

C. elegans RNA-dependent RNA polymerases *rrf-1* and *ego-1* silence *Drosophila* transgenes by differing mechanisms

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Abstract *Drosophila* possesses the core gene silencing machinery but, like all insects, lacks the canonical RNA-dependent RNA polymerases (RdRps) that in *C. elegans* either trigger or enhance two major small RNA-dependent gene silencing pathways. Introduction of two different nematode RdRps into *Drosophila* showed them to be functional, resulting in differing silencing activities. While RRF-1 enhanced transitive dsRNA-dependent silencing, EGO-1 triggered dsRNA-independent silencing, specifically of transgenes. The strain *w; da-Gal4; UAST-ego-1*, constitutively expressing *ego-1*, is capable of silencing transgene including dsRNA hairpin upon a single cross, which created a powerful tool for research in *Drosophila*. In *C. elegans*,

EGO-1 is involved in transcriptional gene silencing (TGS) of chromosome regions that are unpaired during meiosis. There was no opportunity for meiotic interactions involving EGO-1 in *Drosophila* that would explain the observed transgene silencing. Transgene DNA is, however, unpaired during the pairing of chromosomes in embryonic mitosis that is an unusual characteristic of Diptera, suggesting that in *Drosophila*, EGO-1 triggers transcriptional silencing of unpaired DNA during embryonic mitosis.

Keywords *Drosophila* · RNA-dependent RNA polymerase (RdRp) · RNA interference (RNAi) · microRNAs (miRNAs)

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Introduction

Drosophila melanogaster, along with all insects and the vertebrates, lacks genes encoding members of the canonical RdRp gene family, which are required for the systemic spread of gene silencing by RNA interference (RNAi) in fungi, plants, and some animals [1–4]. Two major forms of RNAi exist in the animal system in which RNAi has been most thoroughly studied, *C. elegans*. For transitive post-transcription gene silencing (PTGS), RNAi is triggered by double-stranded RNA (dsRNA), which is processed into small interfering RNAs (termed primary siRNAs, ~20–30 nucleotides long) that target a complementary mRNA, leading to its degradation [5]. Transcriptional, dsRNA-independent gene silencing is involved in epigenetic modification and heterochromatin regulation [6]. *C. elegans* contains multiple RdRp family genes: of these, *rrf-1* [7] is required for systemic dsRNA-dependent RNAi in somatic cells, resulting in the unprimed production of secondary siRNAs [8, 9] that are structurally different to

the primary siRNAs. These secondary siRNAs enhance silencing of genes targeted by the primary dicer-derived siRNA molecules that are complementary to the target sequence. The other RdRp associated with gene silencing, *ego-1*, is required for germline transcriptional gene silencing and heterochromatin assembly [10].

Drosophila possesses the core gene silencing machinery [11–13]. Dicer-2 and Ago-2 are able to generate siRNAs from dsRNA, which act to repress transposon transcripts or endogenous mRNAs [14, 15]. The introduction of transgenes producing hairpin dsRNAs allows cell-specific silencing of *Drosophila* genes [16]. Moreover, a dsRNA-uptake system [17, 18] has recently been shown to allow systemic silencing of viral RNAs in *Drosophila* [19]. Despite these findings, *Drosophila* is incapable of systemic RNAi. We therefore asked whether RNAi in *Drosophila* could be enhanced as observed for systemic RNAi in *C. elegans* by the introduction of *C. elegans* RdRp genes.

We have previously shown that GAL4-mediated expression of transgenic *C. elegans* RdRps in *Drosophila* does not affect morphological development [20]. However, it remained an open question as to whether these *C. elegans* RdRps were active in *Drosophila* at all. We therefore asked whether these RdRps were capable of enhancing RNAi of a specific, known target gene, triggered by a dsRNA corresponding to that gene. In this study we show that *C. elegans* RdRps RRF-1 and EGO-1 silence *Drosophila* transgenes by differing mechanisms: RRF-1 was found to enhance transitive, dsRNA-dependent RNAi of target genes, whereas EGO-1 expression resulted in robust silencing specifically of transgenes that was independent of dsRNA.

Results

RRF-1 and EGO-1 could not be observed to enhance silencing of the endogenous gene, *pebble*

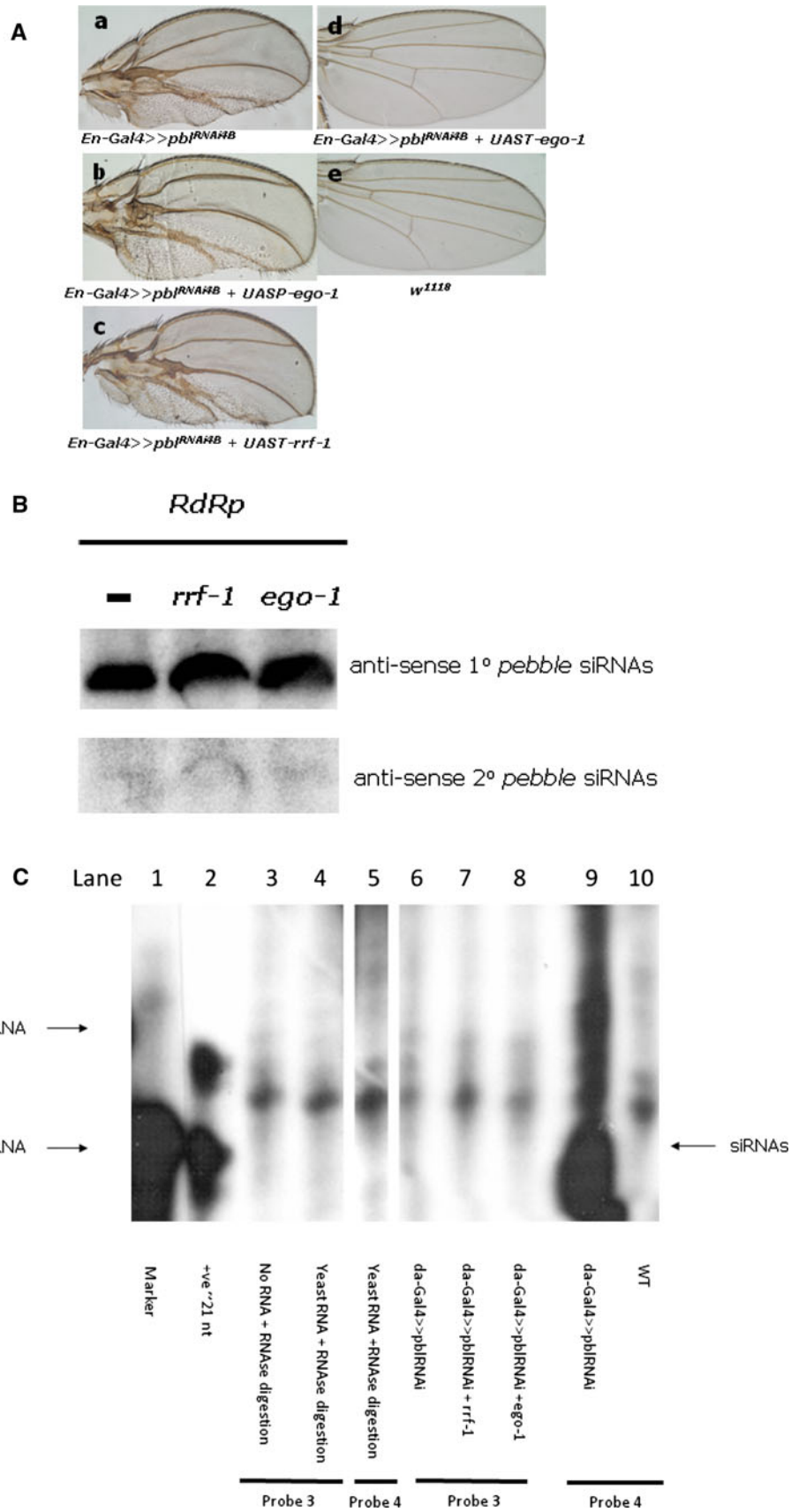
It is known that expression of a dsRNA from a transgenic construct leads to silencing mediated by siRNAs derived solely from dicer-2 activity. In many cases, such silencing is only partial, which provides an assay for enhancement of silencing by the introduction of the *C. elegans* RdRp genes into transgenic flies. To test this, we selected *pebble* [21] as the candidate endogenous gene. This gene encodes a guanine nucleotide exchange factor (GEF) that is important for *Rho1* activation in dividing cells [22] and therefore required for cytokinesis; knockdown produces multinucleate cells, a cell-autonomous phenotype that is easy to observe. Silencing of *pbl* specifically in the posterior compartment of the wing disc by combining an *en-Gal4* construct with *pblRNAi^{4B(3.1)}* [23], a *UAS* construct that expresses *pbl* hairpin dsRNA, resulted in disruption of the

