Phenotypic inheritance induced by hairpin RNA in *Drosophila*

Huaguang Li 1,2* and Yi Lu 1

1CAS-MPG Partner Institute for Computational Biology, Shanghai Institutes for Biological Sciences, Shanghai 200031, China
2Graduate School of the Chinese Academy of Sciences, Shanghai 200031, China

*Correspondence address. Tel: +86-21-54921139; Fax: +86-21-54921126; E-mail: hgli@sibs.ac.cn

Phenotypic inheritance induced by RNA has been documented in mouse and *Caenorhabditis elegans*. Here we report a similar inheritance in *Drosophila*. Mutant phenotypes of eye defects and antenna duplication generated from the crossing of one RNA interference (RNAi) transgenic line harboring one hairpin RNA transgene with a GAL4 driver line were inherited independently of the GAL4 driver. Hairpin RNA injection experiments demonstrated that the hairpin RNA could induce heritable mutant-like phenotypes on the eye and antenna. The penetrance of mutant phenotypes was reduced when the mutants were crossed to *ago1* and *piwi* mutants. Our data suggest that hairpin RNA can induce phenotypic inheritance in *Drosophila*.

**Keywords** phenotypic inheritance; *Drosophila*; hairpin RNA

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**Introduction**

Double-stranded RNA or hairpin RNA is able to trigger cognate mRNA degradation in a process referred to as RNA interference (RNAi) [1,2]. In addition to this post-transcriptional gene silencing, RNAi participates in other processes such as transcriptional gene silencing [3] and chromatin organization [4]. Moreover, RNAi could play a role in phenotypic inheritance as recent studies in *Caenorhabditis elegans* [5] and mouse [6] have revealed that RNAi can induce phenotypic inheritance.

In *Drosophila*, RNAi pathway has also been implicated in post-transcriptional gene silencing [2,7], transcriptional gene silencing [7], and heterochromatin formation [8]. Nonetheless, it remains unknown whether *Drosophila* RNAi machinery has a role on phenotypic inheritance.

In the current work, we present evidence that hairpin RNA can induce phenotypic inheritance in *Drosophila*.

This inheritance is repressed by *ago1* and *piwi* mutations. These findings may provide an insight into the role of hairpin RNA in *Drosophila* phenotypic inheritance.

**Materials and Methods**

**Fly strains and husbandry**

Flies were reared on corn meal/maltose/agar medium at 22°C. *Drosophila melanogaster Canton S* and w1118 are from our laboratory collection. The strains w*;P[w/+mW.hs]=GawB]I-76-D;Ubx9.22/TM6B;Th* (Bloomington No. 1854), w1118;P[w+[+mC]=UAS-lacZ.NZ]20b (Bloomington No. 3955), and cn1P[ry [+t.7.2]=PZ]AGO104845/CyO (Bloomington No. 11388) are from the Bloomington *Drosophila* stock center at Indiana University (Indiana, USA). w1118;piwi1//CyO is from Dr Giacomo Cavalli’s lab (Institute of Human Genetics, Montpellier, France).

**Plasmid construction and fly injection**

Three fragments dE2f1RA (596 bp), dE2f1RB (171 bp), and dE2f1RC (337 bp) from dE2f1 transcript 5’ UTR region were amplified using primer pairs Li32/Li33, Li35/Li36, and Frr60/Li38, respectively. They were cloned into the *Nhel* site of the pWIZ vector [9]. The insert orientation in the vector was identified by sequencing. To generate reverse repeats in the vector, three pairs of primers (Li33/Li34 for dE2f1RA; Li35/Li37 for dE2f1RB; and Li38/Li39 for dE2f1RC) were used to amplify three fragments. They were cloned into the position between the *Spel* site and the *EcoRI* site of pWIZ vector and thus formed RNAi constructs. Fly transformation was performed as described [10]. To construct plasmids for *in vitro* transcription, reverse repeats were cut out of the RNAi constructs and cloned into the *Xbal* and *EcoRI* sites of the vector pTZ19R (Fermentas, Lithuania). The green fluorescent protein (GFP) fragment
was amplified using two pairs of primers (Li28 and Li30; Li29 and Li30) and the pDra construct (our lab collection) as template. pDra contains a 330-bp GFP open reading frame. GFP reverse repeats were constructed in pWIZ vector. They were cut off and then cloned into the pTZ19R vector for in vitro transcription in the same way as three dE2f1 fragments. In vitro transcription, RNA purification, and injection were carried out according to the protocol described in Drosophila protocols [11]. All the primers are listed in Table 1.

