miR-184 controls Dilp8 to regulate pupariation timing in response to developmental perturbations in *Drosophila melanogaster*

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Abstract

Organismal development relies on the precise coordination of growth and developmental timing, controlled by a complex interplay of intrinsic and extrinsic factors. However, the specific mechanisms governing the regulation of these events still need to be fully understood. Post-transcriptional regulation involving microRNAs play a significant role in ensuring the timely expression of factors coordinating growth and development. MicroRNAs are known to fine-tune gene expression by binding to target mRNAs, thereby buffering fluctuations in gene expression, which could otherwise affect normal development. In Drosophila, miR-184 has been implicated in regulating female germline maturation, and early embryonic development. Here, we demonstrate that miR-184 regulates developmental timing by acting in the imaginal discs of Drosophila larva. Through a combination of genetic and molecular analyses, we have identified Drosophila insulin-like peptide 8 (Dilp8), a paracrine factor crucial for regulating developmental timing in response to abnormal growth of larval imaginal discs, as a functional target of miR-184. Furthermore, our research demonstrates that miR-184 plays a critical role in response to imaginal disc damage by inducing Dilp8 expression. These findings uncover a novel post-transcriptional mechanism during Drosophila development that aids in the responses to tissue damage.

Key words: Developmental time point, pupariation, *dilp8*, miR-184, genetic perturbation, developmental perturbation, growth, imaginal discs

Introduction

Normal growth and development are crucial for an organism's functioning and reproductive capacity. Disruptions in these processes can lead to anomalies, including developmental defects, physiological disruptions, metabolic imbalances, and diseases like cancer. Environmental factors, such as nutrition and stress, significantly impact growth and development. In *Drosophila*, growth and developmental timing are interconnected, as reaching a critical body weight is essential for the transition from larval to pupal stages to initiate adult metamorphosis. Studying the links between growth and developmental timing in *Drosophila* can enhance our understanding of similar mechanisms in other animals, including humans, which will provide insights into developmental defects, and suggest potential therapeutic interventions.

In *Drosophila*, transitions between larval instars and from larval to pupal stages are initiated by pulses of the molting hormone ecdysone (Baehrecke, 1996; Yamanaka et al., 2013). Ecdysone is secreted from the prothoracic gland, a part of the ring gland, into the hemolymph and converted into its active form, 20-hydroxyecdysone (20-HE), in the larval fat body and other tissues. This active hormone initiates a transcriptional cascade via the EcR/USP receptor complex, leading to metamorphosis and the cessation of growth (Yamanaka et al., 2013). Ecdysone secretion controls the timing of pupariation, with reduced or delayed ecdysone pulses and defects in ecdysone synthesis delaying development and pupariation (Baehrecke, 1996; Yamanaka et al., 2013). Delay in pupariation leads to extension of the larval feeding period and results in excessive growth. Environmental factors like temperature, crowding, dietary restriction, oxygen availability and toxins also modulate pupariation timing (Beamish et al., 2021; Bonnier, 1926; Klepsatel et al., 2018; Layalle et al., 2008; Turingan et al., 2024). Activity of insulin receptor in the prothoracic gland plays a role in the growth of the gland and ecdysone production. Insulin signaling in the prothoracic gland also regulates ecdysone production by modulating the expression of the microRNA Bantam (Boulan et al., 2013). Recent studies show an antagonistic relationship between 20-hydroxyecdysone and juvenile hormone (JH), with JH inhibiting ecdysteroid biosynthesis and being inhibited by 20-HE in the corpus allatum (Liu et al., 2018). Akh (Adipokinetic hormone), similar to

glucagon, produced in the corpora cardiacum, modulates calcium signaling in the prothoracic gland in response to nutrient stress, thereby regulating ecdysone secretion (Hughson et al., 2021). Thus, the timing of the larva-to-pupa transition is controlled by multiple internal and external factors acting on the prothoracic gland.

A bilateral pair of neurons in the larval central brain innervate the prothoracic gland and release the neuropeptide prothoracicotropic hormone (PTTH), stimulating gland growth and ecdysone production. PTTH mutants exhibit delayed pupariation and increased body size due to prolonged larval feeding. PTTH neurons receive synaptic inputs from upstream neurons, controlling the timing of PTTH release and ecdysone production, thus modulating developmental timing and onset of metamorphosis in Drosophila (Ghosh et al., 2010; McBrayer et al., 2007). The ecdysteroid biosynthetic pathway involves several cytochrome P_{450} enzymes encoded by Halloween genes, which are tightly regulated during ecdysteroidogenesis. The transcript levels of Halloween genes correlate well with the ecdysone titer, implying a stringent transcriptional regulation of these genes during ecdysone synthesis. Disruption in the expression of Halloween genes results in delayed pupariation (Kamiyama & Niwa, 2022). Several transcriptional regulators that act in multiple cell signaling pathways, have been identified to regulate Halloween genes (Danielsen et al., 2014; Kamiyama & Niwa, 2022).

Recently, imaginal discs in larvae have been shown to regulate pupariation timing. Imaginal discs secrete factors such as Dilp8 (Drosophila insulin-like peptide-8), Dpp (Decapentaplegic), and Upd3 (Unpaired-3), which regulate pupariation timing. Dilp8, a relaxin-like molecule, plays a key role in buffering developmental noise and delays the onset of pupariation as a response to defects in imaginal disc growth. Growth perturbations in imaginal discs trigger JNK pathway activity, upregulating Dilp8 levels in the hemolymph (Colombani, Andersen, & Léopold, 2012; Katsuyama et al., 2015). Increase in Dilp8 would trigger a delay in pupariation via its receptor Lgr3, expressed in a small number of neurons in the larval brain that connects PTTH neurons and thereby regulating ecdysone signaling. Thus, Dilp8 acts as a paracrine signal regulating growth and development timing in response to perturbations in the imaginal discs (Colombani, Andersen, & Léopol, 2012, 2012; Garelli et al., 2015; Katsuyama et al., 2015; Vallejo et

al., 2015). Damage to the L2-early L3 larval hindgut also increases Dilp8 expression, delaying pupariation (Cohen et al., 2021). Dpp secreted by the growing imaginal discs inhibits ecdysone biosynthesis and prevents metamorphosis, and reduction in Dpp levels in response to attaining final disc growth allows progression to metamorphosis (Setiawan et al., 2018). Upd3, a cytokine secreted by the imaginal discs, delays pupariation in response to malignant transformation in Drosophila, by upregulating Bantam microRNA and activating JAK/STAT signaling (Romão et al., 2021).

Several microRNAs, that act by buffering fluctuations in target gene expression, were reported to regulate the initiation of metamorphosis. Bantam microRNA, expressed in the prothoracic gland regulates pupariation timing by modulating ecdysone levels (Boulan et al., 2013). Bantam also controls the expression of *jhamt*, a gene involved in JH biosynthesis in the corpus allatum (Qu et al., 2017). miR-8, highly expressed in the corpus allatum, affects cell growth, JH biosynthesis, and pupariation timing (Zhang et al., 2021). Furthermore, several microRNAs have been identified whose expression is differentially modulated by ecdysone during the larval-pupal transition, to play an essential role in regulating metamorphosis (Jin et al., 2020; Lim et al., 2018). These studies highlight the complex regulation of ecdysone signaling by microRNAs during a critical developmental stage. However, no studies have yet reported the role of microRNAs in regulating the onset of metamorphosis in response to disc damage.

miR-184, a microRNA conserved across bilaterians, is maternally deposited and plays a major role during oogenesis and early embryonic development in Drosophila (Iovino et al., 2009). Here, we report the critical role of miR-184 in regulating the developmental timing of Drosophila as miR-184 mutants exhibit delayed pupariation due to reduced ecdysone signaling. miR-184 acts in the imaginal discs, and regulates the pupariation timing, through its target gene *dilp8*. We show that miR-184 regulates Dilp8 levels through the target sites in the 3'UTR of *dilp8* transcript. Our findings also indicate that miR-184 delays the larval-to-pupal transition in response to imaginal disc damage, by enhancing Dilp8 levels. These results confirm a critical role for miR-184 in regulating developmental timing during normal growth and in response to tissue damage.

