An intrinsic tumour eviction mechanism in *Drosophila* mediated by steroid hormone signalling

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Polycomb group proteins are epigenetic regulators maintaining transcriptional memory during cellular proliferation. In *Drosophila* larvae, malfunction of Polyhomeotic (Ph), a member of the PRC1 silencing complex, results in neoplastic growth. Here, we report an intrinsic tumour suppression mechanism mediated by the steroid hormone ecdysone during metamorphosis. Ecdysone alters neoplastic growth into a nontumorigenic state of the mutant *ph* cells which then become eliminated during adult stage. We demonstrate that ecdysone exerts this function by inducing a heterochronic network encompassing the activation of the microRNA *let-7*, which suppresses its target gene *chronologically inappropriate morphogenesis*. This pathway can also promote remission of brain tumours formed in *brain tumour* mutants, revealing a restraining of neoplastic growth in different tumour types. Given the conserved role of *let-7*, the identification and molecular characterization of this innate tumour eviction mechanism in flies might provide important clues towards the exploitation of related pathways for human tumour therapy.

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Polycomb group (PcG) proteins are evolutionarily conserved chromatin regulators modulating histone modifications and suppressing target gene expression, required for the maintenance of cellular memory. PcG proteins can bind to particular genomic regions, where they mediate specific histone modifications and chromatin compaction, thereby suppressing the expression of the target genes in these loci. Dysregulation of PcG genes is associated with various human cancers, but the mechanisms are incompletely understood.

PcG proteins form two major Polycomb repressive complexes, PRC1 and PRC2, to silence the expression of target genes. Previous studies have shown that the PRC1 components can act as tumour suppressors in *Drosophila*. In the developing eye-antennal imaginal discs, for instance, cells homozygous mutant for *ph* overgrow and give rise to neoplastic tumours. These tumours can be transplanted and continue to grow in wild-type adult flies.

Here, we carry out studies to investigate the mechanisms underlying tumour formation and growth in *ph* mutants. Unexpectedly, we observe that the tumorigenic *ph* mutant cells are transformed into non-tumorigenic cells after metamorphosis, and eventually evicted in adult flies. We show that edcsyne signalling is responsible for the transformation of tumorigenicity. By performing transcriptome analyses we identify miRNA *let-7* as a key target of the edcsyne response in this process. We further demonstrate that mis-expression of chronologically inappropriate morphogenesis (chimmo), a direct target of both *Ph* and *let-7*, is required for tumour growth. Furthermore, we show that the *let-7* cascade could also suppress the overgrowth of brain tumours in *brain tumour* (*brat*) mutant flies. Our analyses reveal an intrinsic mechanism that is able to reprogram tumorigenic cells and suppress their malignant growth in adult *Drosophila*.

**Results**

**Conversion of tumorigenic *ph* mutant cells during metamorphosis.**

The *Drosophila* genome encodes two *ph* genes, *ph proximal* (*ph-p*) and *ph distal* (*ph-d*). *ph*505 is a loss of function allele of both genes. Homozygous *ph*505 clones, generated genetically by MARCM (mosaic analysis with a repressible cell marker) and marked by GFP, overgrow and give rise to large tumours in the larval eye-antennal discs at the wandering third instar (Fig. 1a). The morphology of these clones is in sharp contrast to wild-type GFP-expressing clones (Fig. 1b). After transplanting *ph*505 eye disc tumours into wild-type adult hosts (Fig. 1c, arrow), *ph*505 cells continued to proliferate, resulting in the formation of neoplastic tumours (Fig. 1c, d). This indicates that larval *ph*505 cells are tumorigenic and is also consistent with previously reported results. These tumours can recapitulate proliferation after serial retransplantation into new hosts, but they did not give rise to metastatic tumours in other parts of the body (Fig. 1d).