PCR genotyping
Two pairs of primers (YJ20/Li38 and Li55/Li38) were used to check the presence of dE2f1RC reverse repeats in the RNAi construct. YJ20 and Li55 are from the pWIZ vector and are located outside of the reverse repeats. Li22 and Li70 were used to check for GAL4 allele. Frr63 and Frr64 were used to amplify the Rp49 allele. All the primers are listed in the Table 1.

X-gal staining for β-galactosidase
After the crossing of w*;P{f[w/+mW.hs]=GawB} I-76-D,Ubx9.22e1/TM6B,Tb+ with w1118;P{f[w/+mC]=UAS-lacZ.NZ} 20b, embryos were dechorionated manually and fixed in a heptane fix (25 mM Na2HPO4/NaH2PO4, pH 7.2, 12% glutaraldehyde, and 50% heptane). Larvae or flies were dissected in Ringer solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl2, 10 mM Tris–HCl, pH 7.2). Tissues were fixed in 0.7% glutaraldehyde (60 μl of 25% glutaraldehyde in 2 ml of 0.1 M Na2HPO4/NaH2PO4, pH 7.4) for 20 min at room temperature, washed three times with PBS and incubated in staining solution [10 mM Na2HPO4/NaH2PO4, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 3.1 mM potassium ferri cyanide, 3.1 mM potassium ferrocyanide, and 0.5 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)] at 37°C overnight. Stained tissues were washed with PBS to remove redundant color precipitate and checked under the microscope.

Reverse transcription–PCR
Total RNA from wing discs, eye discs, embryos, and adult flies were extracted by Trizol reagent (Invitrogen, Carlsbad, USA). DNA was digested by DNase I. One microgram RNA was used for reverse transcription reaction with random primers (Promega, Madison, USA). RNA was denatured at 70°C for 5 min and chilled on ice for 5 min. RNA was mixed with 5 μl of M-MLV 5×

<table>
<thead>
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<th>Primers</th>
<th>Origin</th>
<th>Localization</th>
</tr>
</thead>
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<tr>
<td>Li22: 5’TACATAAGAGAAGACGTCGC-3’</td>
<td>pGawB</td>
<td></td>
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<tr>
<td>Li28: 5’ ATACCTAGATGTGTTCCAAGAATGGTTTCCA-3’</td>
<td>X83959</td>
<td>418-399</td>
</tr>
<tr>
<td>Li29: 5’ ATACCTAGATGTGTTCCAAGAATGGTTTCCA-3’</td>
<td>X83959</td>
<td>418-399</td>
</tr>
<tr>
<td>Li30: 5’ CAAATAGTTGGAAGGTTGGAAGTTGAT-3’</td>
<td>X83959</td>
<td>89-108</td>
</tr>
<tr>
<td>Li32: 5’ GATTCTAGAGTACAGACTAGCTAGATTG-3’</td>
<td>AE014297</td>
<td>17458660-17458640</td>
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<td>AE014297</td>
<td>17458065-17458084</td>
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<td>AE014297</td>
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</tr>
<tr>
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<td>17486116-17486099</td>
</tr>
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<td>17485946-17485962</td>
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<td>17463815-17463796</td>
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<tr>
<td>Li55: 5’ ATCCTCGAGTTGTTGCGT-3’</td>
<td>pWIZ</td>
<td></td>
</tr>
<tr>
<td>Li70: 5’ GCTGCAACAGGCATAAACAATCTGC-3’</td>
<td>pGawB</td>
<td></td>
</tr>
<tr>
<td>Frn48: 5’ AATTAGACACCTAAGAGATCTTTCATC-3’</td>
<td>AE014297</td>
<td>17452547-17452572</td>
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<td>AE014297</td>
<td>17463479-17463497</td>
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<tr>
<td>Frn64: 5’ CGACACAGTTCAAGAAGCTCTG-3’</td>
<td>Y13939</td>
<td>865-843</td>
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<td>YJ20: 5’ GTAAATCAGATCGAATCAGAACTG-3’</td>
<td>pWIZ</td>
<td></td>
</tr>
</tbody>
</table>

