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A mitochondrial carrier gene, *CG32103*, is highly expressed in the corpora allata in the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae)

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Abstract

Here we describe a novel gene that is highly expressed in the corpora allata, an endocrine organ responsible for synthesizing juvenile hormones (JHs), in the fruit fly, *Drosophila melanogaster* Meigen. We isolated an enhancer-trap line in which the transgene was inserted at the locus, *CG32103*, which encodes a mitochondrial carrier family protein with calcium binding motifs. RNA *in situ* hybridization revealed that *CG32103* is predominantly expressed in the corpora allata in *D. melanogaster* larvae. Putative orthologs of *CG32103* are conserved in many insect species. Mitochondrial carriers are responsible for transporting metabolites across the inner mitochondrial membrane. Given that both mitochondrial membrane transport and cytoplasmic calcium signaling are important for the regulation of JH biosynthesis, we speculate that CG32103 represents a new member of the family of JH biosynthesis regulators in insects.

Keywords

juvenile hormone, corpora allata, mitochondrial carrier, *Drosophila melanogaster*

Introduction

Insect juvenile hormone (JH) is a multifunctional hormone that controls a variety of developmental and physiological events (Nijhout, 1994). In general, JH prevents metamorphosis by modulating the action of the principal insect steroid hormone, ecdysteroids. When JH is present, ecdysteroids cause the insect to repeat the most recent developmental stage, and ecdysteroids must act in the absence of JH in order for metamorphosis to be initiated (Riddiford, 1994). Changes in hemolymph JH titers are primarily regulated by modulations in the synthesis of JH by a specialized endocrine organ, called the corpora allata (CA), but is also regulated by JH catabolism through the action of specific enzymes. These changes must be precisely controlled to ensure proper insect development.

The biosynthetic pathway of JHs in the CA is conventionally divided into two main components (Bellés et al., 2005). In the early step, farnesyl pyrophosphate (FPP), an important intermediate in the biosynthesis of cholesterol and other bioactive terpenoids, is generated via the classical mevalonate pathway, which is common to vertebrates and invertebrates (Goldstein and Brown, 1990). In the late steps, FPP is first hydrolyzed by a pyrophosphatase into farnesol. Farnesol is then successively oxidized by an aldehyde dehydrogenase to become farnesoic acid (FA). Finally, FA is converted to active JH (JH III) by C-10,11 epoxidation by a P450 monooxygenase and methylation of the carboxyl group by an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase (MTase). The ethyl-branched JHs, JH I and JH II, are the predominant JHs in lepidopteran insects and are also synthesized via the same pathway. In contrast, the heteropteran and higher dipteran insects produce the unique JHs that are featured by bisepoxide structures, known as JH III skipped bisepoxide ($JHSB_3$) (Kotaki

et al., 2009) and JH III bisepoxide (JHB_3) (Richard et al., 1989; Bendena et al., 2011), respectively. Therefore, these insects might have some particular JH biosynthesis enzymes as compared to other insects. Because the JHs are highly specific to insects, JH biosynthesis enzymes could be excellent targets for selective insect growth regulators.

Several approaches have been used in the past to facilitate the molecular study of JH synthesis in the CA. Differential displays of mRNA from the silk worm, *Bombyx mori* L., revealed a gene called *JHAMT*, which encodes for the SAM-dependent MTase that is specifically expressed in the CA and is crucial for the termination of JH biosynthesis (Shinoda and Itoyama, 2003). The other approach is construction of an EST database derived from the CA. One of the EST clones from the CA of the German cockroach, *Diploptera punctata* Eschscholtz, represents *Cyp15a1*, which encodes a cytochrome P450 family member that catalyzes the C-10,11 epoxidation reaction (Helvig et al., 2004). In addition, a comparative EST analysis from the CA across insect species has been recently performed in attempt to reveal the gene candidates responsible for the biosynthetic activity in the CA (Noriega et al., 2006). Moreover, the recent advances from insect genome projects have allowed researchers to perform genome-wide and large scale gene expression analyses to identify genes expressed in the CA. For example, a study of the *in situ* gene expression of all of the predicted *D. melanogaster* P450 genes has revealed that *Cyp6g2* is predominantly expressed in the CA (Chung et al., 2009). Information on the *B. mori* genome has also revealed a number of insect orthologs encoding genes that are involved in the classical mevalonate pathway, many of which are strongly expressed in the CA (Kinjoh et al., 2007; Ueda et al., 2009; Kaneko et al., 2011).