Fig. 1 Silencing of the endogenous gene, *pebble*, in *Drosophila* and detection of the primary as well as secondary siRNAs. **a** Expression of canonical *C. elegans* RdRps does not noticeably enhance RNAi-mediated silencing of the *Drosophila* endogenous gene, *pebble*. For activation of the dsRNA expression, flies harboring the *pebble* gene dsRNA construct *pblRNAi^{4B(3.1)}* were crossed with the tissue-specific driver *en-Gal4* in the presence (*b–d*) or absence (*a*) of one of the RdRps, RRF-1 or EGO-1. Compared with the control (*a*), noticeable changes in *pebble* gene silencing in either *b* or *c* were not observed; however, expression of higher levels of EGO-1 from the UAST vector gave a wild-type phenotype (*d*) through comparison with WT (*e*). **b** Northern hybridization did not detect any production of secondary siRNAs. Probe 1, designed based on the *pebble* targeted sequence (Fig. S1), successfully detected 1° siRNAs, while probe 2, designed based on the sequence upstream of the targeted sequence, did not detect any 2° siRNA production regardless of the presence of either of the RdRp genes. We used the RNA samples from *UASP-ego-1* rather than *UAST-ego-1* as *da-gal4*-activated *UASP-ego-1* automatically silenced *pblRNAi^{4B(3.1)}* and gave WT phenotype, i.e., could not suspend the development at pupae stage as occurred in *UASP-rrf-1* and *UASP-ego-1*. **c** Secondary siRNAs detection with RNase protection assays. RNAs was prepared from pupae. Lane 1: RNA marker, lane 2: positive control RNA; lanes 3 and 4 are controls for probe 3 with yeast or no RNA; lane 5 is control for probe 4 with RNase digestion. Lanes 6–8 are the results for detection of 2° siRNAs upstream of the targeted *pebble* sequence (Fig. S1 and S2) with probe 3; *da-Gal4*-activated *rrf-1* (lane 7) or *ego-1* (lane 8) did not generate detectable 2° siRNAs. Lane 9: detection of the primary siRNAs with probe 4, lane 10: no detection in *w¹¹¹⁸*

development of the posterior compartment of the wing. As shown in Fig. 1a, when the RdRp RRF-1 or EGO-1 (in *UASP*) was co-expressed with the hairpin *pbl* dsRNA, no change in the wing phenotype was observed.

The silencing observed normally for the *pebble* gene, i.e., in the presence of only the dsRNA transgene, is quite pronounced, and the resulting morphologies are variable, ranging up to strongly deformed wings [23]. This means it may not be possible to rely solely on observation of morphological changes upon silencing an endogenous gene to assess enhancement of RNAi. Indeed, RRF-1 had no observable impact on silencing of *pebble*. In contrast, co-expression of EGO-1 at the high levels enabled by the *UASP* vector did have an observable impact on silencing, but curiously this was to abolish it, since all progeny with this genotype had a wild-type phenotype (Fig. 1a–d and legend). This was the first observation suggesting that EGO-1 expression directly silenced a transgene, that producing *pebble* dsRNA.

We next tested whether the expression of RdRps affected the production of siRNAs in these RNAi flies. Although RdRp can enhance silencing by producing extra 2° siRNAs upstream of the dsRNA targeting sequence in *C. elegans* [8, 9], neither Northern hybridization, nor RNase protection assays, detected production of 2° siRNAs in these flies, while expression of 1° siRNAs was not affected by the expression of *rrf-1* or of the *UASP-ego-1* transgene (Fig. 1b, c). Since the *pebble* gene is only expressed in a specific tissue for a limited time, it is possible that the level of any 2° siRNAs produced may be too low for detection.



Silencing by RRF-1 and EGO-1 of an exogenous reporter gene, *egfp*

The above work showed modulation of *pebble* silencing to be unsuitable as an experimental system for testing activity of the RdRp transgenes. We therefore asked whether these RdRps could enhance silencing of an exogenous reporter gene, *egfp*, placed under the control of an ubiquitin/*GAL4* construct to ensure expression throughout all tissues and stages. The *egfp* hairpin dsRNA required to generate primary siRNAs targets almost the complete *egfp* sequence, leaving only ~28 nt uncovered at the amino-terminal portion of the EGFP coding sequence [16]. In order to provide additional sequences upstream on the same mRNA to allow testing for generation of secondary siRNAs as a consequence of RdRp action, the EGFP coding sequence was translationally fused 3' to the coding sequence of a *Drosophila* gene, *synaptotagmin* (hereafter *syt*) (*syt.EGFP*) [24], or of a bacterial gene, *NaChBac* (*NaChBac.EGFP*), encoding a Na-channel protein [25].