The primer’s origin (gene, chromosome, or vector) is indicated by its accession number in NCBI or its name. The primer localization is listed according to NCBI. Italic letters indicate recognition sites of restriction enzymes.
reverse transcription buffer, 1 μl of M-MLV reverse transcriptase (200 U/μl, Promega), and 1.25 μl of dNTP (10 mM each) and sterile water was added to a final volume of 25 μl. Reverse transcription program was: 40°C, 10 min; 55°C, 50 min. Primers Li38 and Frr48 were used for PCR to detect dE2f1RC transcript. Rp49 was used as a control for RNA amount. All the primers are listed in Table 1. PCR was carried out by denaturation at 94°C for 2 min and followed by amplification with 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Western blot analysis
Fly embryos were ground in lysis buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol) and insoluble material was removed by brief centrifugation. Embryo lysates were diluted with SDS–PAGE loading buffer, boiled for 5 min, loaded onto 10% SDS–PAGE, and then transferred to PVDF membranes (ROTH, Karlsruhe, Germany). The membranes were blocked with 15% nonfat milk and then incubated with primary antibodies and corresponding secondary antibodies. Detection was performed with ECL advance western blotting detection kit (Amersham, Buckinghamshire, UK). Guinea pig anti-dE2f1 antibody, a kind gift from Dr Terry L. Orr-Weaver [12], was diluted at 1:4000. Mouse anti-a-tubulin (T5168; Sigma, St. Louis, USA) was diluted at 1:8000. Goat anti-guinea pig conjugated with HRP (106-036-003; Jackson ImmunoResearch, West Grove, USA) and goat anti-mouse conjugated with HRP (115-036-006; Jackson ImmunoResearch) were diluted at 1:100,000.

Results
Generation of mutant flies
Three RNAi transformation plasmids harboring reverse repeats matching dE2f1 5' UTR sequence were constructed and were designated as pWIZ-dE2f1RA, pWIZ-dE2f1RB, and pWIZ-dE2f1RC (see “Materials and Methods”). These constructs contain upstream activating sequence (UAS) promoter, which allows the controlled expression of the hairpin RNA if combined with a GAL4 driver. We screened 11 RNAi transgenic lines (3 lines for dE2f1RA, 5 lines for dE2f1RB and 3 lines for dE2f1RC) and found all of them were phenotypically normal. One of pWIZ-dE2f1RC lines with an insertion site at the third chromosome was used in further analyses as we observed intriguing phenotypes when crossing this line with a GAL4 driver strain w*;P[w/+mW.hs]=GawB]I-76-D,UbxB^{w.22)e}/TM6B,Tb+. The homozygous flies of this GAL4 line are lethal. This cross yields heterozygous flies for the transgene and the GAL4 driver with the genetic constitution w;P[w/+mW.hs]=GawB]I-76-D,UbxB^{w.22)e}/TM6B,Tb+ (designated as pWIZ-dE2f1RC/GAL4I-76-D for short). We noted that a small fraction (about 1%) of these heterozygous flies (pWIZ-dE2f1RC/GAL4I-76-D) displayed mutant phenotypes including eye defects and antenna duplication (always at the same side as the deformed eye) [Figs. 1(B,C) and 2(A)] compared with wild-type flies [Fig. 1(A)]. However, we did not observe abnormal phenotypes for pWIZ-dE2f1RA and pWIZ-dE2f1RB transgenic lines when crossed to the same GAL4 or other GAL4 strains. Our expression analysis showed that this GAL4 driver directed LacZ reporter gene expression in the embryo and eye disc but not in the ovary (Fig. 3).