Results

Role of miR-184 in the regulation of pupariation timing

While analyzing the mutants of various *Drosophila* microRNAs for their roles in development, we observed that *miR-184* homozygous mutants exhibited significant delays in larval development, as evidenced by a notable increase in the time taken to puparium formation (Fig. 1A). To further investigate the role of miR-184 in developmental timing, we employed *miR-184 sponge*, a transgenic inhibitor of miR-184. Downregulation of miR-184 in the entire organism by the expression of miR-184 sponge using a *tubGAL4* driver line, resulted in a similar delay in puparium formation (Fig. 1B). This prompted us to investigate the regulation of pupariation timing by miR-184.

miR-184 manages optimal Ecdysone signaling

The expression and release of the steroid hormone ecdysone by the prothoracic gland in developing larvae tightly controls the timing of pupariation. Ecdysone secreted into the hemolymph is converted into its active form, 20-hydroxyecdysone, in peripheral tissues. The active form of ecdysone regulates the expression of various ecdysone response genes, crucial for pupariation, metamorphosis and adult emergence. Given the observed delay in pupariation in *miR-184* depleted larvae, we investigated whether downregulation of *miR-184* leads to a significant decrease in ecdysone levels.

First, we measured the mRNA levels of prothoracicotropic hormone (*ptth)*, phantom (*phm)* and disembodied (*dib)*; genes that promote ecdysone biogenesis. PTTH activates the pulse of ecdysone preceding pupariation, while Phm and Dib are hydroxylases encoded by Halloween genes crucial for ecdysone biosynthesis. Downregulation of *miR-184* in larvae significantly decreased the expression of *ptth*, *phm*, and *dib* (Fig. 2A). Next, we measured the transcript levels of key ecdysone response genes - *Ecdysone inducible protein 74* (*E74*), *Ecdysone inducible protein 75* (*E75*), *Broad-complex* (*BR-C*), and *Fatbody protein-1* (*Fbp1*). The mRNA levels of these ecdysone response genes were also found to be lower in response to reduced *miR-184* levels (Fig. 2B). These results indicate that miR-184 plays an essential role in maintaining optimal levels of ecdysone signaling in developing larvae and regulates the pupariation time point. Next,

we focused on identifying the tissue where miR-184 acts in regulating ecdysone signalling.

miR-184 acts in the imaginal discs to regulate pupariation timing

We investigated whether miR-184 activity in the prothoracic gland plays a role in regulating the pupariation time point. Surprisingly, no delay in pupariation time point (Fig. 3A) was observed when *miR-184* was downregulated in the prothoracic gland using *miR-184 sponge*. These results indicate that the phenotype observed in *miR-184* mutants is not due to the reduction of miR-184 levels in the prothoracic gland. Since juvenile hormone (JH) levels are also known to negatively regulate the expression and release of ecdysone, we further examined whether miR-184 levels in the corpus allatum, the source of JH in *Drosophila* larvae, play a role. However, no change in the pupariation time point was observed when *miR-184* was downregulated in the corpus allatum using *aug21GAL4* and *miR-184 sponge* (Fig. 3B).

Recent studies report that adult imaginal discs in larvae play a role in regulating the timing of pupariation, especially in response to tissue perturbations. We found that downregulation of miR-184 in the imaginal discs using *rnGAL4* and *miR-184 sponge* resulted in a significant delay in the pupariation time point (Fig. 3C). These findings strongly suggest that miR-184 acts in the imaginal discs in the larvae to regulate the timing of pupariation. As microRNAs function by regulating target gene expression, we sought to identify miR-184 target genes in the imaginal discs that influence pupariation timing.

miR-184 regulates larval to pupal transition timing via its target *dilp8*

Recent research has highlighted the crucial role of imaginal discs in controlling the timing of pupariation by synthesizing and releasing key factors such as DILP8, Dpp, and Upd3 (Colombani, Andersen, & Léopol, 2012; Garelli et al., 2012; Romão et al., 2021; Setiawan et al., 2018). Among these factors, *dilp8* has been predicted as a target of miR-184 according to two independent microRNA target prediction programs (Fig. S1). Given that microRNAs function as post-transcriptional regulators of gene expression, we hypothesized that the levels of *dilp8* transcripts would be elevated in a *miR-184*

loss-of-function background. Indeed, we found that the *dilp8* mRNA levels were higher in both *miR-184* knockout (KO) mutant larvae (Fig. 4A) and in response to whole body downregulation of *miR-184* (Fig. 4B), indicating that *dilp8* could be a target of miR-184. These observations suggest that miR-184 likely plays a role in restricting *dilp8* expression, during larval development and control pupariation timing.

Further, to demonstrate that *dilp8* is a direct target of miR-184 acting through the 3' untranslated region (UTR) of the *dilp8* mRNA, we tested whether miR-184 affects the expression of a *dilp8-3'UTR-GFP* reporter. We observed reduced expression of the *dilp8-3'UTR-GFP* reporter in imaginal discs where *miR-184* was overexpressed using *ptcGAL4* driver (Fig. 4C-D'), providing strong evidence that miR-184 regulates *dilp8* expression post-transcriptionally via its 3'UTR.

To confirm the functional relevance of *dilp8* as a target of *miR-184*, we conducted experiments to determine if reducing *dilp8* levels could rescue the pupariation delays observed in *miR-184* loss-of-function mutants. Surprisingly, we found that removing one functional copy of *dilp8* was sufficient to rescue the pupariation delay seen in *miR-184* homozygous knockout (KO) mutant larvae (Fig. 4E). Similarly, coexpressing *dilp8* RNAi with *miR-184* sponge led to a comparable rescue in the pupariation time point (Fig. 4F). These results provide strong support for the regulation of pupariation time point during normal development by miR-184 through post-transcriptional control of *dilp8*.

miR-184 **controls a developmental checkpoint in the face of perturbations**

Previous research has shown that in response to developmental stresses, such as excessive cell death or hyperplastic growth, DILP8 levels increase significantly (Katsuyama et al., 2015). The surge in DILP8 interferes with ecdysone synthesis, causing a delay in pupariation, which offers additional time for tissue repair or regeneration (Garelli et al., 2015; Vallejo et al., 2015). To delve deeper into the regulatory relationship between *miR-184* and *dilp8*, we explored whether miR-184 is involved in responding to developmental perturbations in managing the pupariation time point. We hypothesized that miR-184 might contribute to the induction of DILP8 leading to pupariation delays in response to developmental perturbations. To investigate this,

we induced tissue damage in imaginal discs by expressing a cold-sensitive version of the type 2 ribosome-inactivating protein Ricin-A (RA^{CS}), a cytotoxic protein. The expression of *Ricin-A*, has previously been shown to induce cell death and increase the expression of *dilp8*. Consistent with previous findings, the expression of *Ricin-A*^{CS} in the imaginal discs led to an increase in *dilp8* expression and caused a significant delay in the pupariation time point (Fig. 5A, B). Imaginal disc growth perturbations and tissue damage has previously been shown to trigger an increase in JNK signaling, which leads to elevated *dilp8* levels crucial for developmental delays in *Drosophila* larvae. To confirm if the expression of *RA*^{CS} alters JNK signaling and to ensure the specificity of *RA*^{CS} activity, we measured the expression of *puc* (*puckered*) and *mmp1* (*matrix metalloproteinase 1*), target genes of JNK signaling in the wing imaginal discs. The expression of *RA*^{CS} in the imaginal discs induced the expression of *puc* and *mmp1* (Fig. 5C,D), indicating heightened JNK signaling in response to imaginal disc damage.

Despite the well-established dynamic expression pattern of miR-184 during larval development and its regulation by nutrient levels and in tumorous conditions, no studies have addressed the regulation of *miR-184* levels during developmental perturbations. However, our experiments suggest a role for *miR-184* in the induction of *dilp8* expression levels in response to developmental perturbations. Therefore, to determine if *miR-184* is modulated in response to such perturbations, we measured *miR-184* levels in the imaginal discs after expressing *RA*^{CS}. Interestingly, we observed a decrease in *miR-184* levels in the wing imaginal discs in response to the expression of *RA*^{CS} (Fig. 5E), which correlates with the increased expression of *dilp8* (Fig. 5F). These findings suggest a regulatory mechanism where *miR-184* is downregulated in response to developmental stressors, which leads to increased *dilp8* expression allowing damage repair through delaying pupariation.