In contrast to the observation with the endogenous *pebble* gene, we observed that dsRNA-dependent silencing (Fig. 2b, i) of the *egfp* gene was significantly enhanced in the presence of either of the RdRps (Fig. 2c, e, j, l). To investigate if the corresponding EGFP dsRNA was required for the silencing, we set up parallel crosses omitting only the dsRNA, while keeping all other genes and drivers (i.e., EGFP, RdRp, *da-Gal4*) unchanged. Surprisingly, EGO-1 still totally silenced the *egfp* gene (Fig. 2g, m) while RRF-1 did not (Fig. 2d, k), in the absence of the dsRNA; this observation was confirmed using different RdRp lines and a line in which *syt.EGFP* and the EGFP dsRNA are present on different chromosomes (Supplementary Figure S6). This result was further confirmed by another reporter gene, *rfp*, encoding the red fluorescence protein (RFP) [26] silencing by EGO-1 without the corresponding *rfp* dsRNA was observed (Fig. 2p), while no silencing was found by RRF-1 alone (Fig. 2o). qPCR experiments confirmed the RdRp transgenes were expressed (Supplementary Figure S11A and B), and Northern blot and hybridization confirmed the *egfp* expression (Supplementary Figure 10). Taken together, these results indicate that EGO-1 is an independent silencer that can autonomously silence transgenes; on the other hand, RRF-1 is only capable of enhancing dsRNA-dependent silencing.

Enhancement of silencing by RdRp releases suspension of development due to an exogenous gene, *NaChBac*, or a dsRNA hairpin

We next asked whether a sensitive test could be developed using a biologically active gene expressed in all tissues and stages, as was the case for the reporter gene *egfp*. For this purpose, we used the bacterial voltage-gated sodium

channel, *NaChBac*, which as described above, is available as a translation fusion to EGFP. Expression of this fusion transgene in *Drosophila* has previously been restricted to specific neuron tissues [27]. However, general activation of the *NaChBac* gene with *da-Gal4*, which activated EGFP in first instar larvae as shown in Fig. 2h, was also observed to suspend *Drosophila* development at that stage. This finding established a novel experimental system for testing silencing of a biologically active transgene.

We then asked whether dsRNA-induced partial silencing of the *NaChBac.EGFP* gene, could overcome this developmental block. Indeed, silencing of the fusion gene, as shown by the reduction in EGFP fluorescence (Fig. 2i), allowed larvae to develop up to the second instar, (Fig. 3a-1), at which point further development was again suspended, consistent with the expected partial silencing of the *NaChBac* transgene. However, when combined with either one of the RdRps, total silencing was achieved (Fig. 3b, c); this, in turn, released the development suspension conferred by *NaChBac*, and the larvae were observed to complete full development to adults. While EGO-1 expression allowed completion of development irrespective of the presence of dsRNA, RRF-1 had a similar effect only in the presence of the dsRNA transgene (Fig. 3a-3). These observations confirmed the utility of this novel system for RNAi investigation in *Drosophila*.

Although the limited extent of *pebble* expression had earlier been a factor restricting its utility for studies of RNAi enhancement, we unexpectedly found that general activation of the *pblRNAi^{4B(3.1)}* dsRNA hairpin construct with *da-Gal4* suspended fly development at the pupal stage (Fig. 3c-i, ii). This allowed experiments to ask whether EGO-1 could block expression of this dsRNA hairpin completely to overcome this biological effect. Silencing of the *pebble* hairpin by *en-Gal4*-activated EGO-1 had earlier been found to release its silencing of the *pebble* gene in the wing disc (Fig. 1a-d), co-expression of EGO-1 was indeed observed to release *pebble* repression in all tissues, therefore rescuing full development (Fig. 3c-i, ii) to the adult stage.

RRF-1, but not EGO-1, enhances transitive RNAi pathway by producing extra secondary siRNAs

We next asked whether the RdRps produced 2° siRNAs [7–9]. Production of 2° siRNAs is dependent upon presence of 1° siRNAs, as detected in all flies expressing the EGFP dsRNA hairpin (Fig. 4b, d). Both sense and anti-sense 2° siRNAs were detected in flies expressing *syt.EGFP* and the EGFP dsRNA hairpin (Fig. 4a, c, blue boxes, respectively) in the absence of either *rrf-1* or *ego-1*; their origin, however, remains unknown in the absence of a proven endogenous RdRp. In contrast to observations with the sense 2° siRNAs (Fig. 4c), expression of RRF-1 enhanced anti-sense 2° siRNA levels in flies (Fig. 4a, red