The inheritance of the mutant traits
We then inbred pWIZ-dE2f1RC/GAL4I-76-D flies showing eye defects, and observed that a portion of the offspring displayed lethality at embryonic and larval stage, as well as eye defects and antenna duplication identical to their parents. Intriguingly, during further successive rounds of inbreeding, the penetrance of the mutant phenotypes among the offspring increased.

Figure 1 Reduced eye and antenna duplication of mutants (A) The eye of wild-type fly (Canton S). (B) Reduced eye of pWIZ-dE2f1RC/GAL4I-76-D. (C) Antenna duplication (arrow) in pWIZ-dE2f1RC/GAL4I-76-D. (D) Reduced eye and antenna duplication (arrow) in the affected heterozygous pWIZ-dE2f1RC fly derived from the cross of pWIZ-dE2f1RC/GAL4I-76-D with w^{1116}. (E) Reduced eye and antenna duplication (arrow) induced by dE2f1RC hairpin RNA injection.
gradually from the initial 1% to 42% in the sixth generation [Fig. 2(A)]. Few mutants with wing defects were also recovered. The wing trait, however, lasted for only one generation. The initial transgenic flies pWIZ-dE2f1RC do not show the mutant phenotypes, as we have raised a large amount of these transgenic flies (more than 6000) and all of them appeared phenotypically normal (Fig. 4). This reveals that the mutant phenotypes are not caused by the insertion of pWIZ-dE2f1RC construct into fly genome.

We then outcrossed single female or male in the sixth generation showing the eye phenotype to the control line (w1118). By analysis of the eye color (Fig. 4) and PCR genotyping of the offspring, we can retrospectively trace the genotypes of the mutant parents. We performed such outcrosses, and found that the genotype of the mutant parent was pWIZ-dE2f1RC/GAL4I-76-D. This indicates that during the six round of inbreeding most, if not all of the mutant flies, are of the pWIZ-dE2f1RC/GAL4I-76-D genotype. When analyzing the F1 progeny of the outcross, we found that a fraction of those harboring the pWIZ-dE2f1RC construct only (without the GAL4 driver) also displayed the mutant eye trait [Fig. 1(D)], albeit at a lower penetrance (around 3%) [Fig. 2(B)]. They displayed yellow eye color like heterozygous pWIZ-dE2f1RC (Fig. 4), and their genetic constitution was confirmed by PCR genotyping [Fig. 2(C)]. The F1 progeny carrying the GAL4 driver only never displayed the eye trait (Fig. 4). We then inbred the heterozygous pWIZ-dE2f1RC flies showing the mutant eye phenotype. The penetrance of the mutant phenotypes increased from 3% to 15% over three generations of inbreeding [Fig. 2(B)]. Intriguingly, we did not find homozygous pWIZ-dE2f1RC flies which should display the red eye color (Fig. 4). This implies that pWIZ-dE2f1RC homozygotes derived from such inbreedings are lethal.

We noted that when pWIZ-dE2f1RC/GAL4I-76-D mutant flies were outcrossed to w1118, the F1 progeny that showed the mutant eye phenotype all carried the pWIZ-dE2f1RC construct [Fig. 1(D)]. Consistently, in the two subsequent rounds of inbreeding using these heterozygous pWIZ-dE2f1RC mutant flies, all the offspring that showed the mutant eye trait harbored the pWIZ-dE2f1RC construct. All of these imply that the mutant traits and lethality are closely associated with the pWIZ-dE2f1RC construct. Since initial pWIZ-dE2f1RC transgenic flies do not show these mutant phenotypes (Fig. 4), we reason these phenotypes were induced by the hairpin RNA driven by GAL4.
Mutant phenotypes might be independent on dE2f1
To examine whether these mutant phenotypes were attributed to the knock-down of dE2f1RC transcript, we carried out RT–PCR to compare dE2f1RC level in mutants and w1118. As shown in Fig. 5(A), there was no significant difference in embryos, eye discs, and adults between mutants and w1118. Western blot analysis showed that there was no detectable difference between mutant and w1118 embryos [Fig. 5(B)]. These imply the mutant phenotypes and their inheritance were not arising from dE2f1RC down-regulation.