Here, we report the identification of a novel gene that is highly expressed in the CA using an alternative method of gene hunting, an enhancer-trap strategy in the fruit

fly, *Drosophila melanogaster* Meigen. Many genes exhibiting spatial and temporal specific expression patterns have already been identified in *D. melanogaster* using enhancer-trap methods, where a transgenic construct is inserted into a chromosome to identify enhancers for certain genes in the genome (Durick et al., 1999). We isolated one enhancer-trap line in which a transgene was inserted at the *CG32103* locus, which encodes a mitochondrial carrier family protein with calcium binding motifs. *CG32103* is predominantly expressed in the CA in both the larval and adult stages. We propose that CG32103 is a candidate molecule for JH biosynthesis that acts through the regulation of mitochondrial activity.

Materials and methods

Animal strains

All *D. melanogaster* flies were reared on standard agar cornmeal medium at 25 ºC under a 12 h light/12 h dark photoperiod. *yw* and *w* were used as a wild type. *UAS-GFP.S65T* was obtained from the *Drosophila* Genetic Resource Center at the Kyoto Institute of Technology. *AUG21-GAL4*, in which the *GAL4* transgene is active in the CA cells (Siegmund and Korge, 2001; Adám et al., 2003) was kindly provided from Dr. G. Korge. Double strand RNA mediated RNA interference (RNAi) for *CG32103* was performed using the *UAS-CG32103-inverted repeat* strain *P{KK100089}*$^{VIE\text{-}260B}$ that was obtained from the Vienna *Drosophila* RNAi Center.

Generating an enhancer-trap *GAL4* line

We created a transgenic DNA construct that expresses the *GAL4* gene under the promoter of *CG13687*, which is a *D. melanogaster* ortholog of *prothoracicotropic hormone* (*ptth*) (McBrayer et al., 2007). The 603-bp upstream region of *CG13687* was obtained using genomic PCR with the following primers: 5′-GCGGCCGCTGGGAGACATAGTGAGCTCATA-3′ and 5′-GGATCCATCCATTACGGTTCGTCACCTGGAC-3′. The PCR product was subcloned into the pGEM-T vector (Promega) and then sequenced. The pGEM-T vector containing the *ptth* promoter was then digested with *Not*I and *BamH*I. This digested fragment was subconed into a *Not*I/*BamH*I-digested pPTGAL plasmid, which is a CaSpeR family P-element vector that contains the *GAL4* gene (Sharma et al., 2002), leading to a *ptth-p*-pPTGAL construct that expresses the yeast transcription factor gene, *GAL4*, under the control of the *ptth* promoter. Eight independent *D. melanogaster* transformants that contained *ptth-p*-pPTGAL were obtained using standard protocols. When crossed with *UAS-GFP.S65T*, we confirmed that all of the 8 lines showed *GAL4* expression in two pairs of neurons in the brain, which correspond to the *ptth*-expressing neurons (Fig. 1a). Among these 8 lines, one strain, designated as *CG32103&ptth-GAL4*, showed a prominent level of *GFP* expression in the CA in addition to the *ptth*-positive neurons after it was crossed with the *UAS-GFP.S65T* fly line (Fig. 1a).

Determining the insertion site of *ptth-p*-pPTGAL

To recover the genomic sequences flanking the *ptth*-pPTGAL vector in *CG32103&ptth-GAL4*, we performed an inverse PCR, essentially as previously described (Huang et al., 2000). The amplified sample was then subcloned into the pCR2.1 vector (Invitrogen), and the DNA sequences were determined.