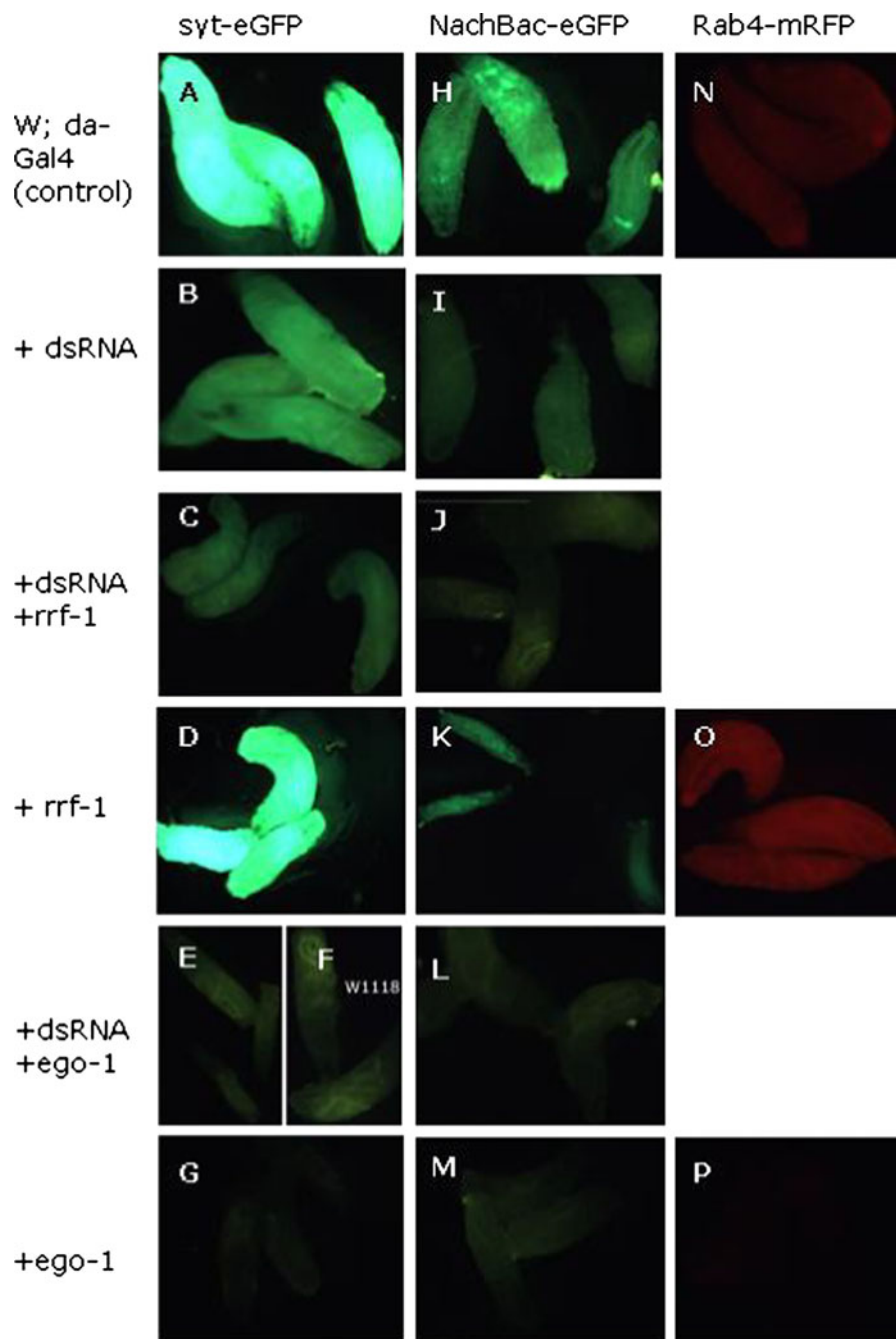
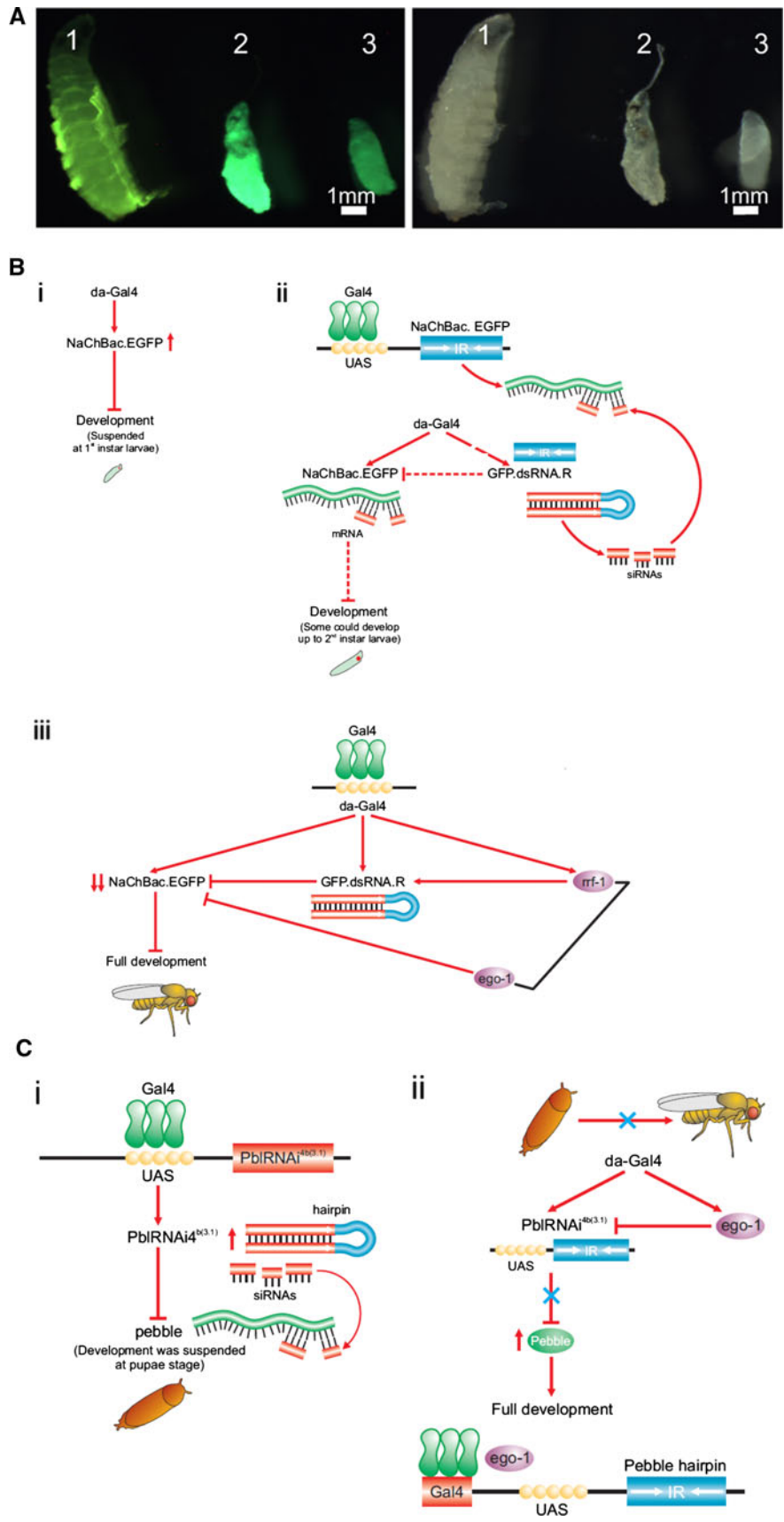


Fig. 2 *C. elegans* RdRp RRF-1 enhances dsRNA-triggered silencing of exogenous target genes, whereas silencing of exogenous genes by EGO-1 under the control of *UAST* is dsRNA-independent. Crosses for obtaining the larvae illustrated above are shown in Supplementary Methods S2, online supplement. Each cross was set up in at least for four replicates, and about 12 1^o instar larvae from each cross were randomly selected and checked for consistency of the fluorescence signal; three of these were randomly selected for photography. **a**, **h**, and **n** are positive controls for fluorescence from *NaChbac.eGFP*, *syt.eGFP*, or *Rab4-mRFP*, respectively; **b** and **i** show a reduction of green fluorescence signals, indicating that the EGFP transgenes were partially silenced with the introduction of the corresponding EGFP dsRNA; **c** and **j** show a dramatic further

reduction in the green fluorescence signal after combination with the RdRp gene, *rrf-1*, indicating a dramatic enhancement of silencing of the EGFP gene. Addition of the *rrf-1* gene resulted in no silencing enhancement in the absence of the dsRNA trigger for either GFP [**d**, **k** or for the RFP line (**o**)]. In contrast, total silencing of fluorescent protein gene expression was observed in the presence of *ego-1*, irrespective of whether the EGFP dsRNA hairpin was present (**e**, **l**) or not (**g**, **m**); the RFP gene was also silenced totally in the absence of any dsRNA trigger (**p**). Expression of the EGFP-linked *NaChBac* gene resulted in suspended larval development at the 1^o instar stage (**k**). **f** is the negative control from *w¹¹¹⁸* under a GFP filter (using a Leica MZ11 fluorescence microscope); for the RFP gene a TXR filter was used