Injection of the hairpin RNA induced the phenotypic inheritance
To test if similar heritable mutant traits can be induced by the same hairpin RNA, we injected purified dE2f1RC hairpin RNA from in vitro transcription into the posterior of w1118 embryos of an age less than 0.5 h after egg laying. The flies developing from injected embryos (“F0”) were all phenotypically normal while morphological abnormalities appeared in the F1 generation from single crosses of each F0 fly to w1118. Totally, 5 out of 113 F0 flies produced mutant offspring (“F1”) at the penetrance of about 1% (Table 2). Each F1 mutant fly was crossed to its phenotypically normal siblings. The mutant phenotypes were also found in the next generation (“F2”) in three out of five strains. Control embryos injected with GFP (105 F0 flies), dE2f1RA (102 F0 flies), or dE2f1RB (120 F0 flies) hairpin RNA did not produce such mutant phenotypes in the subsequent generation. Therefore, dE2f1RC hairpin RNA serves as the trigger of the observed mutant eye and antenna phenotypes.

piwi and ago1 mutations repressed the phenotypic inheritance
To investigate whether the inheritance of phenotypic traits required the RNAi machinery, we chose heterozygous pWIZ-dE2f1RC mutants with deformed eyes to cross to the piwi mutant line (w1118;piwi1/CyO)

Figure 5 RT–PCR of dE2f1RC, western blot analysis of dE2f1 protein and the effect of the piwi mutation on the penetrance of mutants (A) RT–PCR of dE2f1RC on embryos, adults, and eye discs from w1118 (left lane of each group) and affected pWIZ-dE2f1RC/ GAL4I-76-D (right lane of each group). Rp49 served as a control for RNA amount. (B) Western blot analysis of dE2f1 protein in w1118 embryos (left lane) and pWIZ-dE2f1RC/GAL4I-76-D mutant embryos (right lane). Alpha tubulin acts as the control of protein loading amount. (C) The comparison of mutant penetrance (including both lethality and eye defects) from two crossings which were indicated underneath each plot of the histogram. The asterisk indicated the significant difference (P < 0.01). Column 1, w1118; pWIZ-dE2f1RC/++; column 2, w1118;piwi1/+; pWIZ-dE2f1RC/++.

| Table 2 The inheritance of phenotypes induced by injection of dE2f1RC hairpin RNA |
|----------------------------------|----------------------------------|-------------------------------|-----------------|-----------------|
| F0 code (gender) | F1 mutant (gender) | F1 phenotype | F2 mutant (gender) | F2 phenotype |
| BG (♂) | 1/136 (♀) | Reduced eye and antenna duplication | 1/76 (♂) | Reduced eye |
| CO (♂) | 1/88 (♂) | Wing nicking | 1/130 (♂) | Reduced eye |
| CW (♂) | 1/123 (♂) | Wing nicking | 0 | |
| DN (♀) | 1/116 (♂) | Small wings | 1/123 (♀) | Reduced eye |
| DS (♀) | 1/65 (♂) | Wing nicking | 0 | |

The injected flies were coded by double letters according to the sequence of hatching. The numbers of mutants in comparison with total adults after crosses of the injected flies to w1118 flies are indicated. F2 flies were derived from crosses of F1 mutant flies with non-mutant siblings.
Table 3 The amount of offspring from the crossing of w1118;piwi1/CyO (♀) with heterozygous pWIZ-dE2f1RC (♂)