Next, we tested whether the reduction of *miR-184* expression during developmental perturbations is responsible for the induction of *dilp8* levels and pupariation delays. As expected, downregulation of *dilp8* using RNAi in the *RA*^{CS} overexpression background resulted in decreased *dilp8* expression (Fig. 6A) and a comparable rescue of the pupariation delay (Fig. 6C). When *miR-184* was co-overexpressed with *RA*^{CS}, we

observed a significant decrease in *dilp8* expression (Fig. 6B) and a partial rescue of the pupariation delay (Fig. 6C). Thus, our experiments demonstrate that the reduction of *miR-184* levels in response to imaginal tissue damage in *Drosophila* larvae leads to increased *dilp8* expression, resulting in delays during larval development.

From these observations we hypothesized that the JNK signaling pathway might regulate *miR-184* levels in the imaginal discs. Next, to test this, we blocked basal levels of JNK signaling in the imaginal discs by expressing *UAS-bskDN* (a dominant negative form of *basket, Drosophila* JNK) and measured the levels of *miR-184* in the wing imaginal discs. Interestingly, blocking endogenous JNK signaling led to an increase in the expression of *miR-184* in the wing imaginal discs (Fig. 6D). These experiments demonstrate that JNK signaling modulates *miR-184* expression. Our data also raises the possibility that activation of JNK signaling in response to imaginal tissue damage lowers *miR-184* levels leading to upregulation of *dilp8* levels and delay pupariation.

Discussion

Coordination between growth and developmental timing is crucial for achieving the final body size as determined by the genetic makeup of a particular species. Final body size is also influenced by environmental factors, which act on gene expression and signaling pathways that regulate growth and developmental timing. These extrinsic factors are subject to fluctuations, and various biological systems have built-in buffering mechanisms to mitigate their effects. One powerful mechanism for limiting the impact of environmental fluctuations on gene expression is post-transcriptional regulation by microRNAs. Among several Drosophila microRNAs, miR-184 is a maternally deposited microRNA (Iovino et al., 2009) expressed in a highly dynamic pattern throughout different developmental stages (Li et al., 2011).

Here, we demonstrate that miR-184 controls the timing of the critical developmental transition from larval to pupal stages by acting in the imaginal discs. We tested the role of *dilp8*, a predicted target gene of miR-184, in the developmental delays in *miR-184* mutants. Dilp8, a paracrine factor in Drosophila, induces a delay in pupariation timing, in response to imaginal disc damage. Blocking *miR-184* expression led to higher expression of *dilp8* transcripts, and suppressing *dilp8* levels using RNAi in this background alleviated the effects on pupariation timing. Defects in pupariation timing which we observed in response to the lack of *miR-184* was caused by reduced ecdysone signaling. Further, our results show that miR-184 can act on the 3'UTR of *dilp8* messenger RNA and regulate Dilp8 expression. Our experiments confirm that Dilp8 levels are under stringent regulation of miR-184 during normal larval development, and a loss of this regulation would lead to delays in pupariation. Earlier studies have suggested the importance of regulating *dilp8* levels during development, as higher expression levels of *dilp8* has led to pupariation delays (Colombani, Andersen, & Léopol, 2012; Garelli et al., 2012; Jones et al., 2016). Thus, we report a novel post-transcriptional mechanism of Dilp8 regulation by a microRNA, crucial for ensuring proper developmental timing.

The role of Dilp8 in ensuring developmental stability was recently reported by several groups. Loss of Dilp8 increased wing asymmetry, suggesting the role of Dilp8 in

buffering developmental noise. During normal development, the Hippo pathway maintains the expression of Dilp8 and manages the symmetric growth of tissues (Boone et al., 2016). At the onset of pupariation, ecdysone-triggered Dilp8 signaling from the cuticle epidermis is crucial for coordinating behavioral, morphogenetic, and neuromotor functions (Heredia et al., 2021). Pacman/XRN1, a cytoplasmic exoribonuclease, is crucial in regulating the expression of Dilp8 post-transcriptionally, influencing developmental timing (Jones et al., 2016). Additionally, dNOC1, a nucleolar protein necessary for rRNA maturation, suppresses JNK signaling and maintains low *dilp8* levels during normal development (Destefanis et al., 2022). These reports highlight the importance of maintaining Dilp8 levels for proper development. Our observations confirm that the regulation of Dilp8 levels is critical for normal developmental timing and proposes a novel post-transcriptional regulatory mechanism for maintaining Dilp8 levels via miR-184.

Lgr3, the receptor for Dilp8, expressed in a small number of neurons in the larval brain, plays a significant role in the developmental delays caused by Dilp8 in response to tissue damage. Lgr3 neurons directly contact the PTTH and insulin-producing neurons, regulating developmental timing and growth. Through the Lgr3-PTTH neuronal circuitry, Dilp8 regulates ecdysone synthesis in the prothoracic gland in response to tissue damage and induces developmental delays (Jaszczak et al., 2016). Here, we show that loss of *miR-184* triggers *dilp8* levels, reducing the expression of PTTH gene, and ecdysone signaling, which could be responsible for the pupariation delays observed.

Furthermore, it has been recently reported that in response to growth perturbations Dilp8 activates a developmental checkpoint, which induces a delay in developmental timing and growth of unaffected tissues. Several signaling pathways regulate Dilp8 during tissue damage or stress. In early regenerating discs, JAK/STAT signaling induces Dilp8 expression, which delays the onset of pupariation in response to disc fragmentation (Katsuyama et al., 2015). Malignant disc tumors showed enhanced secretion of Dilp8 under the regulation of JNK signaling (Romão et al., 2021). Blocking JNK signaling in a damaged imaginal disc affected Dilp8 induction and the eclosion delay (Colombani, Andersen, & Léopol, 2012; Katsuyama et al., 2015). In this paper, we

show that in response to perturbations in the imaginal tissue, *miR-184* levels decrease, leading to higher expression of *dilp8* and delaying pupariation. Lowering *miR-184* levels is likely due to increased JNK signaling in response to tissue damage.

miR-184 plays a role in the female germline and early developmental processes (Iovino et al., 2009). It has also been demonstrated that miR-184 is a nutrient-sensitive microRNA. miR-184 was reported to be expressed at higher levels in low-nutrient conditions and is essential for survival during these conditions (Fernandes et al., 2022; Gendron & Pletcher, 2017). When overexpressed, the epithelial tricellular junction protein Gliotactin regulates its own levels through the upregulation of miR-184 via BMP signaling (Sharifkhodaei et al., 2016). Additionally, miR-184 levels were demonstrated to decrease in the wing disc tumors (Shu et al., 2017), though the significance of this was not understood.

Dilp8 levels in the imaginal discs in *Drosophila* were demonstrated to be a new checkpoint for developmental progression. During regenerative growth, Dilp8, dMyc, and Wg levels were shown to be constrained by the *brain tumor* (*brat*) gene in the regenerating tissue to ensure proper patterning, cell-fate specification, and differentiation in *Drosophila* (Abidi et al., 2023). Dilp8 levels also play a role during metamorphosis in maintaining symmetry of adult organs in *Drosophila* (Boone et al., 2016; Garelli et al., 2012). Recently, Dilp8, through its receptor Drl, was shown to be involved in the transdetermination of the Drosophila imaginal disc (Nemoto et al., 2023). In adult *Drosophila*, Dilp8 levels in the ovarian follicle cells of female flies were found to regulate ovulation (Liao, 2020). In *Aedes aegypti*, microRNA miR-277 was discovered to control lipid metabolism and reproduction by regulating its target *Dilp8* (Ling et al., 2017). It remains to be addressed if the miR-184 - Dilp8 regulatory relationship also acts in such developmental contexts.