◀ **Fig. 3** Partial silencing of *NaChBac:eGFP* fusion gene releases suspension of larval development at the 1° instar larvae stage. **a** *Left panel* fluorescence images; *right panel* bright light images of the same larvae. Partial silencing of the EGFP-linked *NaChBac* gene by introduction of the EGFP dsRNA hairpin gene (1), allows larval development beyond the 1° instar stage whereas development is suspended in larvae expressing EGFP-linked *NaChBac* (2). Introduction of *rrf-1* in the absence of the dsRNA hairpin had no effect on larval development (3). However, the partial silencing achieved by introduction of the EGFP dsRNA is insufficient for full development to adult stage. The *green fluorescence* signal is decreased (1) compared with no such silencing in 2 and 3. **b** Schematic model proposed for the genetic interactions observed for EGFP silencing. (i) The general driver *da-Gal4* activates the *NaChBac:eGFP* fusion gene, resulting in the suspension of *Drosophila* development at the 1° instar stage. (ii) Activation by the general driver *da-Gal4* of the EGFP dsRNA hairpin gene, as well as of the *NaChBac:eGFP* fusion gene, results in partial silencing of the latter and a reduction of the *NaChBac:eGFP* protein product, partially releasing the developmental arrest, and allowing some larvae to develop further, to the 2° instar stage. (iii) When RRF-1 was introduced, EGFP dsRNA-dependent silencing of the *NaChBac:eGFP* was dramatically enhanced, allowing larvae to go through full development towards the adult fly stage. In contrast, EGO-1 directly and completely silences the *NaChBac:eGFP* transgene, independent of the corresponding dsRNA, allowing full development of *Drosophila* to occur. **c** Schematic model proposed for the genetic interactions observed for *pebble* silencing. (i) The general driver *da-Gal4* activates the *pblRNAi^{4B(3.1)}* dsRNA construct, whose expression silences the *pebble* gene, resulting in development being suspended at the pupal stage. (ii) General activation of *pblRNAi^{4B(3.1)}* as well as EGO-1 (in *UAST*), resulted in *pblRNAi^{4B(3.1)}* expression being repressed by EGO-1, which in turn released *pebble* silencing imposed by *pblRNAi^{4B(3.1)}* and subsequently led to full development

box), confirming that RRF-1 enhances RNAi in *Drosophila* by the transitive pathway.

In contrast, 1° siRNAs were reduced and 2° siRNAs abolished in the flies with EGO-1 and the EGFP dsRNA (Fig. 4, yellow boxes). The abolition of even the 1° EGFP siRNAs in the presence of EGO-1 (Fig. 4b, yellow box), suggested that EGO-1 simultaneously silenced the *egfp* hairpin dsRNA transgene as well as the actual EGFP-transcript (since no dsRNA was required for silencing of this transgene). Silencing of a dsRNA-producing transgene would explain the original observation (Fig. 1, above) that EGO-1 abolished silencing of the endogenous *pebble* gene.

Transgene silencing by EGO-1

The dsRNA-independent silencing of transgenes, including those producing dsRNA hairpins, by EGO-1 appears to be transcriptional. Evidence that the silencing of the eGFP transgene is transcriptional is that no mRNA was detected (Supplementary Figure S10), no siRNA was produced (Fig. 4a), and the silencing was irreversible (Supplementary Figure S7). Nuclear run-on experiments to confirm transcriptional silencing failed, due to the difficulty of obtaining RNA from the development-suspended larvae and the limited expression level of transgenes. This

targeted silencing of transgenes, but not endogenous genes, is consistent with the absence of morphological or other obvious phenotypic changes evident in *Drosophila* expressing this gene [20]. Silencing of transgenes by EGO-1 cannot be attributed to titration of the GAL4 transactivator required for transgene expression. The *ego-1* and *rrf-1* genes, and the other genes dependent on GAL4, were expressed from exactly the same expression cassettes, carrying the same complement of UAS sequences. The two RdRp genes nonetheless showed totally different effects.

EGO-1 is involved in silencing of unpaired DNA in *C. elegans* [10]. Since the *Drosophila* transgenes are actually unpaired in the progeny flies described above, we hypothesized that this is what has made them subject to EGO-1 silencing. For example, the progeny of the cross in Fig. 2g (in which EGO-1 was observed to cause silencing of EGFP) are heterozygous: *w; da-gal4/+; T-ego-1/UAST-syt-eGFP*. To test this hypothesis, we asked whether the transgene was silenced when paired, by crossing a line homozygous for *syt-eGFP* and *ego-1* with a line homozygous for *da-Gal4* and heterozygous for *syt-eGFP* (Supplementary methods S3). The EGFP was indeed silenced in the progeny flies heterozygous for *syt-eGFP* (Fig. 5a) but not in those homozygous for this gene (Fig. 5b), supporting the hypothesis of unpaired DNA silencing.

Silencing by EGO-1 may involve methylation and/or rearrangements of the promoter region of the transgenes

There is ongoing interest in the extent to which DNA methylation occurs in *Drosophila*, with little evidence for methylation after early embryonic stages [28]. Since the silencing of transgenes by EGO-1 appeared not to be reversible, we next asked whether expression of EGO-1 had altered the chromatin status by stimulating any methylation of DNA. To address this, we first performed Southern blot and hybridization in the EGFP coding region as well as its promoter region. The sizes of fragments detected in the EGFP coding region were consistent with the expected sizes indicated in the diagrams (Supplementary Figure S12) except that the small fragments <100 bp were not detected (Fig. 6a). However, in the promoter region of transgenes, the *HpaII* and *HhaI* cutting pattern between the samples with or without EGO-1 obviously altered (Fig. 6a), a possible explanation is that the expression of EGO-1 altered the methylation or demethylation status in the promoter region.

In light of the lack of resolution obtained by the Southern experiment, we next performed bisulfite sequencing to analyze individual cytosine residues in the promoter regions of the transgenes (Fig. 6b). The promoter

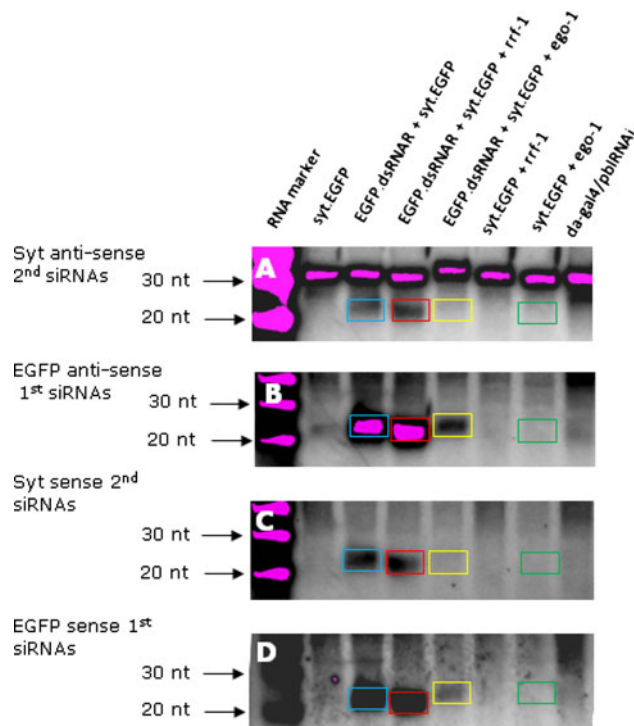


Fig. 4 Northern blot and hybridization for detection of the primary and secondary siRNAs in flies with the transgene *egfp* RNAi. A diagram illustrating the probes and target sequence is provided in Fig. S3 and S4. **a** Detection of the *syt* anti-sense 2° siRNAs with *syt* sense probe, compared with control (*EGFP.dsRNA* + *syt.eGFP*) without either *rrf-1* or *ego-1*, in which the 2° siRNAs (blue) were produced by endogenous non-canonical RdRp activity, the 2° siRNA production in the presence of RRF-1 (*red*) was dramatically increased, while siRNA production in the presence of EGO-1 was dramatically reduced and difficult to see with naked eye (*yellow*), indicating an enhanced transitive RNAi pathway in the presence of RRF-1 but not EGO-1. The detection of *syt* sense 2° siRNAs in **c** is consistent with the results shown in **a**. In comparison, in **b**, EGFP anti-sense 1° siRNAs without RdRp (*blue*) and with RRF-1 (*red*) were detected at a similar signal strength, but production of these siRNAs was dramatically decreased (*yellow*) when EGO-1 was present, indicating an independent, direct silencing of the EGFP dsRNA hairpin transgene by EGO-1, which is consistent with the EGFP sense 1° siRNAs detection in **d**. In addition, independent silencing by EGO-1 was achieved not through producing 1° or 2° sense or anti-sense siRNAs (*green boxes* in all panels)