In the head, these marked *ph*505 cells formed grape-like, single-layered epithelial structures (Fig. 1f; Supplementary Fig. 1a). In contrast to *brat* (Supplementary Fig. 1b), *let-7* expression levels of a group of genes was upregulated in the *ph*505 samples, groups of genes with similar expression showed a significantly differentially expressed genes. Applying k-means clustering on the gene expression profiles across wild-type, tumour and metamorphed samples, groups of genes with similar profiles can be found (Fig. 2f; Supplementary Fig. 3). Gene ontology (GO) term analysis of the resulting five groups revealed that transcription regulators and genes required for metabolic processes were upregulated in the tumours, but genes involved in neuronal differentiation were downregulated (Fig. 2f; Supplementary Fig. 3). Cell-cycle-related genes were downregulated in *ph*505 cells in addition to the differentiation-related genes (Fig. 2f; Supplementary Fig. 3), when compared to a wild-type transplanted disc. Lower levels of neuronal marker Elav can corroborate this (Fig. 1f). We next focused on genes that are known to respond to edcsyne (Supplementary Fig. 4). Expression levels of a group of genes was upregulated in the *ph*505 cells but decreased in the transplanted tumours (Fig. 2g). Among these is *let-7-C*, which is a polycistronic locus encoding three miRNAs including *let-7*. As the mammalian homologues of *let-7* are underexpressed in various cancer types, we decided to further investigate the potential role of *let-7* in altering *ph*505 tumorigenicity.

Edcsyne controls the transformation of *ph*505 tumour cells. 20-hydroxyedcsyne (edcsyne) is the key molting steroid hormone controlling metamorphosis of flies. Edcsyne is produced as a series of brief low-level pulses during embryonic and early larval stages. Near the end of third larval instar, a mid-level pulse of edcsyne triggers pupariation. The expression of edcsyne increases and reaches the peak level around 48 h after pupa formation. Afterward, edcsyne expression gradually decreases to a low basal level, which is then maintained during adulthood. To assess the role of edcsyne in altering the oncogenic potential of *ph*505 cells, we ectopically expressed a dominant-negative form of the edcsyne receptor (EcR) in *ph*505 mutant cells (*ph*505; *UAS-EcRDN*). In contrast to metamorphed *ph*505 cells (Fig. 1e, g), *ph*505; *UAS-EcRDN* cells continued to grow in the adults and resulted in the accumulation of large tumours throughout the body (Fig. 2a). In the head, the tumour mass (Fig. 2b, arrow) could reach a similar size as the adult brain (Fig. 2c, arrowhead). As a result, these flies showed a significantly reduced lifespan (Fig. 2d) and *ph*505; *UAS-EcRDN* cells were able to give rise to neoplastic tumours after transplantation (Fig. 2e). A knockdown of EcR co-receptor Ultraspiracle (Usp) using transgenic RNAi (*ph*505; *UAS-RNAi-usp*) showed similar results substantiating edcsyne involvement (Supplementary Fig. 2). Hence, the manifestation of the edcsyne pulse at metamorphosis appears directly responsible for suppressing the tumorigenic character of larval *ph*505 cells. Conversely, a cell-autonomous block of edcsyne signalling retains the tumorigenicity of *ph*505 cells in the intact adult flies.

To better understand how edcsyne exerts its tumour-suppressor function in *ph*505 cells, we performed transcriptome analyses by miRNA sequencing. RNA from metamorphed *ph*505 cells in adults, 4, 8, 14 weeks old tumours in adult hosts after weekly re-transplantations, and wild-type transplanted discs as control were extracted and sequenced. In total, we identified 2015 significantly differentially expressed genes. Applying k-means clustering on the gene expression profiles across wild-type, tumour and metamorphed samples, groups of genes with similar profiles can be found (Fig. 2f; Supplementary Fig. 3). Gene ontology (GO) term analysis of the resulting five groups revealed that transcription regulators and genes required for metabolic processes were upregulated in the tumours, but genes involved in neuronal differentiation were downregulated (Fig. 2f; Supplementary Fig. 3). Cell-cycle-related genes were downregulated in metamorphed *ph*505 cells in addition to the differentiation-related genes (Fig. 2f; Supplementary Fig. 3), when compared to a wild-type transplanted disc. Lower levels of neuronal marker Elav can corroborate this (Fig. 1f). We next focused on genes that are known to respond to edcsyne (Supplementary Fig. 4). Expression levels of a group of genes was upregulated in the metamorphed *ph*505 cells but decreased in the transplanted tumours (Fig. 2g). Among these is *let-7-C*, which is a polycistronic locus encoding three miRNAs including *let-7*. As the mammalian homologues of *let-7* are underexpressed in various cancer types, we decided to further investigate the potential role of *let-7* in altering *ph*505 tumorigenicity.
Ecdysone-induced let-7 suppresses the growth of ph505 tumour. In Drosophila, let-7 expression is induced by ecdysone at the start of pupariation, and the expression of let-7 correlates with the dynamic changes of the ecdysone level during metamorphosis. A first step was to confirm mature let-7 levels in the various samples using the Taqman quantitative PCR assay. As previously reported, the expression of mature let-7 was low at the wandering third larval instar but increased significantly to a high level at 48 h after pupa formation (Fig. 3a). Indeed, let-7 expression was also elevated in the metamorphed ph505 cells, but not in the ph505 transplanted tumours (Fig. 3a). Moreover, let-7 level in ph505; UAS-let-7DN cells collected from adults remained low (Fig. 3a), indicating let-7 was not induced when ecdysone signalling was blocked.