RNA *in situ* hybridization

In situ hybridization was performed as described (Lehmann and Tautz, 1994; Niwa et al., 2004). Digoxigenin (DIG)-labeled RNA probes were synthesized using a DIG RNA labeling kit (Roche) with T3 and T7 RNA polymerase (Invitrogen). For the *CG32103* probe, the *D. melanogaster* EST clone, RE56970 (GenBank Accession Number AY119650) (Stapleton et al., 2002), was used as a template.

Reverse transcription (RT)-PCR

Total RNA was extracted from the ring glands of the wandering 3rd instar larvae of *D. melanogaster* using RNA iso plus (TaKaRa). The extracted RNA was treated with DNase I (TaKaRa), followed by single-stranded cDNA synthesis using Rever Tra Ace qRT kit (ToYoBo). For obtaining data represented in Fig. 2a, PCR was performed using AmpliTaq Gold 360 Master mix (Life Technologies). *rp49* was used as a loading control as described previously (Foley et al., 1993). To distinguish among three isoforms of *CG32103* (Fig. 2a), specific primers for the isoform A (A-forward: 5′-GCCAAGAAGAGGATGACTTCATCG-3′, A-reverse: 5′-CCGATATCAGCTCCTCCAAGTCC-3′), the isoform B (B-forward: 5′-TGAAGAGTTCTGCGTGAATCGC-3′, B-reverse: 5′-CCGATATCAGCTCCTCCAAGTCC-3′), and the isoform C (C-forward: 5′-GGAAGTGAAACAGTGCAAAAGCG-3′, C-reverse: 5′-CCAGGCGCGTCTTTAATACTTCC-3′) were used, giving 836-, 879-, and 587-bp fragments, respectively. A PCR condition was 40 cycles of 95 ºC for 30 sec and 65 ºC

for 30 sec. A quantitative RT-PCR (qRT-PCR) was performed as previously described (Shinoda and Itoyama, 2003). Specific primers for qRT-PCR were: A-forward (described above) and A-reverse-qRT (5′-GTGTTGGGCTATCAGCTGCTCTG-3′) for the isoform A; B-forward and B-reverse-qRT (5′-GTGTTGGGCTATCAGCTGCTCTG-3′) for the isoform B; and C-forward and C-reverse-qRT (5′-TTCATATCCTCGCCAATGTCGAG-3′) for the isoform C. Relative expression levels were quantified by the ΔΔCt method (Livak and Schmittgen, 2001). The level of each transcript was normalized to *rp49* in the same sample.

Results and Discussion

We originally investigated the gene expression pattern of the *D. melanogaster CG13687/ptth* gene (McBrayer et al., 2007). To do this, we monitored the expression of a transgene under the control of the *ptth* promoter region. We created a transgenic strain that carried a construct consisting of the *ptth* promoter region fused to the yeast transcription factor gene, *GAL4*. We then crossed this transgenic strain with *UAS-GFP* flies and examined the expression pattern of *GAL4* using the *GAL4/UAS* system (Brand and Perrimon, 1993). In the course of establishing the transgenic lines (see details in Materials and methods), we found that one transgenic line showed active expression of *GAL4* not only in the *ptth*-positive neurons but also in the CA during larval development (Fig. 1a). This raised the possibility that the *GAL4* vector might have trapped an enhancer of a gene that is predominantly expressed in the CA.