regions from the transgenes *syt.eGFP*, in the absence of EGO-1 (S4), in the presence of EGO-1 (S12 and S20), or only that from EGO-1 (S29, S33, and S34), were compared using VectorNTI (Invitrogen) and CYMATE (GMI) [29]. In sample S29, a fragment between ~151 and 375 bp was found to be heavily methylated in all three patterns CGN, CHG, and CHH, sequence analysis showed that it was comprised of 5 × Gal4 UAS and the first half of HS promoter (Supplementary Figure S13). This indicated that the effects of expression of EGO-1 were not universal in all cells. Moreover, it was striking that a fragment between ~151 and 260 bp in sample S20 was deleted, possibly as a

consequence of EGO-1 modulation of the transgene's expression. Further sequence analysis confirmed that this deleted fragment contained 5 × Gal4 UAS, which is critical in the GAL4-UAS system for successful transgene expression. It should be noted that the observed incidence was relatively low, with only one sample in ten being affected. Deletion of a portion of the transgene promoter was observed as a possible consequence of EGO-1 being co-expressed.

Discussion

In this study, we show that *C. elegans* RNA-directed RNA polymerases RRF-1 and EGO-1, are capable of activity when introduced into *Drosophila* as transgenes. By taking advantage of the partial silencing induced by dsRNA, we investigated whether these RdRps were capable of enhancing RNAi. In accordance with their different roles in *C. elegans*, we confirmed their ability to function by mechanisms dependent and independent, respectively, of dsRNA.

Novel insights from RdRp-enhancement of endogenous and trans-gene silencing

The use of transgenes encoding reporter proteins allowed testing for enhancement of dsRNA-dependent silencing by RRF-1. Beyond the experiments using only the reporter genes, work with two biologically active target genes was required to assess how effective the enhancement of RNAi was. The endogenous target gene used initially, *pbl* [21], was silenced quite effectively by dsRNA. The pronounced, if variable, deformation caused by this silencing meant it was not possible to confirm any enhancement when the *en-Gal4* construct was used to drive the dsRNA and RRF-1 transgenes. Use of the *da-Gal4*-activator, on the other hand, to drive the dsRNA transgene enabled general silencing of *pbl* at a level sufficient to kill all progeny at the pupa stage. While this is of interest as the first demonstration that general silencing of *pbl* is lethal, it was not possible to assess any enhancement by RRF-1.

To demonstrate that RRF-1 could enhance RNAi of a biologically active gene required establishment of a novel system for RNAi investigation in *Drosophila*, involving modulation of *NaChBac.eGFP* silencing. The voltage-gated Na-channel protein [25] encoded by *NaChBac* has previously been used to study neuron excitation in *Drosophila* [27, 30] but only with its expression restricted to specific tissues and neurons. Our finding that general activation of the sodium channel *NaChBac* (as a translational fusion protein with EGFP) suspends development at the first instar larval stage generates a valuable research

Fig. 5 Silencing by unpaired DNA mechanism. The crosses used to generate the flies are provided in the Supplementary Methods S3. The flies were checked for genotype and the consistency of EGFP expression/non-expression. Three representative flies were then selected for photography under the same conditions and the same fluorescence microscope parameter. EGFP was silenced in the heterozygous flies (a) but not in the homozygous ones (b)

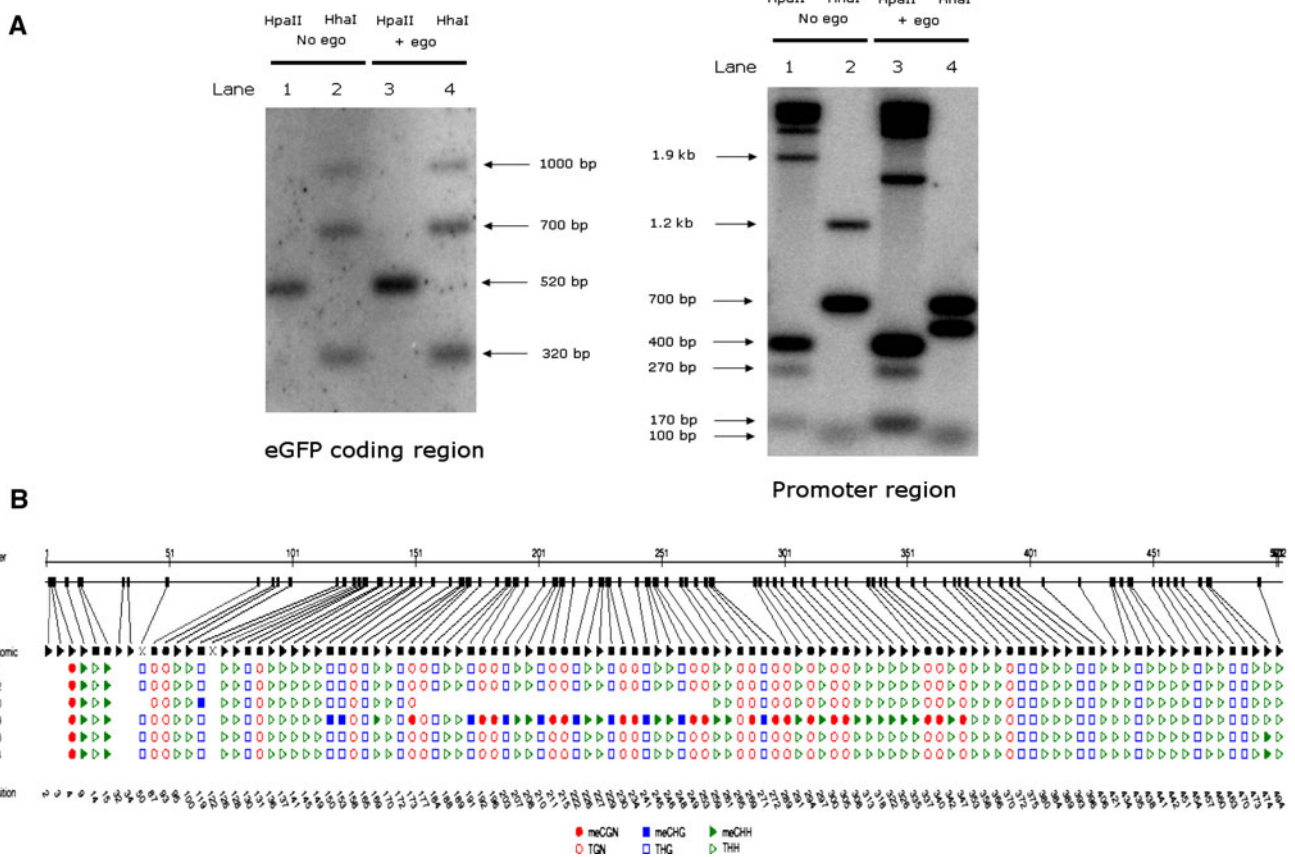
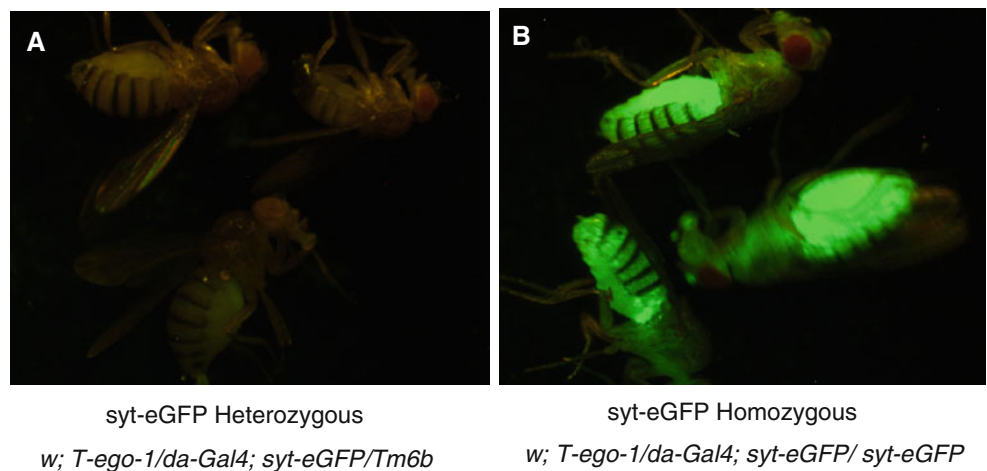