In conclusion, our results reveal a novel mechanism by which miR-184 regulates Dilp8 levels in the imaginal discs, which aids in (i) fine-tuning developmental timing during normal development by maintaining appropriate ecdysone signaling and (ii) actively adjusting developmental timing in response to perturbations, enabling damage response (Fig. 6E, F). Our findings open up several questions about how signaling

pathways are regulated post-transcriptionally in response to developmental disturbances to aid in normal adult emergence. Further research is needed to explore whether similar regulatory mechanisms exist in other tissues and higher species during tissue damage responses.

Figure legends:

Figure 1. *miR-184* plays a role in the regulation of pupariation time point

(A) miR-184 mutants display delayed pupariation, pupariation rates of w^{1118} (control) (n=142) and *miR-184* homozygous null mutants (n=104). Data from three independent experiments are shown; ($p<0.0001$). (B) Downregulation of miR-184 leads to delayed pupariation, pupariation of *tubGal4, UAS-scramble* (control) (n=955) and *tubGal4, UAS-miR-184-sponge* (n=617). Data from three independent experiments are shown; ($p<0.0001$). Data points plotted are the percentage of larvae pupariated, LogRank test was performed to determine statistical significance.

Figure 2. miR-184 levels maintain proper Ecdysone signalling

(A) Expression of Ecdysone biosynthesis genes in response to ubiquitous downregulation of miR-184; using *tubGal4, UAS-miR-184-sponge* and *tubGal4, UAS-scramble* (control), from three independent experiments are shown. Fold change of transcript levels of *ptth*, *phm* and *dib* are shown, normalized to *rp49*. Total RNA was extracted from whole larvae. (B) Expression of Ecdysone response genes in response to ubiquitous downregulation of miR-184 using tubGal4; *tubGal4, UAS-scramble* (control) and *tubGal4, UAS-miR-184-sponge*, data from three independent experiments are shown. Fold change of transcript levels of *E74*, *E75A*, *E75B*, *BR-C,* and *fbp1* are shown, normalized to *rp49*. Total RNA was extracted from whole larvae. Bars graphs plotted are average/mean values, error bars represent SEM; a two-way ANOVA was performed to determine statistical significance; * $p < 0.05$, ***$p<0.001$, ****$p<0.0001$.

Figure 3. miR-184 levels in the imaginal discs regulate pupariation timepoint

(A) Downregulation of miR-184 in prothoracic gland leads to minimal effects on pupariation time point. *phmGal4, UAS-miR-184-sponge*, (n=206) and *phmGal4, UAS-scramble* (control) (n=265), three independent experiments are shown; p<0.0001. (B) Downregulation of miR-184 in the corpus allatum leads to minimal effects on pupariation time point. *aug21Gal4, UAS-miR-184-sponge* (n=601) and *aug21Gal4, UAS-scramble* (control) (n=292), six independent experiments are shown; p<0.0001. (C) Downregulation of miR-184 in the imaginal discs leads to a delay in pupariation timepoint. *rotundGal4, UAS-miR-184-sponge* (n=151), and *rotundGal4, UAS-scramble* (control) (n=182), three independent experiments are shown; p<0.0001. Data points plotted are the percentage of larvae pupariated, LogRank test was performed to determine statistical significance.

Figure 4. miR-184 regulates developmental time point via its target gene *dilp8*

(A) *dilp8* mRNA levels in w^{1118} (control), and *miR-184* homozygous null mutants from four independent experiments are shown; p=0.0281. Total RNA was extracted from whole larvae. (B) *dilp8* mRNA levels in *tubGal4, UAS-scramble* (control),and *tubGal4, UAS-miR-184-sponge*, four independent experiments are shown; p=0.0428. Total RNA was extracted from whole larvae. Bars graphs plotted are average/mean values, error bars represent SEM, Welch's test was performed to determine statistical significance (C) *dilp8* 3'UTR-GFP reporter assay, reporter GFP expression (anti-GFP, green) in control (*ptcGal4/+)* and (D) reporter GFP expression (anti-GFP, green) in *ptcGal4/+, UAS-miR-184 OE.* (C') Patched expression (anti-Ptc, red) in *ptcGal4/+* (control) and (D') Patched expression (anti-Ptc, red) in *ptcGal4/+, UAS-miR-184* (overexpression). (E) Rescue of pupariation delay, *w1118* (control) (n=486); *miR-184-/-* (homozygous mutant) (n=104) and *miR-184-/-; dilp8-/+* (rescue) (n=173), from three independent experiments are shown. (F) Rescue of pupariation delay, *tubGal4, UAS-scramble* (control) (n=255) and *tubGal4, UAS-miR-184-sponge* (miR-184 downregulation) (n=136) and *tubGal4, UAS-miR-184 sponge, dilp8-RNAi* (rescue) (n=76), three independent experiments are shown. Data points plotted are percentage pupariated, LogRank test was performed to determine statistical significance. * p < 0.05, ***p<0.001, ****p<0.0001.

Figure 5. Effects of Ricin-induced developmental perturbation

(A) *dilp8* mRNA levels increased when Ricin-A was expressed in the imaginal discs, *rnGal4/+* (control) and *rnGal4, UAS-EGFP; UAS-RA^{cs},* three independent experiments are shown; p<0.0243. Fold change of transcript levels of *dilp8* is shown, normalized to *rp49*. Total RNA was extracted from the whole larvae. Bars graphs plotted are average/mean values, error bars represent SEM, and a Welch's t-test was carried out to determine statistical significance (B) Pupariation delays were observed when Ricin-A^{cs} was expressed in the imaginal discs, pupariation rates of *rnGal4/+* (control), n=107 and *rnGal4, UAS-EGFP; UAS-RA^{cs},* n=159, three independent experiments are shown; p<0.0001. Data points are percentage pupariated, LogRank test was performed to determine statistical significance. (C-D) JNK target levels increase when Ricin-A was expressed in the imaginal discs, (C) *puckered* mRNA levels in *rnGal4/+* (control) and *rnGal4, UAS-EGFP, UAS-RA^{cs}*, four independent experiments are shown; p= 0.0383. (D) *mmp1* mRNA levels in *rnGal4/+* (control), and *rnGal4, UAS-EGFP, UAS-RA^{cs}*, three independent experiments are shown; p=0.0172. (E) miR-184 levels are affected in response to Ricin-A expression in the imaginal discs, *rnGal4/+* (control) and *rnGal4, UAS-EGFP, UAS-RA^{cs}*, four independent experiments are shown; p=0.001. Fold change of transcript levels of *puc* and *mmp1* are shown, normalized to *rp49*. Fold change of transcript levels of *miR-184* is shown, normalized to *2SrRNA*. Total RNA was extracted from the wing imaginal discs. Bars graphs plotted are average/mean values, error bars represent SEM, Welch's t-test was performed to determine statistical significance. (F) *dilp8* mRNA levels increase when Ricin-A was expressed in the imaginal discs, *rnGal4/+* (control) and *rnGal4, UAS-EGFP, UAS-RA^{cs},* three independent experiments are shown; p=0.0417. Fold change of transcript levels of *dilp8* is shown, normalized to *rp49*, Total RNA was extracted from the wing imaginal discs. Bars graphs plotted are average/mean values, error bars represent SEM, and a Welch's t-test was performed to determine statistical significance.* p < 0.05, ***p<0.001, ****p<0.0001.