To further characterize the role of let-7, we reduced the activity of endogenous let-7 in ph505 cells using a transgenic miRNA sponge (ph505; UAS-let-7-SP) (Fig. 3a). We observed that ph505; UAS-let-7-SP cells maintained the tumorigenic growth in the adults (Fig. 3b). After dissection and transplantation, ph505; UAS-let-7-SP cells collected from adults retained tumour formation in the hosts (Fig. 3c), indicating that let-7 is necessary to mediate the suppression of tumorigenic growth of ph505 cells. Next, we overexpressed let-7 in ph505 cells. Overexpression of either the entire let-7-C (ph505; UAS-let-7-C) or let-7 alone (ph505; UAS-let-7) could strongly suppress tumour growth in the eye-antennal discs (Supplementary Fig. 5a, b). The average tumour volume was reduced to 21% in ph505; UAS-let-7 larvae (Fig. 3d). The effect of let-7 overexpression appeared to be independent of apoptosis, as overexpression of let-7 did not lead to elevated cell death in the eye-antennal discs (Supplementary Fig. 5c, d). Moreover, ph505; UAS-let-7 cells did not give rise to tumours after transplantation into adult hosts (n > 50).

let-7-C also encodes another two miRNAs, miR-100 and miR-125. We next tested if these two miRNAs might also play a role in transforming the tumorigenic ph505 cells. First, unlike let-7-SP, ph505; UAS-miR-100-SP cells stopped growing and disappeared in the adult flies (Supplementary Fig. 6a). Immunostaining showed that the apoptosis cell marker cDCP-1 was expressed in the ph505; UAS-miR-100-SP cells (Supplementary Fig. 6b), suggesting that the ph505; UAS-miR-100-SP cells can still be transformed into nontumorigenic cells and undergo apoptosis. For miR-125 we observed that the ph505; UAS-miR-125-SP cells stopped growing in the adult flies, but were still visible in these flies 2 weeks after eclosion (Supplementary Fig. 6c, d), unlike the metamorphed ph505 cells that eventually are eliminated. Immunostaining showed that ph505; UAS-miR-125-SP cells did not express the apoptosis cell marker cDCP-1 (Supplementary Fig. 6d), nor did they express the mitosis marker PH3 (Supplementary Fig. 6e). Furthermore, when we transplanted adult ph505; UAS-miR-125-SP cells into host flies (Supplementary Fig. 6f), they did not give rise to neoplastic tumours, indicating the ph505; UAS-miR-125-SP cells are not tumorigenic anymore. However, even at 4 weeks after transplantation, the ph505; UAS-miR-125-SP cells were still present (Supplementary Fig. 6g). These results show that ph505; UAS-miR-125-SP cells neither proliferate nor undergo apoptotic cell death.
Because miRNA sponges inhibit miRNA/mRNA interaction by sequestration, the function of targeted miRNAs might not be disrupted completely. Therefore, we tested if the miRNA sponges used in our experiments were functional. In flies, the zinc finger transcription factor chinnio is a known target of let-7 and miR-125.18 Using quantitative PCR, we determined the expression of chinnio, and some computationally predicted targets of miR-125 and miR-100, as there are no experimentally validated miRNA/mRNA interactions for these two miRNAs. The expression of chinnio indeed increased in ph505; UAS-let-7-SP cells (Supplementary Fig. 6h), but did not in ph505; UAS-miR-125-SP cells (Supplementary Fig. 6i). However, the expression of the two computationally predicted targets, Zasp52 and RecQ4, increased in ph505; UAS-miR-125-SP cells (Supplementary Fig. 6i). On the other hand, the expression of predicted targets of miR-100 did not increase in ph505; UAS-miR-100-SP cells. These results indicate that the let-7 sponge and miR-125 sponge appear to be functional in ph505 cells, whereas the miR-100 sponge may not.