We then examined the position of the insertion of the *GAL4*-transgene construct. Inverse PCR analysis revealed that this strain contained one copy of pCaSpeR that was inserted at position 12,366,232 of the genomic scaffold of

182 chromosome 3L, which is in the region of the *CG32103* gene (Fig. 2a). To examine
183 whether the endogenous *CG32103* transcript is expressed in the CA, we performed
184 RNA *in situ* hybridization. *CG32103* expression was predominantly detected in the CA
185 but was not found in the prothoracic gland or the corpolla cardiaca of 3rd instar larvae
186 (Fig. 1b). Predominant expression of *CG32103* in the CA was also observed in embryos
187 (Fig. 1c), consistent with the data obtained from the enhancer-trap line.
188 The *D. melanogster* genome project predicts that *CG32103* has three different
189 splice variants, designated the isoforms A, B and C (Fig. 2a). We performed RT-PCR
190 analysis with isoform-specific primers to distinguish which isoform(s) are expressed in
191 the CA. We found that all three isoforms were expressed in the ring gland containing the
192 CA (Fig. 2b). We further conducted qRT-PCR analysis to examine the spatial expression
193 pattern of *CG32103* in various tissues from the wandering 3rd instar larvae, a stage at
194 which transcription of *jhamt* in the CA is high in *D. melanogaster* (Niwa et al., 2008).
195 Expressions of all three isoforms were detected in most of tissues in the wandering 3rd
196 instar larvae as well as adult testes and ovaries, while expression levels of each isoform
197 varied among the tissues (Fig. 2c). In fact, the expression of the isoform C in the fat
198 body and intestine in the 3rd instar larvae were more than 10-times higher than that in
199 the ring gland (Fig. 2c). These data indicate that *CG32103* is significantly but not
200 specifically expressed in the CA, which is comparable to the feature of some
201 mevalonate pathway genes whose expression are detected in not only the CA but also
202 other tissues in *B. mori* (Kinjoh et al., 2007).
203 We next examined the temporal expression profile of *CG32103* in the CA by
204 monitoring GFP signals in progenies of the *CG32103&ptth-GAL4* flies crossed with
205 *UAS-GFP*. The GFP signal in the CA was detected throughout larval stages (Fig. 3).
206 The GFP signals was relatively weak in the CA of the 1st instar larvae (Fig. 3a), and

207 gradually increased in the later instar larvae (Fig. 3b-d). These results suggest that the
208 overall change in the expression level of *CG32103* does not show a clear correlation
209 with changes in hemolymph JH titers during *D. melanogaster* development (Sliter et al.,
210 1987), which is contrast to the temporal transcriptional regulation of *jhamt* (Shinoda
211 and Itoyama, 2003; Niwa et al., 2008). In addition, a recent high-throughput RNA
212 sequencing project (Graveley et al., 2011) has reported that *CG32103* transcript is
213 almost constantly detected from embryos to the mid-stage pupae, while the slightly
214 higher expression is detected in the late pupal stage
215 (http://flybase.org/reports/FBgn0052103.html). Therefore, CG32103 might contribute to
216 the regulation of JH biosynthesis at the posttranscriptional level.
217 A BLAST search using the deduced protein sequence of isoforms A and B of
218 CG32103 (Fig. 2a) revealed that this protein is made up of 4 EF-hand motifs and a three
219 times repeated module of ~100 amino acids that contains two transmembrane domains
220 and the characteristic signature of mitochondrial carriers (Fig. 4a, b). These features are
221 characteristics of the evolutionarily conserved family of calcium-dependent
222 mitochondrial carriers (CaMCs) (Indiveri et al., 1997). Mitochondrial carriers are
223 responsible for the transport of metabolites across the inner mitochondrial membrane
224 (Palmieri, 2004). CaMCs are a subgroup of the mitochondrial carriers that are
225 characterized by a long N-terminal extension that harbors EF-hand calcium-binding
226 motifs that face the intermembrane space. In contrast to the isoform A and B, the short
227 isoform C (Fig. 2a) possesses the mitochondrial carrier domains but lacks the EF-hand
228 motifs. Putative insect orthologs of *D. melanogaster CG32103* are present in several
229 insect genomes, including *Acromyrmex echinatior* Forel (GenBank accession no.
230 EGI64450), *Aedes aegypti* L. (XP_001649449), *Anopheles gambiae* Giles (XP_557186),
231 *B. mori* (*BGIBMGA002002*;

http://sgp.dna.affrc.go.jp/KAIKObase/kaikogaas_gene_browse/cgi-bin/genechina.pl?seq_id=BGIBMGA002002), *Nasonia vitripennis* Walker (XP_001603181) and *Tribolium castaneum* Herbst (XP_001811057), implying that the role of *CG32103* is highly conserved across insect species.