Figure 6. Role of miR-184 in Ricin-induced developmental perturbation

(A-B) Rescue of *dilp8* levels in the wing imaginal discs expressing Ricin-A, (A) *rnGal4/+* (control); *rnGal4, UAS-EGFP, UAS-RA*^{cs} and *rnGal4, UAS-miR-dilp8-RNAi*, *UAS-RA*^{cs}*,* three independent experiments are shown; (B) Ricin-A, *rnGal4/+* (control); *rnGal4, UAS-EGFP, UAS-RA*^{cs}; and *rnGal4, UAS-miR-184-OE, UAS-RA*^{cs} three independent experiments are shown, fold change of transcript levels of *dilp8* is shown, normalized to *rp49*, Total RNA was extracted from whole larvae. Bars graphs plotted are average/mean values, error bars represent SEM, a Brown-Forsythe and Welch's ANOVA test was performed to determine statistical significance (C) Rescue of pupariation delays in response to Ricin-A expression in the imaginal discs, *rnGal4/+* (control) (n=298); *rnGal4, UAS-EGFP, UAS-RA*^{cs} (n=139); *rnGal4, UAS-miR-184-OE, UAS-RA*^{cs} (n=272) and *rnGal4, UAS-miR-dilp8-RNAi, UAS-RA*^{cs} (n=233). Three independent experiments are shown; $p<0.0001$. Data points are percentage pupariated, LogRank test was performed to determine statistical significance. (D) Blocking JNK signaling in the imaginal discs leads to the induction of miR-184 levels, *UAS-bskDN/+* (control) and *rnGal4, UAS-bskDN,* four independent experiments are shown; $p=0.0308$. Fold change of transcript levels of *miR-184* is shown, normalized to *2SrRNA*. Total RNA was extracted from the wing imaginal disc. Bars graphs plotted are average/mean values. Error bars represent SEM. * $p < 0.05$, ***$p<0.001$, ****$p<0.0001$. (E-F) Function of miR-184 in growth and development. Role of miR-184 during normal growth (E) and a developmental perturbation (F).

Experimental Methodology:

Fly stocks

The fly stocks were reared in food containing standard laboratory cornmeal medium in an incubator maintained at 25°C, 70 % humidity, and a 12:12 Light: Dark (LD) cycle. 1 L of the Drosophila medium contains 58.2 g cornmeal, 50.8 g dextrose, 23.6 g yeast, 8 g agar, and 3 g nipagin (10% in 100% ethanol). *w1118* (6326), and *UAS-miR-184-sponge* (61396) lines were obtained from BDSC. The *miR-184KO* line (116326) was obtained from DGRC. The UAS Scramble-sponge was a kind gift from Dr. Tudor Fulga (Weatherall Institute, Oxford, UK). The *UAS-dilp8RNAi* and *dilp8*^{MI00727} were a kind gift from Dr. Pierre Leopold's lab. The *tub-GAL4, dilp2-GAL4, cg-GAL4, phm-GAL4,* and *btl-GAL4* were a kind gift from Dr. Stephen Cohen's lab. The *rn-GAL4* and *UAS-RA*^{cs} were a kind gift from Dr. Marco Milan's lab. The *dilp8 3'UTR-GFP* reporter line was created as described (Varghese & Cohen, 2007).

Pupariation time point assay

Pupariation time point assay measures the time taken from hatching to pupariation. All the larvae hatched before the peak hatching was picked and discarded. The rest of the eggs are kept in a 25°C incubator for one hour. First instar larvae are collected within a tight time window of one hour. Collected larvae are reared in a 25°C incubator for the next few days, until their pupariation. The number of larvae pupariated is quantified every 4 hours. Logrank statistical Analyses were carried out in Oasis2 (Han et al., 2016). Pupariation experiments involving the use of UAS-RAcs were carried out at 29°C.

Sample collection for RNA isolation

Larval samples were collected ~116-124 hrs post-hatching and plunged into liquid nitrogen. The samples were then transferred to -80°C for storage. For the imaginal discs RNA isolation: 20 Larvae per set were collected ~92-100 hrs post-hatching and were then dissected in PBS solution. The wing imaginal discs were isolated and stored in TriZol immediately post-dissection before proceeding for total RNA extraction.

qRT-PCR

Total RNA was extracted from samples each using the TriZol Reagent, as per the manufacturer's instruction and pelleted using chloroform and isopropanol. The pellet was washed with 70% ethanol and centrifuged at 15,000 rpm. The pellet was dried and dissolved in 40µl of Molecular biology-grade water (Himedia). Any contaminating DNA was degraded with the help of a QIAGEN RNase-Free DNase set as per the manufacturer's instructions. Following this, the RNA was re-extracted and dissolved in 40µl of Molecular biology-grade water (Himedia). Takara Perfect Real-time kit was used to convert similar quantities of the RNA to cDNA. The cDNA was used for qPCR quantification using TB Green Premix Ex Taq (Tli RNaseH Plus) in a CFX96 machine (Bio-Rad). The level of the genes was normalized to the level of actin. The ΔCt values generated were analysed using the Student's t-test in GraphPad Prism.

microRNA qRT-PCR

Total RNA was extracted from L3 larvae using the TriZol extraction method (described above). miR-184 was reverse transcribed and the qPCR was performed as per instructions of the Takara miR-X kit.

Table 1: List of primers used for qRT-PCR

Sr. No.	Primer	Sequence
1	2SrRNA Forw. primer	CATTCATGCTTGGACTACATATGGTTGAGG
2	miR-184 Forw. primer	TGGACGGAGAACTGATAAGGGC
3	Rp49 Forward primer	GCTAAGCTGTCGCACAAA
4	Rp49 Reverse primer	TCCGGTGGGCAGCATGTG
5	dilp8 Forward primer	GGACGGACGGGTTAACCATT
6	dilp8 Reverse primer	CATCAGGCAACAGACTCCGA
7	Phm Forward primer	GGATTTCTTTCGGCGCGATGTG
8	Phm Reverse primer	TGCCTCAGTATCGAAAAGCCGT
9	dib Forward primer	TGCCCTCAATCCCTATCTGGTC
10	dib Reverse primer	ACAGGGTCTTCACACCCATCTC

11	ptth Forward primer	TGAAGGTTTGCACGAGATGG
12	ptth Reverse primer	CTGTGGGATGTGGAGTGCTG
13	E74 Forward primer	GATGGGCAGCAGGCTAAGACT
14	E74 Reverse primer	CTTCACCTCTTGCCTCGTCTT
15	E75A Forward primer	ACCACAGCACCACCCATTT
16	E75A Reverse primer	TGTTTGGCGGTAGTTTCAGG
17	E75B Forward primer	CAACAGCAACAACACCCAGA
18	E75B Reverse primer	GCTGGGAATTGATTCGGATTT
19	Br-c Forward primer	AACACCCGGTCATACTGCTG
20	Br-c Reverse primer	ATGGCTGTGTGTGTCCTCTG
21	Fbp1 Forward primer	CAACAGCAACAACACCCAGA
22	Fbp1 Reverse primer	CAGCTGGTCGCACGTCTTAAC

Antibody stainings and immunofluorescence

For every genotype, non-wandering early third instar larvae were dissected in 1x ice-cold PBS and fixed in 4% paraformaldehyde (PFA) (Cat# P6148, Sigma-Aldrich) solution for 20 minutes at RT. Following the removal of PFA, the tissues were washed using PBT (1× phosphate-buffered saline + 0.1% Triton X-100 [Bio-Rad Cat. No. 161-0407]). After adding the blocking solution (PBT + 0.1% BSA [Cat. no. A2153 from Sigma-Aldrich]), the samples were allowed to sit at room temperature for forty-five minutes on a nutator to ensure efficient blocking. The samples were then incubated with anti-ptc antibody (DSHB antibody 1:200 dilution with blocking solution) and anti-GFP antibody (polyclonal antibody 1:500 dilution with blocking solution) (Cat. no. A-6455 from Invitrogen) overnight at 4°C while being rotated continuously. Following a thorough PBT wash, the tissues were left to incubate for two hours at room temperature with the secondary antibody. Anti-mouse IgG-conjugated with Alex-Fluor 633 and anti-rabbit IgG-conjugated with Alex-Fluor 488 diluted with blocking solution were used to fluorescently label the samples respectively. The samples were thoroughly cleaned and mounted using a drop of Thermo Fisher Scientific's SlowFade Gold Antifade Reagent (Cat. no. S36939) after two hours. The samples were visualized and imaged by a

confocal microscope (Leica DM6000B). ImageJ software was used to analyse the images further.