To further evaluate the role of miR-125 and miR-100, we carried out overexpression experiments. First, overexpression of miR-125 (ph505; UAS-miR-125) could partially reduce the tumour volume in the eye-antennal discs (Supplementary Fig. 5a). However, when we transplanted the ph505; UAS-miR-125 cells into wild-type hosts, we could still observe the formation of tumours in 52% of the hosts (n = 31), indicating ph505; UAS-miR-125 cells are still tumorigenic. Similarly, overexpression of miR-100 (ph505; UAS-miR-100) only slightly reduced the tumour volume in the eye-antennal discs (Supplementary Fig. 5a) and did not alter the tumorigenicity.

Taken together, these analyses show that let-7 is the key regulator in the let-7-C. The miRNA appears indispensable and needed for suppressing the tumorigenic character of ph505 cells. While our tests do not identify a significant involvement of miR-100, miR-125 does appear to have a certain role in the elimination of the transformed cells. Previous work has revealed that there are cross-regulatory relationships among the three miRNAs, which may explain the complicated phenotype of the ph505; UAS-miR-125-SP cells described above.

Ph and let-7 target chinnio is required for ph505 tumour growth. To further elucidate the mechanism of how let-7 controls the tumorigenesis of ph505 cells, we searched for genes that are bound by Ph in their promoter region and are let-7 targets. Among others, we identified the gene chinnio (Fig. 3e). The expression of chinnio was significantly increased in the ph505 tumours but decreased in the metamorphed ph505 cells (Fig. 2f, black curve). Immunostaining showed that Chinnio was ubiquitously expressed in the transplanted tumours (Fig. 3f) but was not detectable in metamorphed ph505 cells (Fig. 3g). In the eye discs,
Chinmo was expressed in the ph505 mutant clones, as well as the neighbouring heterozygous cells (Supplementary Fig. 7a). However, at 48 h after pupa formation, when edcsyne level was at the peak and let-7 was highly expressed (Fig. 3a), the expression of Chinmo became undetectable (Supplementary Fig. 7b). Another known let-7 target is the abrupt gene22, which has been shown to promote the development of some types of tumours in the eye-antennal discs23. However, by immunostaining we did not detect the expression of Arupt in the transplanted ph505 tumours, in the eye-antennal discs, as well as in the metamorphed ph505 cells. This indicates that abrupt does not play a significant role in regulating the tumorigenesis of ph505 cells.

To test the function of Chinmo, we overexpressed the protein in the tumour cells (ph505; UAS-chinmo) and observed that these cells continued to grow in the adult flies (Fig. 3h) and gave rise to tumours after transplantation (Fig. 3i). Furthermore, when chinmo was knocked down (ph505; UAS-RNAi-chinmo), the average volume of the ph505; UAS-RNAi-chinmo clones in the eye-antennal discs was reduced to 60% (Fig. 3d, Supplementary Fig. 5e). After transplantation of ph505; UAS-RNAi-chinmo eye discs, tumours formed in only 16% of the hosts (n = 67), significantly less frequent than after transplantation of larval ph505 eye discs (79%, n = 74). These results show that chinmo is an important downstream effector of let-7 in regulating the proliferation of ph505-driven tumours.

Ecdysone-induced let-7 suppresses the growth of brain tumours. Because let-7 expression is induced by edcsyne in a variety of tissues during metamorphosis16, we next tested if let-7-dependent tumour suppression also acts in a different tissue. In Drosophila, brat gene mutations lead to the formation of malignant brain tumours24. To generate such tumours, we used wor-Gal4 ase-Gal80 to knockdown brat (UAS-RNAi-brat) in the type II neuroblasts25. Compared to the wild-type adult brain (Fig. 4a), brat RNAi resulted in the growth of large brain tumours in all
larvae and hatching adult flies (Fig. 4b). However, when let-7 was overexpressed in brat-negative cells (UAS-RNAi-brat; UAS-let-7), a strong suppression of tumour growth in all adult brains was observed (Fig. 4c). Chinmo has recently been shown to play a key role in sustaining the tumour growth in all adult brains (Fig. 4d, arrows), but not in the tumour cells in UAS-RNAi-brat brains (e, arrowheads).