Both mitochondrial transport and calcium signaling play essential roles in the production of juvenile hormone in the CA. During the early step of the JH biosynthetic pathway, FPP is generated via the classical mevalonate pathway in the mitochondria (Bellés et al., 2005). The importance of mitochondrial transport in JH biosynthesis has been proposed by a study on the peptide hormones known as allatostatins, which regulate JH synthesis by inhibition (Sutherland and Feyereisen, 1996). Allatostatins stimulate intracellular calcium signaling via the allatostatin receptors, which are G-protein coupled receptor family members (Birgul et al., 1999; Lenz et al., 2000; Larsen et al., 2001). In addition, an increased level of cytosolic calcium concentration activates the biosynthesis of JH in the CA (Gilbert et al., 2000; Stay and Tobe, 2007). Because CG32103 possesses both the mitochondrial carrier domain and calcium binding motifs, it might transmit calcium signals to the mitochondria and control JH biosynthesis activity in the CA. Interestingly, the transport activities of some CaMCs are affected by cytosolic or extramitochondrial calcium levels (Nosek et al., 1990; Palmieri et al., 2001; Lasorsa et al., 2003).

To evaluate a functional importance of *CG32103*, we performed a transgenic double strand RNA mediated interference (RNAi) experiment (Kennerdell and Carthew, 2000). We used a transgenic RNAi strain in which double-stranded RNA molecules corresponding to *CG32103* were generated using an inverted repeat construct by *GAL4/UAS* system. To knock down *CG32103* specifically in the CA, the *UAS* line was crossed with *CG32103&ptth-GAL4 GAL4* or the CA-specific driver *AUG21-GAL4*

257 (Siegmund and Korge, 2001; Adám et al., 2003). The *CG32103* RNAi animals, however,
258 exhibited no visible effect on *D. melanogaster* development (data not shown). It is
259 important to point out that RNAi, in general, results in partial, but not complete,
260 loss-of-function animals. Alternatively, it is possible that this is due to a peculiarity of
261 the cyclorrhaphous Diptera, including *D. melanogster*, where JHs do not have the
262 typical 'status quo' effect on larval development (Gilbert et al., 2000; Liu et al., 2009;
263 Riddiford et al., 2010). In order to determine the function of CG32103 in more detail,
264 isolation and study of genetic null mutants of *CG32103* are necessary.
265 It would be also intriguing to examine what substance CG32103 actually
266 transports from the mitochondria in the CA. According to a previous study (Sutherland
267 and Feyereisen, 1996), one of the proposed mechanisms of inhibition is that allatostatins
268 affect the export of citrate from the mitochondria, implying that a mitochondrial citrate
269 transporter would be involved in this step. It is therefore worth examining whether
270 CG32103 can act as a citrate transporter in mitochondria, although the previously
271 characterized mammalian CaMCs only include aspartate/glutamate carriers (Palmieri et
272 al., 2001) and ATP-Mg/Pi carriers (del Arco and Satrustegui, 2004; Fiermonte et al.,
273 2004). On the other hand, a gene called *DmCIC* has been characterized as the citrate
274 transporter (Carrisi et al., 2008). We examined the expression pattern of *DmCIC*, but
275 could not detect a predominant expression in the CA (data not shown).
276 Combined with the previous study (Harvie et al., 1998), our study
277 demonstrates the power of the enhancer-trap screen to identify a gene that is expressed
278 in tiny organs that are hard to isolate manually, such as the CA. Recently, the
279 enhancer-trap technology has been made available for other insect species, including the
280 red flour beetle, *Triborium castaneum*, and the silkworm, *Bombyx mori* (Lorenzen et al.,
281 2007; Uchino et al., 2008). Additional investigations using the enhancer-trap resources

in these insect species would facilitate the identification and characterization of genes that function in the CA when combined with other molecular biological and bioinformatic approaches.