<table>
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<tr>
<th>Crossing number</th>
<th>w1118;CyO/+</th>
<th>w1118;CyO/+;pWIZ-dE2f1RC/+</th>
<th>w1118;piwi1/+</th>
<th>w1118;piwi1/+;pWIZ-dE2f1RC/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>178</td>
<td>139</td>
<td>177</td>
<td>176 (5)</td>
</tr>
<tr>
<td>B</td>
<td>205</td>
<td>163</td>
<td>201</td>
<td>192 (7)</td>
</tr>
<tr>
<td>C</td>
<td>155</td>
<td>119</td>
<td>156</td>
<td>151 (5)</td>
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</tbody>
</table>

The number of each genotype of fly from three individual crossings presented as A, B, and C were counted and shown. The number in the parenthesis indicates the mutant flies with eye defects.

Table 4 The amount of offspring from the crossing of w1118 (♀) with heterozygous pWIZ-dE2f1RC (♂)

<table>
<thead>
<tr>
<th>Crossing number</th>
<th>w1118</th>
<th>w1118;pWIZ-dE2f1RC/+</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>482</td>
<td>441 (23)</td>
</tr>
<tr>
<td>B</td>
<td>500</td>
<td>458 (28)</td>
</tr>
<tr>
<td>C</td>
<td>446</td>
<td>402 (22)</td>
</tr>
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</table>

The number of each genotype of fly from three individual crossings presented as A, B, and C were counted and shown in the table. The number in the parenthesis indicates the mutant flies with eye defects.

Table 5 Crossings of male pWIZ-dE2f1RC/GAL4I-76-D with female w1118, piwi and ago1 mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>pWIZ-dE2f1RC/GAL4I-76-D (♂)</th>
<th>Progeny affected</th>
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<tr>
<td></td>
<td></td>
<td>One eye reduced</td>
</tr>
<tr>
<td>w1118 (♀)</td>
<td>17/754</td>
<td>2/754</td>
</tr>
<tr>
<td>piwi1 (♀)</td>
<td>4/785</td>
<td>1/785</td>
</tr>
<tr>
<td>ago10455 (♀)</td>
<td>1/850</td>
<td>0</td>
</tr>
</tbody>
</table>

The fraction indicates mutants out of total adults after crossings.

Discussion

In this study, we present an intriguing phenotypic inheritance in Drosophila that the mutant phenotypes induced by the hairpin RNA transgene driven by GAL4 can be transmitted to the next generations independently of the GAL4 driver [Figs. 1(D) and 2(B)]. We consider possibilities that contribute to this phenotypic inheritance. One possibility is that the hairpin RNA induces these mutant phenotypes and their inheritance, as injection of the hairpin RNA can induce heritable mutant-like phenotypes on the eye and antenna [Fig. 1(E) and Table 2]. Alternatively, the inheritance of these mutant phenotypes could require the involvement of PIWI and AGO1 [Fig. 5(C), Tables 3 and 5], both of which participate in epigenetic regulation [7,8,13,14]. Therefore, epigenetic regulation might be involved in the inheritance. The mechanism of RNAi-induced phenotypic inheritance has been primarily investigated in Caenorhabditis elegans [6], and it is found that mutations for chromatin remodeling genes affect the phenotypic inheritance, suggesting
chromatin remodeling is responsible for the inheritance. Nonetheless, how RNA induces chromatin remodeling remains to be further determined in detail.

Given that there was no significant difference of dE2f1 between mutants and w1118 [Fig. 5(A,B)], the inheritance was thus not related to the down-regulation of dE2f1. Indeed, the phenotype of antenna duplication in pWIZ-dE2f1RC/GAL4I-76-D mutants has not been documented in previously published dE2f1 mutants [15], even in the dE2f1RNAi mutants [16]. These mutant phenotypes might be due to RNAi off-target effect.

In summary, we have provided evidences that hairpin RNA can induce phenotypic inheritance in Drosophila. This phenomenon is reminiscent of studies in C. elegans and mouse, which have also shown that RNA can induce the inheritance of certain phenotypes [5,6]. All of these could suggest RNA play an important role on phenotypic inheritance.

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References