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AUTHOR CONTRIBUTIONS

Conceptualization: JF and JV; Methodology: JF, MN, AMMP and JV; Investigation: JF, MN and AMMP; Data acquisition: JF, MN and AMMP; Data analysis: JF and MN; Writing – Original Draft: JF; Writing – Review & Editing: JV; Funding Acquisition: JV; Supervision: JV.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure 1

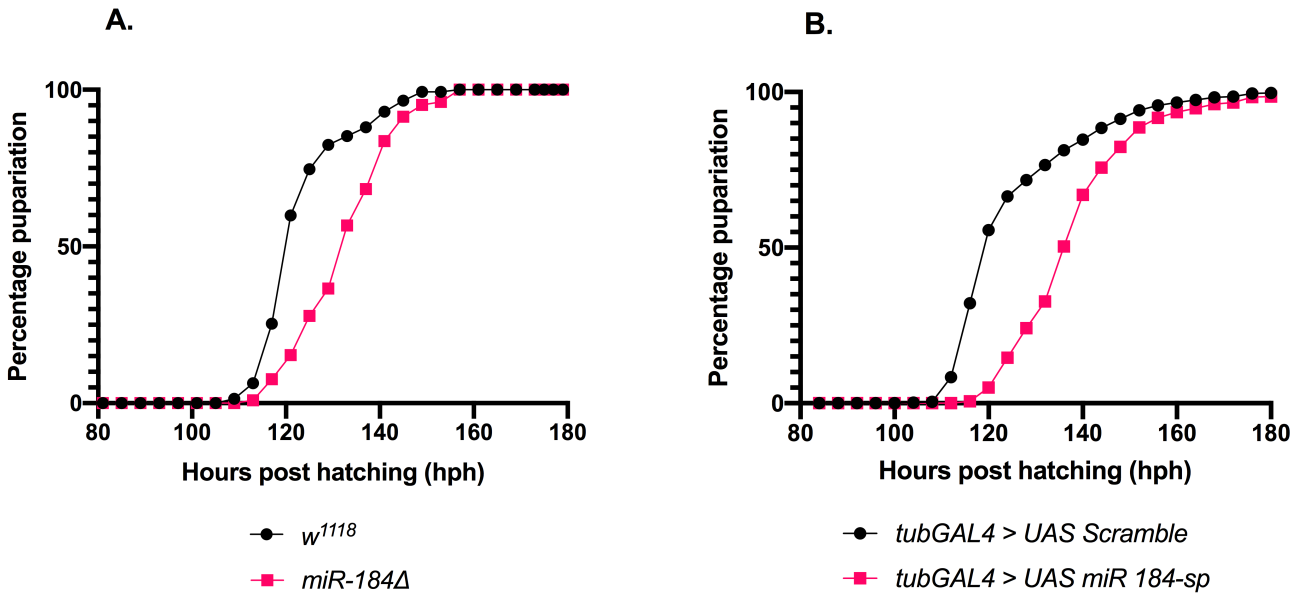

A.
Percentage pupariation
100
50
0
80
100
120
140
160
180
Hours post hatching (hph)
w1118
miR-184Δ
B.
Percentage pupariation
100
50
0
80
100
120
140
160
180
Hours post hatching (hph)
tubGAL4 > UAS Scramble
tubGAL4 > UAS miR 184-sp

Figure 2

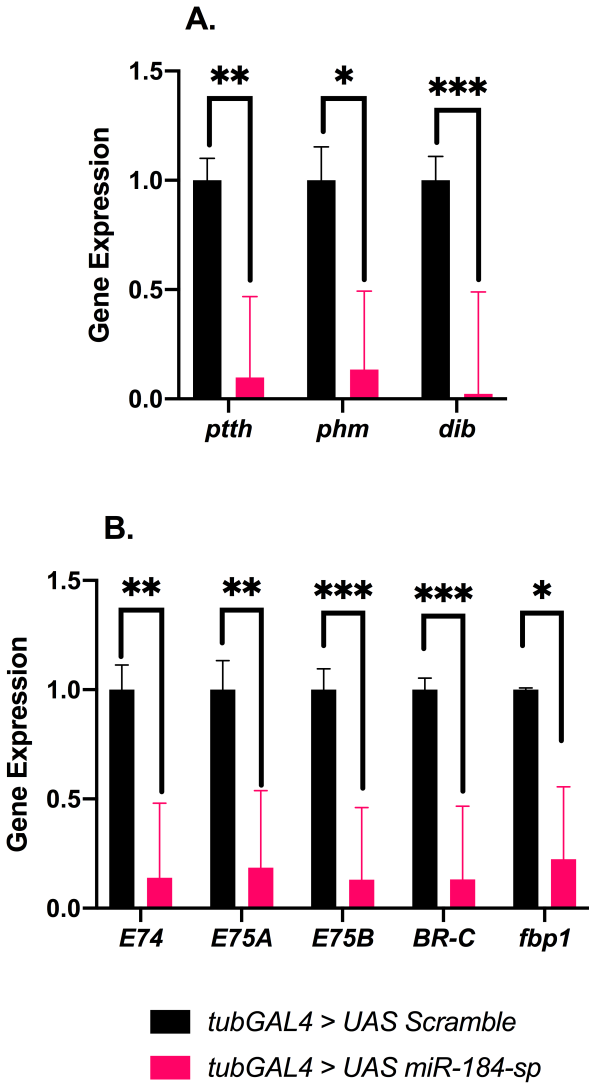

A.
Gene Expression
1.5
1.0
0.5
0.0
**
*
***
ptth
phm
dib
B.
Gene Expression
1.5
1.0
0.5
0.0
**
**
***
***
*
E74
E75A
E75B
BR-C
fbp1
tubGAL4 > UAS Scramble
tubGAL4 > UAS miR-184-sp

Figure 3

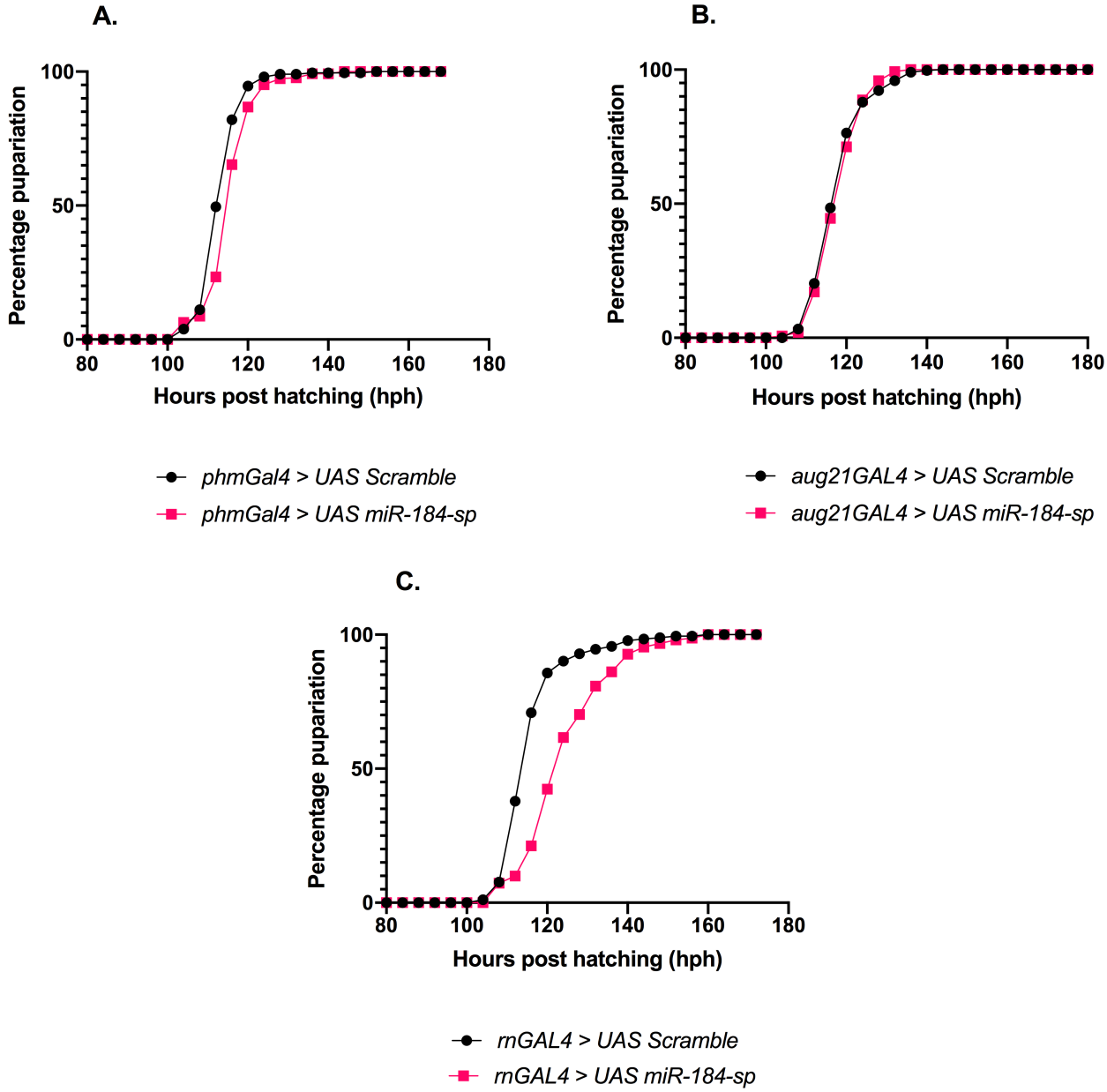
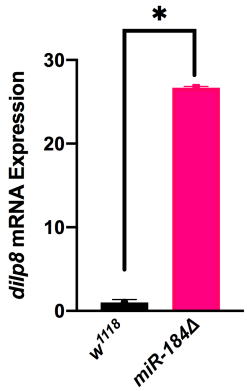
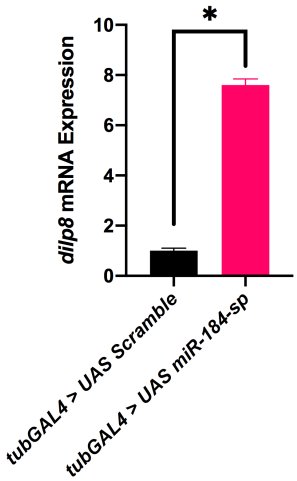
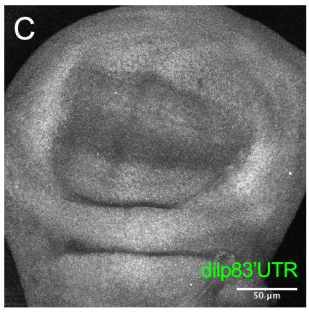
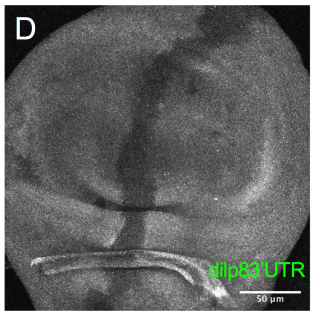