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457 **Figure legends**

458

459 **Fig. 1.** (**a**) GFP expression pattern of *ptth&CG32103-GAL4>UAS-GFP* in wandering
460 3rd instar larvae. The ring gland and brain complex are shown. The corpora allata (CA)
461 is marked by an arrowhead, and the *ptth*-positive neurons are marked by arrows. Scale
462 bar: 50 μm. (**b, c**) Endogenous expression of *CG32103* as detected by RNA *in situ*
463 hybridization. Arrowheads indicate the CAs. (**b**) The ring gland-brain-ventral nerve
464 cord complex of the 3rd instar larvae is shown. Background staining with the antisense
465 probe in the brain and ventral nerve cord was also observed with the sense probe (data
466 not shown). (**c**) Dorsal view of a stage-16 embryo. The anterior is to the left. A dot-like
467 signal in the posterior side of the embryo was a non-specific signal, as this signal was
468 not reproducibly detected in our experiments (data not shown). Scale bars: 100 μm.

469

470 **Fig. 2.** Gene and protein structures of CG32103. (**a**) The genomic and exon-intron
471 structures of *CG32103* corresponding to 69B on the chromosome 3L genome scaffold
472 are shown. *CG32103* splice variants and their isoform nomenclatures are described in
473 FlyBase (http://flybase.org/reports/FBgn0052103.html). The gray and white boxes
474 indicate the open reading frames and untranslated regions, respectively. The insertion
475 into the pPTGAL vector construct carrying the *ptth* promoter is also illustrated. (**b**)
476 Reverse transcription (RT)-PCR analysis to detect mRNAs of isoform A, B and C of
477 *CG32103* in the ring gland. *rp49* was a loading control. (c) Quantitative RT-PCR
478 analysis of the *CG32103* transcript in several tissues from the wandering 3rd instar
479 larvae and adults. RG, ring gland; FB, fat body; SG, salivary gland; IN, intestine; ID,
480 imaginal discs; TS, testis; OV, ovary. The normalized expression level of each isoform
481 in the ring gland is set as 1. Each error bars represent the standard deviation (S. D.)

from three independent samples. Expression levels of the isoform C in the fat body and intestine are represented by actual calculated values (± S. D.).

Fig. 3. Temporal GFP expression in *CG32103&ptth-GAL4>UAS-GFP* flies. All fluorescence images were taken for the same exposure time and processed identically. Dashed lines outline the ring glands. Lower panels are DIC images. (a) The 1st instar larva. (b) The 2nd instar larva. (c) The early 3rd instar larva that did not wander. (d) The wandering 3rd instar larva. Scale bar, 50 μm.

Fig. 4. Protein structure of CG32103. **(a)** The amino acid sequence shown corresponds to isoforms A and B. The protein domains were predicted by SMART (Schultz et al., 2000). Domains 1–4 are EF-hand motifs. Domains A, B and C are modules of ~100 amino acids that contain two transmembrane domains and the characteristic signature of mitochondrial carrier proteins. Note that isoform C lacks all of the 4 EF-hand motifs but still retains the 3 mitochondrial carrier modules. (**b**) A schematic representation of the predicted protein structure of CG32103. The topological model of the mitochondrial carrier protein was adopted from Palmieri et al. (2004). EF1–EF4 represent the EF-hand motifs. Dashed lines separate each module of the mitochondrial carrier protein.