Fig. 6 Methylation detection and rearrangement of the promoter region of the transgenes. **a** Southern hybridization detected the cutting pattern unchanged in the EGFP coding region, and altered in the transgenes' promoter regions, respectively. DNA samples in lane 1: *w; da-Gal4/+; UAST-syt.eGFP/+* cut with *HpaII*; lane 2: *w; da-Gal4/+; UAST-syt.eGFP/+* cut with *HhaI*; lane 3: *w; da-Gal4/+; UAST-syt.eGFP/T-ego-1* cut with *HpaII*; lane 4: *w; da-Gal4/+; UAST-syt.eGFP/T-ego-1* cut with *HhaI*. The cutting pattern in EGFP coding

region was not affected by expression of the EGO-1 gene (left); in contrast, the pattern in transgenes' promoter regions was altered by the co-expression of EGO-1 (right). **b** Bisulfite sequencing analysis in the promoter regions of the transgenes in the presence or absence of EGO-1. Sample S4: *syt.eGFP* only without *ego-1*, S12, S20: *Syt.eGFP* with *ego-1*, S29, S33, S34: *ego-1* only. "N stands for any nucleotide and "H" is either A, C, or T

tool. Previously, over-expression of a potassium channel protein (Kir2.1) in *Drosophila* has also been shown to prove lethal [30]. The RRF-1-enhanced silencing of the

NaChBac.eGFP gene allowed full completion of development, whereas dsRNA-induced partial silencing resulted in larvae developing only up to the 2° instar.

Does *ego-1* induce mitotic silencing of unpaired DNA?

During meiosis, homologous chromosomes are paired to allow recombination and segregation. DNA sequences that are unpaired during meiosis (e.g. as a result of chromosomal rearrangements) are silenced in *C. elegans* germline cells by a process in which the RdRp EGO-1 has been implicated [10, 31]; in *Neurospora crassa* its orthologue SAD-1 displays similar activity [32]. The observed silencing of transgenes elicited by EGO-1 in *Drosophila* occurs in the absence of meiosis, e.g. in the direct progeny of the *w; da-Gal4; UAST-ego-1* × *w; +; UAS-syt.eGFP* crosses. The silencing of a transgene like *UAS-syt.eGFP* by EGO-1 may however be explained by an unusual aspect of mitosis in *Drosophila*: the mitotic pairing of homologous chromosomes in somatic cells that has long been known to occur in Diptera [33]. Since the transgene (*UAS-syt.eGFP*) is unpaired in the progeny of this cross, it would be susceptible to detection by an EGO-1-induced process during mitotic pairing of chromosomes that would then result in its silencing.

Mitotic pairing underlies genetic phenomena such as transvection [34], which affects gene function [35, 36]. Work in *Drosophila* has shown transvection may be evident as silencing of paired genes (pairing-sensitive silencing, PSS) or of unpaired genes (un-paired silencing, UPS) [37–39]. Transvectional PSS affects genes carrying Polycomb group (PcG) response elements (PRE), which are involved in homeotic gene regulation [40–43]. While these studies, interestingly, suggested a role for RNAi pathway genes in pairing-sensitive transcriptional gene silencing (TGS), more recent work has found long ncRNAs to be involved in PRE silencing, so that the role of the RNAi pathway genes and short RNAs in pairing-sensitive TGS remains largely unclear [44, 45]. Interestingly, Kavi and Birchler [46] have recently suggested that the RNA polymerase polIII may be associated with RNAi during heterochromatin silencing. Such suggestions are consistent with the observation that polIII in the yeast *Schizosaccharomyces pombe* has RdRp activity [47].

The EGO-1-dependent silencing observed for unpaired genes in the present work is unlikely to be related to UPS. This specific form of transvection was observed under quite different experimental circumstances, in a study using a transgene driven by a copia LTR promoter which showed silencing was dependent on the histone acetyl transferase *mof* [48]. This was therefore a specific finding for a particular type of transgene, and required a promoter and a histone acetylation activity that cannot be linked to what is known about EGO-1 and its role in meiotic silencing. Overall, the differences between transvection, and the TGS observed in the present study, make it very unlikely that our observations result from transvection. This leads to the possibility that

ego-1 may therefore trigger mitotic silencing of these unpaired transgenes by a mechanism similar to that used in meiotic silencing of unpaired DNA in *C. elegans*.

Conservation of pathways required for *ego-1* induced silencing of unpaired DNA

The small RNA based gene silencing mechanism controlled by EGO-1 in *C. elegans* [31, 49] involves histone H3K9 dimethylation, which requires a specific histone methyltransferase (HMTase). Similarly in *Drosophila*, the HMTase encoded by *Su(Var)3-9* plays a central role in heterochromatin gene silencing [50]. Indeed, the heterochromatin *Su(Var)3-9* HMTases are highly conserved in eukaryotes [51], and introduction of the human gene into *Drosophila* allows partial rescue of silencing defects due to *Su(Var)3-9* mutation [52].