A.
Percentage pupariation
100
50
0
80
100
120
140
160
180
Hours post hatching (hph)
phmGal4 > UAS Scramble
phmGal4 > UAS miR-184-sp
B.
Percentage pupariation
100
50
0
80
100
120
140
160
180
Hours post hatching (hph)
aug21GAL4 > UAS Scramble
aug21GAL4 > UAS miR-184-sp
C.
Percentage pupariation
100
50
0
80
100
120
140
160
180
Hours post hatching (hph)
rnGAL4 > UAS Scramble
rnGAL4 > UAS miR-184-sp

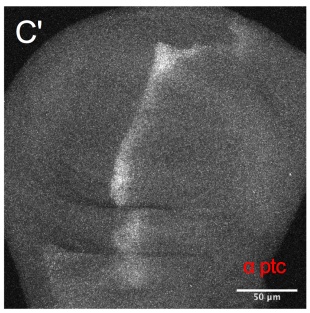
Figure 4

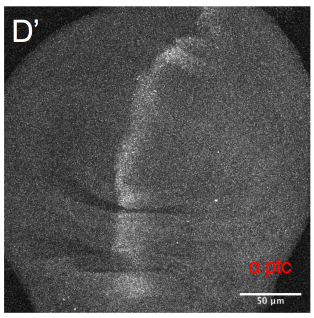

A.
*
dilp8 mRNA Expression
30
20
10
0
w1118
miR-184Δ

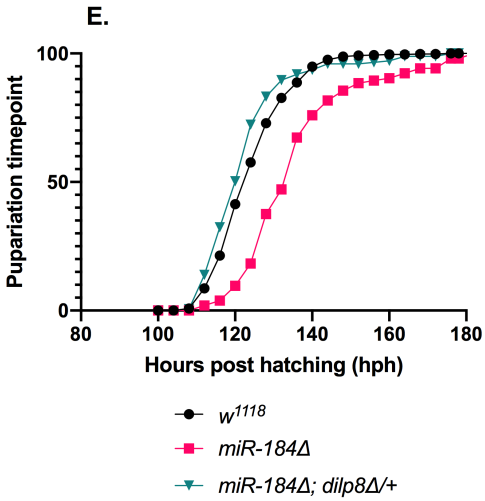

B.
*
dilp8 mRNA Expression
10
8
6
4
2
0
tubGAL4 > UAS Scramble
tubGAL4 > UAS miR-184-sp


C
dilp83'UTR
50 µm


D
dilp83'UTR
50 µm


C'
α ptc
50 µm


D'
α ptc
50 µm


E.
Pupariation timepoint
100
50
0
80
100
120
140
160
180
Hours post hatching (hph)
w1118
miR-184Δ
miR-184Δ; dilp8Δ/+

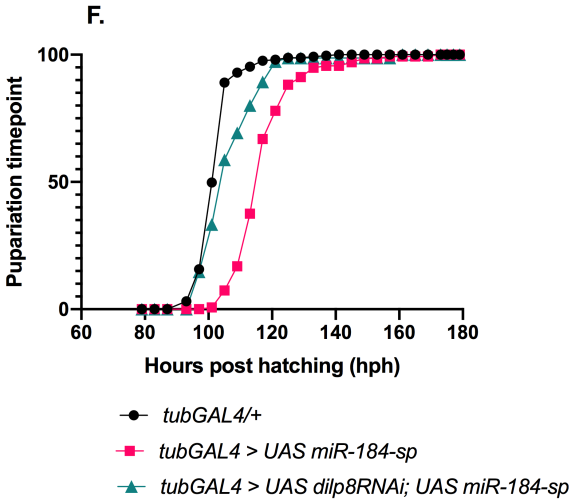

F.
Pupariation timepoint
100
50
0
60
80
100
120
140
160
180
Hours post hatching (hph)
tubGAL4/+
tubGAL4 > UAS miR-184-sp
tubGAL4 > UAS dilp8RNAi; UAS miR-184-sp