Fig. 1
Niwa and Enya

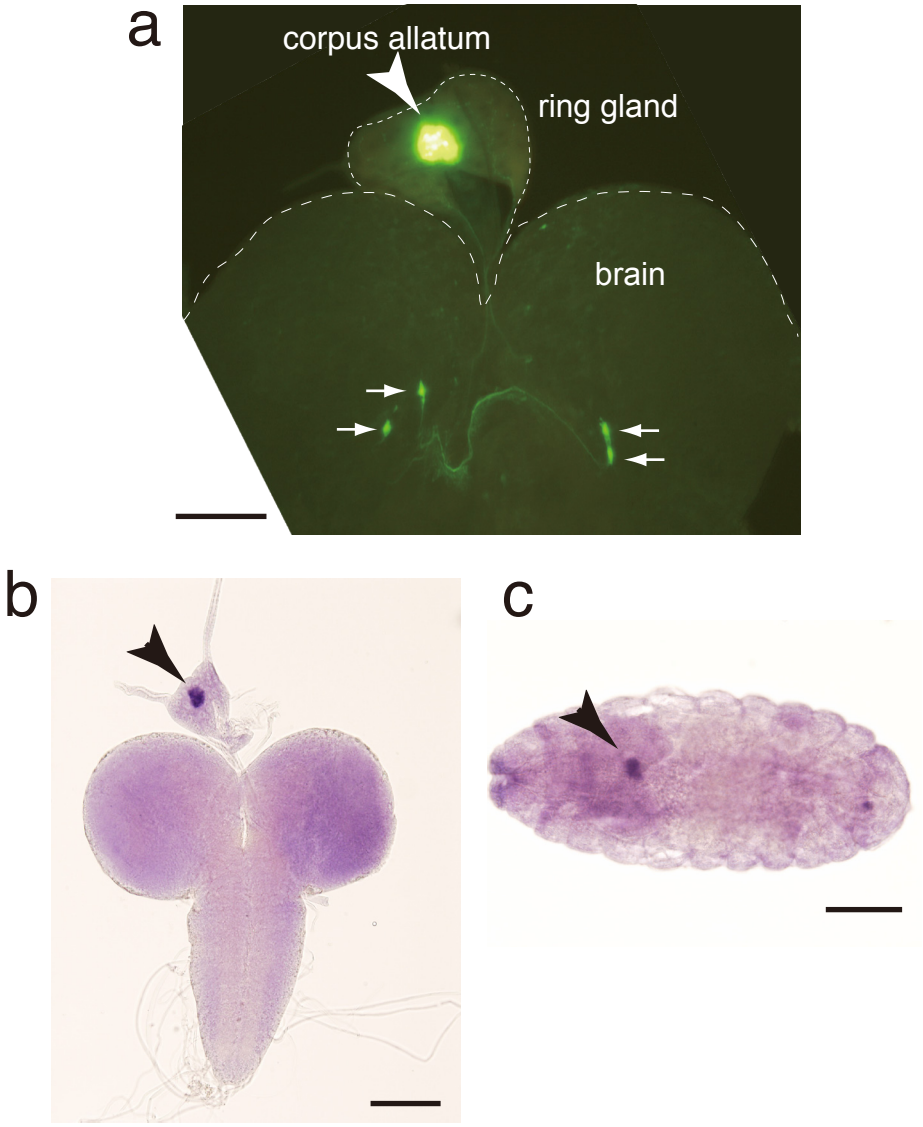

Fig. 2
Niwa and Enya

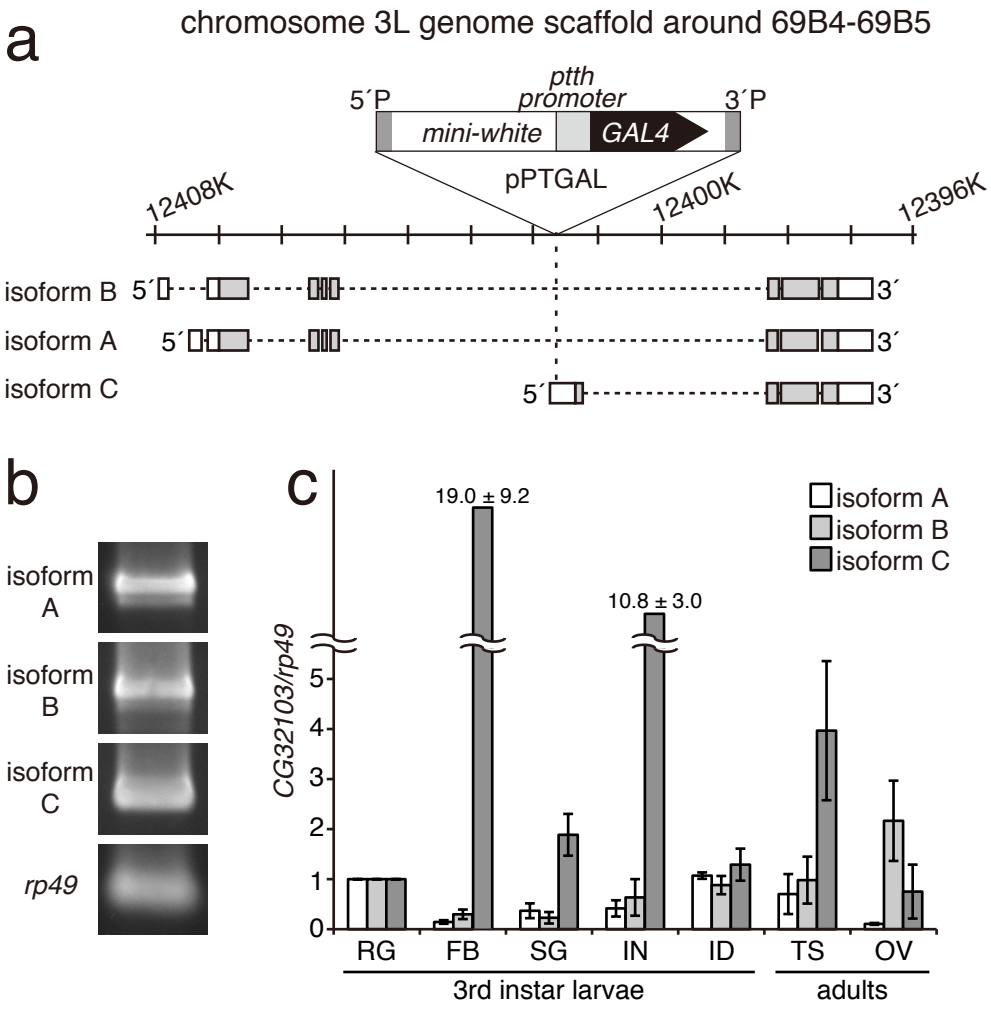

Fig. 3