The H3K9 HMTases have furthermore been shown to direct DNA methylation in fungi, plants and mammals [53, 54]. However methylation in *Drosophila* DNA remains controversial [55], having previously only been reported restricted to the early stages of embryonic development [56]. Our observation of possible methylation associated with EGO-1-dependent silencing is a further example of how the introduction of heterologous transgenes can be used in the study of this question. Previously, introduction of a mammalian *Dnmt3a* showed it to be active in *Drosophila* [57]; the biological effects evident upon DNA hypermethylation required the H3-K9 HMTase *Su(Var)3-9*, suggesting that further insights into possible methylation associated with EGO-1-dependent silencing could come from use of mutants in this *Drosophila* gene. In *Drosophila*, H3K9me2 marks are associated with the GREEN type of chromatin identified by [58]. A link between H3K9 trimethylation and postembryonic DNA methylation in *Drosophila*, mediated by the methyl cytosine binding domain protein dSETDB1, was recently identified by [59]. Finally, it will also be of interest to extend this study to ask whether the introduction of RdRps provides *Drosophila* with a novel genome-defense or RNA-based immunity capability against DNA or RNA viral genomes.

Materials and methods

Constructs and fly strains

Constructs for RdRp expression were as described in Duan et al. [20]. Fly strains used in this study in Supplementary Table 1 are from Ozdros, Australia (<http://www.ozdros.com>), Strains in Supplementary Table 2 are from Bloomington stock centre, USA, and strains in Supplementary

Table 3 were created in this study using strains from Supplementary Table 1, Supplementary Table 2, or strains as described in Duan et al. [20].

Total RNA isolation

Adult flies, pupae, or 1^o instar larvae were collected for total RNA extraction with TRIzol reagent (Invitrogen), RNA quality and quantity were checked with a NanoDrop[®] Spectrophotometer ND-1000 (ThermoFisher Scientific) and an Agilent 2100 Bioanalyser (Agilent Technologies).

Quantitative real-time-PCR and result normalization

Initially, RT-PCR was performed to obtain the first strand cDNA with SuperScript[™] III First-Strand Synthesis SuperMix (Invitrogen), qPCR with the cDNA then was performed using iTaq[™] SYBR[®] Green Supermix with ROX (BIO-RAD) on a machine 7900 HT Fast Real-Time PCR system (Applied Biosystems). Four biological replicates were included for each sample. A normalized value for absolute expression in each line was obtained by calculating the mean from the four replicates followed by a deduction of the mean values from no RT controls before divided by the *actin* mean value.

Small RNAs detection

Small RNAs was detected based on small RNA Northern blotting and hybridization [60, 61]. In general, ~20 µg total RNA for each sample was run on a 15 % polyacrylamide gel, and electroblotted onto Amersham Hybond[™]-N⁺ membrane (GE Healthcare), RNA was fixed on the membrane by UV crosslinking with 1200J using a UVP CL-1000 UV crosslinker. The membrane was hybridized overnight with isotope-labeled probes before exposure in a phosphor screen and detected with a FLA-5000 PhosphorImager (Fujifilm). Extended exposure time was required for weak signals. For re-probing, the membrane was washed with 0.1 % boiling SDS to remove the hybridized probe.

Small RNA probe preparation

Probes for small RNA hybridization were labeled with T7 RNA polymerase (Promega) and P³²-UTP (PerkinElmer) based on template DNA containing a T7 RNA polymerase promoter. The labeled RNA was treated with RNase-free DNase (Promega), and purified using a GE Healthcare Radiolabeled Probe Purification Kit G-50 (GE Healthcare) according to the manufacturer's instruction. For siRNA detection, probes were fragmented to ~50 nt with 200 mM carbonate solution before adding for hybridization.

RNase protection assay

The RNase protection assay was performed according to the instructions in the mirVana[™] miRNA detection kit (Ambion). The hybridization reaction solutions were run on a 45 ml 15 % acrylamide gel followed by exposure with X-ray film in a cassette at -80 °C for 3–5 days (depending on the strength of the signals) before development on a AGFA CP1000.

Southern blotting and hybridization

Restriction fragment was detected based on Southern blotting [62]. In brief, genomic DNA was extracted, and followed by purification with phenol/chloroform extraction, ~15 µg gDNA for each sample was used for restriction digestion with *HapII* or *HhaI*, extended digestions (up to 3 h) were required until a pre-check on the gel showed that a smear was formed in each lane indicating the majority of gDNA was digested, DNA was run on a 1 % agarose gel. The gel was then depurinated in 250 mM HCl and denatured for 2 × 15 min, followed by neutralization at least 30 min. The gel was blotted overnight onto an Amersham Hybond[™]-N⁺ membrane (GE Healthcare) and UV-cross linked as Northern membrane preparation. The probes were radioactively labeled with ++P³²-CTP (PerkinElmer) using a Ready-to-go bead (Amersham Biosciences), and cleaned on an Illustra[™] probe Quant[™] G50 Micro column (GE Healthcare) according to the manufacturer's instructions. The membrane was hybridized overnight with the probe, and signal was detected with a FLA-5000 PhosphorImager (Fujifilm).

mRNA Northern blotting and hybridization

mRNA Northern hybridization was similar as Southern blotting and hybridization except that total RNA and 1.2 % formaldehyde gel were used. The membrane was hybridized with DNA probes labeled with Ready-to-go DNA labeling Beads (-dCTP) (GE Healthcare).

Bisulphite PCR and sequencing

The method for bisulphite PCR was based on Wang et al. [63]. Initially each DNA sample (2–5 µg) was treated with bisulphite using MethylEasy[™] Xceed (Human Genetic Signature) according to the manufacturer's instruction. A hot-start PCR was performed using AmpliTaq Gold[®] 360 DNA polymerase (Applied Biosystems). Nested PCR product was checked and purified from the gel, followed by cloning into the pGEM-T easy vector (Promega) and transformed into DH10BT (Invitrogen). Plasmids were prepared from each individual colony and sequenced with

M13 primer through Micromon (Monash University, Australia).

Sequence analysis

The sequencing results were initially analyzed by aligned to *Drosophila* pUAST expression vector using VectorNTI (Invitrogen), and individual Cytosine methylation was confirmed by non-“C to T” conversion, methylation pattern were analyzed by CYMATE (GMI) [29].

Fluorescence microscopy

The EGFP- or RFP-expressing larvae were collected from vials using 20 % sucrose, a pre-check (~100) with a UV fluorescence microscope (Leica MZ11 fluorescence microscope) was performed to show the fluorescence signals were consistent in flies with the same genotype, and about 12 larvae were randomly selected for photography using the program QCapture. For the EGFP gene, a EGFP2 filter was used at the excitation wavelength of 480 nm (excitation filter) and emission wavelength of 510 nm (barrier filter), a TXR filter was used for the RFP gene images.

Transgenic flies accession: The RdRp transgenic flies were deposited in Ozdros, Australia (<http://www.ozdros.com>).

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Conflict of interest The authors declare no competing financial interests.

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