Figure 5

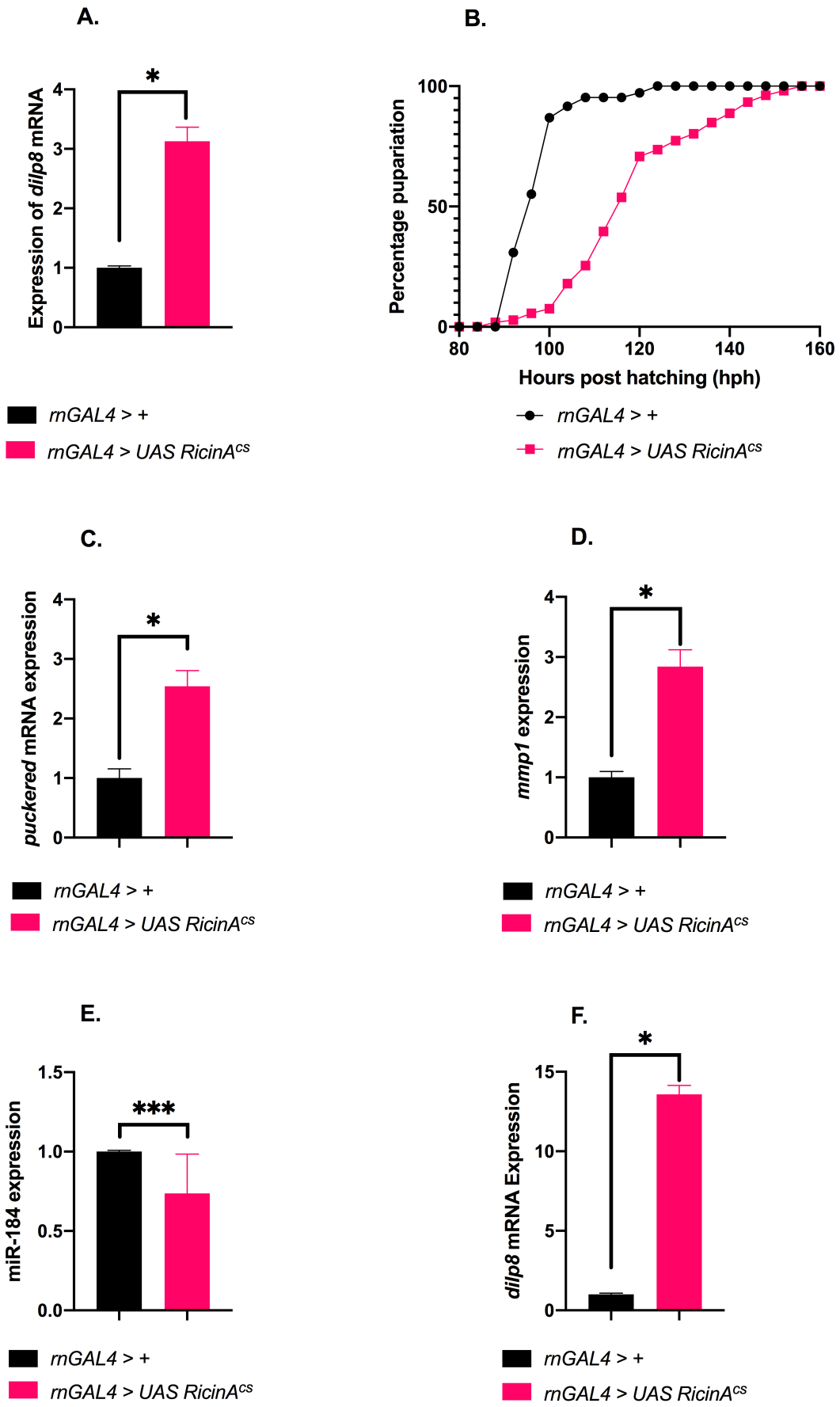
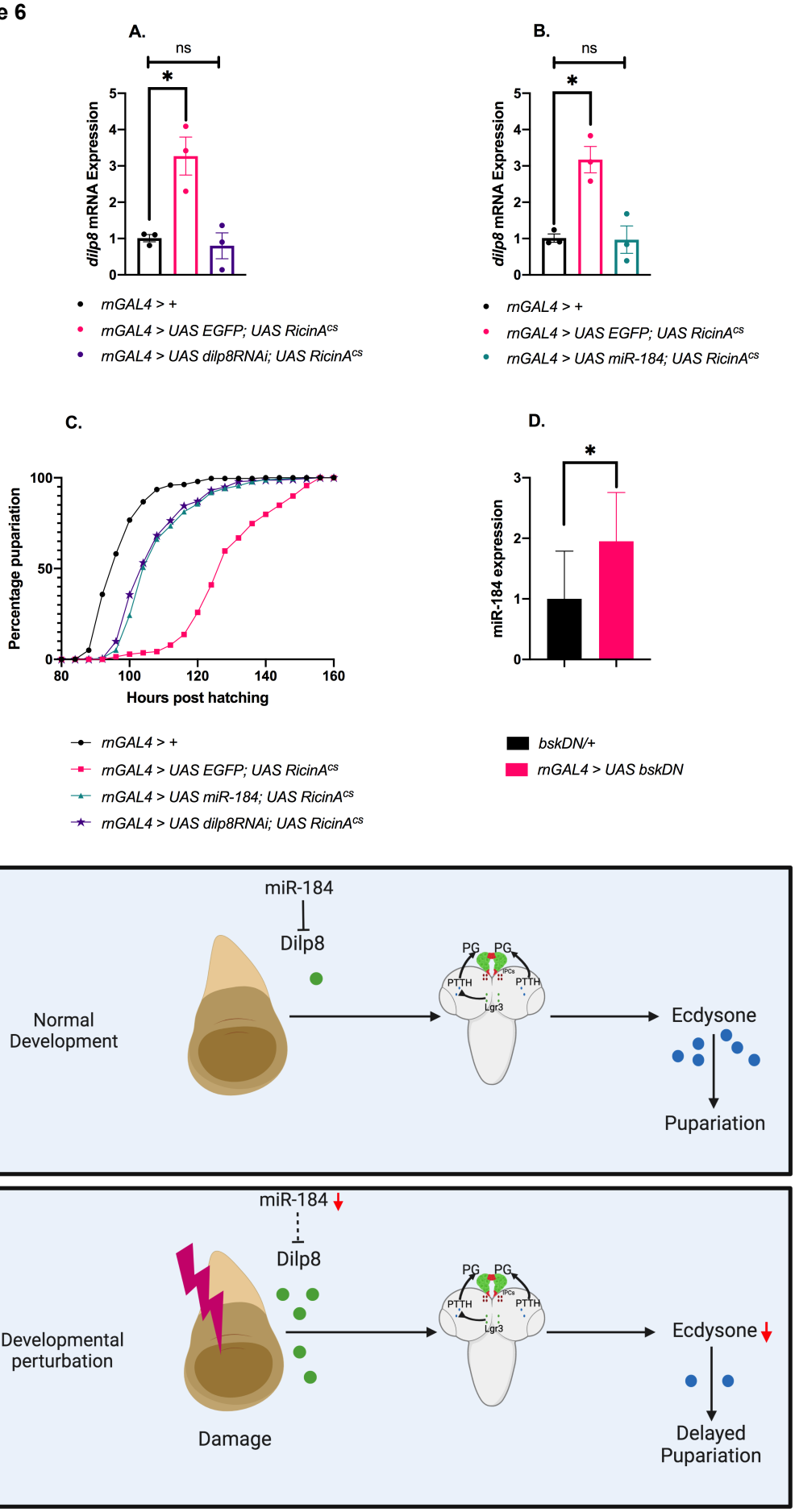

A.
Expression of dilp8 mRNA
0
1
2
3
4
*
rnGAL4 > +
rnGAL4 > UAS RicinA^CS
B.
Percentage pupariation
0
50
100
80
100
120
140
160
Hours post hatching (hph)
rnGAL4 > +
rnGAL4 > UAS RicinA^CS
C.
puckered mRNA expression
0
1
2
3
4
*
rnGAL4 > +
rnGAL4 > UAS RicinA^CS
D.
mmp1 expression
0
1
2
3
4
*
rnGAL4 > +
rnGAL4 > UAS RicinA^CS
E.
miR-184 expression
0.0
0.5
1.0
1.5
***
rnGAL4 > +
rnGAL4 > UAS RicinA^CS
F.
dilp8 mRNA Expression
0
5
10
15
*
rnGAL4 > +
rnGAL4 > UAS RicinA^CS

Figure 6


A.
ns
*
dilp8 mRNA Expression
0
1
2
3
4
5
mGAL4 > +
mGAL4 > UAS EGFP; UAS RicinA^cs
mGAL4 > UAS dilp8RNAi; UAS RicinA^cs
B.
ns
*
dilp8 mRNA Expression
mGAL4 > +
mGAL4 > UAS EGFP; UAS RicinA^cs
mGAL4 > UAS miR-184; UAS RicinA^cs
C.
Percentage pupariation
0
50
100
80
100
120
140
160
Hours post hatching
mGAL4 > +
mGAL4 > UAS EGFP; UAS RicinA^cs
mGAL4 > UAS miR-184; UAS RicinA^cs
mGAL4 > UAS dilp8RNAi; UAS RicinA^cs
D.
*
miR-184 expression
0
1
2
3
bskDN/+
mGAL4 > UAS bskDN
E.
miR-184
Dilp8
Normal
Development
PG
PG
PTTH
PTTH
Lgr3
Ecdysone
Pupariation
F.
miR-184
Dilp8
Developmental
perturbation
Damage
PG
PG
PTTH
PTTH
Lgr3
Ecdysone
Delayed
Pupariation