The number of tumour cells is significantly reduced. Scale bar is 50 μm. i Model for the intrinsic tumour eviction mechanism mediated by ecdysone-induced miRNA let-7 in ph505 tumours. In the drawing, ecdysone refers to 20-HE titres during prepupal and pupal stages11,12; chinmo and let-7 refer to the mRNA expression in the eye-antenna discs. Genotypes: a, d w UAS-dicer2; wor-Gal4 ase-Gal80; UAS-mCD8::GFP; b, e, f w UAS-dicer2; wor-Gal4 ase-Gal80/UAS-RNAi-brat; UAS-mCD8::GFP/i; c w UAS-dicer2; wor-Gal4 ase-Gal80/UAS-RNAi-brat; UAS-mCD8::GFP/UAS-let-7; g, h w UAS-dicer2; wor-Gal4 ase-Gal80/UAS-RNAi-brat; UAS-mCD8::GFP/UAS-EcRB1.

Discussion

Deregulation of PcG gene expression has been associated with various types of human cancer3,28. For example, loss of expression of the human ph homologue has been linked to the formation of osteosarcoma29,30. As the molecular mechanisms of PcG proteins in human cancers are largely unknown, understanding the tumour-suppressor function of PcG genes in Drosophila therefore could provide insights in human cancer biology. During
the past decades imaginal discs have been used as a powerful paradigm to investigate mechanisms underlying the formation and progression of several types of tumour, including Ras-, PcG-, or Hippo pathway-induced tumours. In addition, it is worth noting that the let-7 consensus sequence is identical from C. elegans to humans, suggesting that let-7 may control functionally conserved targets in regulating proliferation and differentiation during development. 

In various types of human cancer, downregulation of one or more let-7 members has been observed. Moreover, induced expression of let-7 in cancer cell lines can suppress cell proliferation and tumour growth. In the human genome, the let-7 family consists of more than ten members. However, the transcriptional regulation, spatial and temporal expression, and their tissue-specific and/or redundant functions of the let-7 family in human are far more complicated and still remain elusive.

Here, we identified an intrinsic mechanism reprogramming tumorigenic to non-tumorigenic cells of at least two different tumour types, by marking the cells for destruction in adult Drosophila. We found that the steroid hormone-induced miRNA let-7, acting as a key regulator of this mechanism, is interestingly conserved in let-7 and its target genes, including chinnio, have been shown to act as heterochronic genes that regulate developmental transitions. Other findings from our lab indicate that ph505 tumour cells are reprogrammed from the original larval imaginal disc identity to an early embryonic state. By artificially overexpressing a differentiation factor, these cancer cells can be induced to lose their neoplastic state, however. It appears as if these tumour cells are trapped in an immature condition, unable to differentiate. Pulses of ecdysone are a major timer of developmental transitions in cells, we attempted to generate let-7 knockdown efficiency. To test the roles of let-7 in tumour suppressive mechanisms by inducing let-7-controlled heterochronic gene networks to enforce cellular differentiation in epigenetically derailed tumours (Fig. 4i). Indeed, differentiation therapy is considered a promising approach for curing human cancers. However, the strategy has been applied only in limited cases. As such, our identification of an innate tumour eviction mechanism in flies based on these principles may provide new ideas how such cancer treatments could be further improved in human patients.

Methods

**Fly genetics.** Fly strains were maintained on standard medium. All genetic experiments were performed at 25°C, except that the brat-RNAi experiments were completed at 29°C to increase the knockdown efficiency. To test the roles of candidate genes (let-7, chinnio, etc.) in the tumorigenic growth of ph505 mutant cells, we attempted to generate fly strains carrying double mutations including the candidate genes in combination with ph505. However, we were unable to establish double mutant strains of ph505 and another gene mutant. Therefore, in this study, we used transgenic fly strains expressing either RNAi or miRNA sponge to reduce the activities of the target genes.

