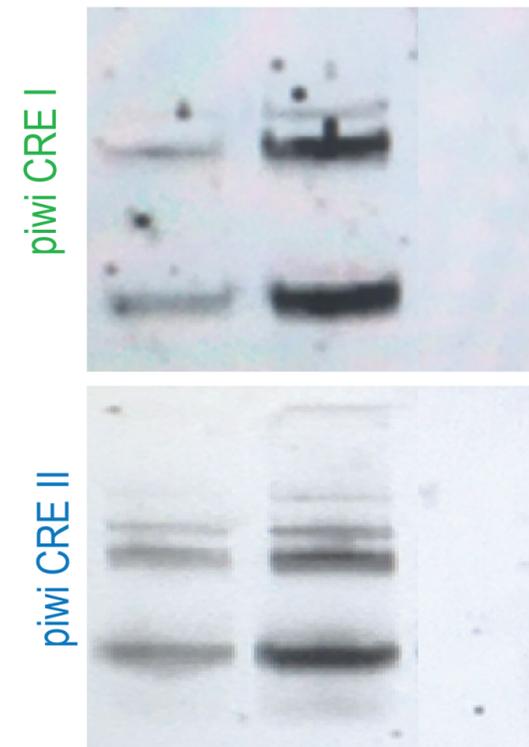
D



piwi CRE+ probe

cricket brain protein

piwi CRE+ probe	+	+	-
cricket brain protein	-	+	+



5' TGATGTTCAACTTGACGTAACCCATGTGGG 3'

5' GCATAGTTTTTTGACGTAAGCAAATAATA 3'

Supplementary material for **oskar acts with the transcription factor Creb to regulate long-1 term memory in crickets**

Link to download: [\[supplements/513429_file06.zip\]](#)