Niwa and Enya

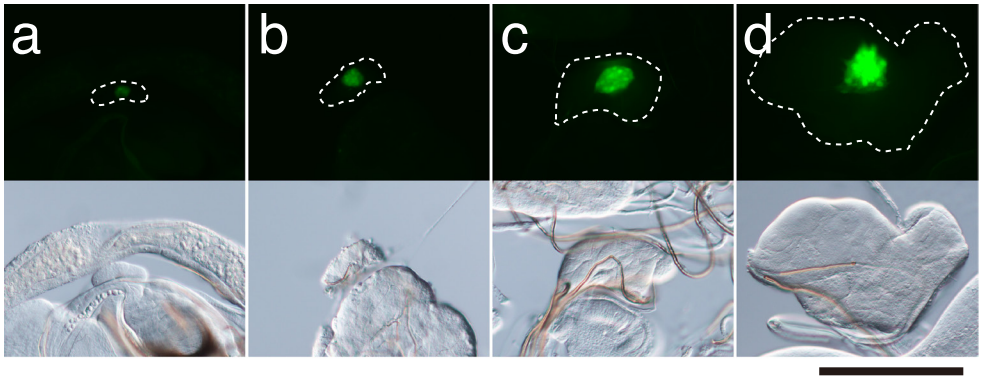

Fig. 4
Niwa and Enya

a

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