To generate homozygous ph505 MARCM clones, virgin females of ph505 FRT19A/FM7 act-GFP were crossed with tub-Gal80 FRT19A; ey-GAL4 UAS-GFP males. For the control MARCM clones, virgin females of w FRT19A were crossed with tub-Gal80 FRT19A; ey-GAL4 act-STOP;Gal4 UAS-GFP males. Because the ey-GAL4 is expressed not only in the eye-antennal discs, but also in all leg discs and the genital disc, clones were observed in these tissues as well. GFP-labelled ph505 cells can be seen in the heads, larval thorax, and abdomen in the adult flies. Other MARCM clones using different transgenic strains in combination with ph505 or FRT19A were generated in the same way by crossing corresponding virgin females with tub-Gal80 FRT19A; ey-GAL4 act-STOP;Gal4 UAS-GFP males.

**Fly strains.** The following fly strains were used in this study: (1) Orec-R, (2) w1118, (3) ph505 FRT19A/FM7 act-GFP, (4) y w FRT19A, (5) tub-Gal80 FRT19A; ey-GAL4 act-STOP;Gal4 UAS-GFP, (6) w; UAS-EcR-ΔN (BL-3541), (7) w; UAS-EcR-ΔN (BL-9450), (8) y; UAS-RNAi-sup (BL-27258), (9) w; UAS-let-7-C (N. Sokol), (10) y w; UAS-let-7 (L. Johnston), (11) w; UAS-mir-100 (C. Gendron), (12) y w; UAS-let-125 (L. Johnston), (13) w; UAS-let-7-SP, UAS-let-7-SP/TM6B (BL-61365), (14) w; UAS-mir-100-SP/YC3O, UAS-mir-100-SP (BL-61391), (15) w; UAS-mir-125-SP/TM6B (BL-125-SP), (16) w; UAS-mir-125-SP (BL-65234), (17) y w; UAS-RNAi-chimno/TM3, SB (BL-33638), (18) y w; UAS-mCherry:Atg8A; Dr/TM3 Ser (BL-37750), (19) w; UAS-dicer2; w; Gal4-aie-Gai86; UAS-mCD8:: GFP, (20) w; UAS-RNAi-brat (VDRC-105054), (21) w; UAS-EcR-B1 (BL-6469).

Immunohistochemistry and Antibodies. All the tissues (larval discs, larval brains, metamorphosed ph505 cells, adult brains, and transplanted tumours) were dissected in cold PBS on ice, fixed in 2% paraformaldehyde (in 1× PBS) for 25 min at room temperature, and washed several times in PBST (1× PBS with 0.5% Triton X-100). Tissues (larval discs, larval brains, or metamorphosed ph505 cells) were incubated overnight with primary antibodies at 4°C, followed by several washes at room temperature, incubated with secondary antibodies at 4°C overnight. Adult brains and transplanted tumours were incubated with primary antibodies for 48 h at 4°C, followed by several washes at room temperature, incubated with secondary antibodies for 48 h at 4°C. After several washes, all tissues were incubated with DAPI (1:200 in PBST) at room temperature for 20 min. Tissues were then mounted in Vectashield and stored at −20°C before analysis.

Primary antibodies used in this study were chicken anti-green fluorescent protein (GFP) (1:1000; Abcam ab13970); mouse anti-Elav (1:30; DSHB 9F8A); rabbit anti-cleaved DCP-1 (1:300; Asp216, Cell Signaling Technology 95785); rabbit anti-I-PH3 (1:1000; Millipore 06-570); rat anti-Chimino (1:500; from N. Sokol); mouse anti-EcR-B1 (1:1000; DSHB AD44); mouse anti-n-cad (1:50; from J. Pielage); Pax2/5/8 (1:500; from L. Johnston), Alexa 555, Alexa 568, and Alexa 647-conjugated anti-chicken, -rabbit, -rat, or -mouse IgG (all 1:500; Molecular Probes).

**Microscopy.** Immunofluorescent images were recorded on a confocal microscope (TCS SP5; Leica). Adult fly pictures were taken on a Leica MZ16 or a Nikon SMZ1270 microscope. Images were processed using ImageJ, Imaris, Photoshop, and Adobe Illustrator. To measure the volume of tumour clones, confocal images of eye-antennal discs from wandering third instar larvae of the corresponding genotypes were collected and processed in Imaris.

**Transplantation.** Transplantation experiments were carried out as previously described. In brief, 4–6 day-old adult w1118 females were used as hosts (n = 20 for each transplantation). The host flies were immobilized on ice-cold metal plate with a piece of double-sided sticky tape, and then put in a petri dish. The dissected eye discs or other tumour tissue from adult flies were cut into small pieces and each piece was transplanted into the abdomen of one host using a custom-made glass needle. All transplantation was made under a GFP microscope to ensure labelled cells were injected into the hosts. After transplantation, host flies were allowed to recover at room temperature for 1–2 h in fresh standard Drosophila medium before transferred to and maintained at 25°C.

**Surviviorship measurement.** To measure the surviviorship of ph505 and ph505, UAS-EcR-ΔN adult flies, newly eclosed adults were collected and separated into three groups (for ph505 flies, n = 20 in each group; for ph505, UAS-EcR-ΔN flies, n = 25 in two groups and n = 20 for the third group). Flies were maintained at 25°C, flipped into fresh food every 2 days, and the number of living flies were counted on each Monday and Friday during the course of experiment. The numbers of flies were imported into Excel and the survivorship at different times was calculated. The survival curve was produced in Excel.

**Quantitative PCR.** As previously reported, the expression of let-7 is low at wandering third larval instar but high during pupal stage in response to ecdysone, we dissected eye-antennal discs together with brains from larvae (wt L3) or pupae (wt pupa) as controls. We collected metamorphosed ph505 cells from adults, transplanted tumours from hosts, ph505, UAS-EcR-ΔN cells from adults, and ph505, UAS-let-7-SP cells from adults. RNA was extracted from all these samples with mirVana miRNA Isolation Kit (Invitrogen), and reverse transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) with let-7 RT primer and 25 RNA reference RT primer. qPCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) using each let-7 and 25 RNA TaqMan Small RNA Assay primer mix (Thermo Fisher Scientific).

**Transcriptome analyses.** Transcriptome analyses were performed by mRNA sequencing. RNAs were isolated from the metamorphosed ph505 cells, as well as RNAs from the ph505 tumour transplanted in adult hosts after 4 times, 8 times, and 14 times weekly retransplantation. As a control, we used the wild-type discs that were transplanted into the abdomen of adult hosts and extracted RNAs 1 day after their transplantation. This step was necessary as earlier evidence showed that the transplantation process itself introduced differential expression of a number of genes. RNA was extracted using an Arcturus PicoPure RNA Isolation kit (Arcturus) and sequenced on an Illumina NextSeq500 for control and tumour samples and NextSeq500 for metamorphosed samples.
Bioinformatics analyses. Short reads were aligned to BDGP dm6 genome assembly using TopHat 2.0.12\(^2\) with parameters “--very-sensitive” for Bowtie 2.2.3. From the aligned reads, differential expression was called using R 3.3.1 with maSigPro 1.46.0\(^{36}\). Genes were stratified into expression profile clusters with k-means clustering. Subsequent biological process gene ontologies for each cluster were found with topGO\(^{37}\). Respective p values were calculated with Fisher’s exact test. For the hierarchical clustering (UPGMA) underlying the heat map, we used library normalized log2 counts per million reads determined by edgeR 3.16.5\(^{40}\) on genes involved in the edysone response as listed by Flybase with scaling per sample/column.

Experimental design. All fly genetic experiments were repeated at least three times. Each transplantation experiment was performed at least twice independently. RNA samples for the transcriptome analysis were collected two or three times. Each transplantation experiment was performed at least twice independently. RNA samples for qPCR were extracted from the mixture of 5–10 independent animals and qPCR was performed biologically twice.

Sample size was not predetermined before the experiments. Sample size in each experiment was randomized based on the number of viable flies and the amount of other materials. No data were excluded. The investigators were not blinded to group allocation during data collection and analysis.

Data availability. All deep-sequencing data pertaining to this study can be found on GEO GSE101455. Materials and all relevant data from this study are available from the corresponding author upon reasonable request.

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Author contributions
Y.J. and M.S. designed and conducted all the experiments, under the supervision of R.P. T.B.S. and M.S. performed the bioinformatics analyses. Y.J., M.S., T.B.S. and R.P. analysed the results, created the figures, and wrote the paper.

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