

# Supporting Information

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## SI Materials and Methods

**Drosophila Stocks.** Detailed genotypes of the flies used in this study are shown in Table S1. For studies of developmental fate, flies were allowed to lay eggs for 1 h on grape juice agar plates supplemented with yeast paste. Newly hatched larvae were transferred to Petri dishes (Falcon) filled with a standard *Drosophila* medium. Larvae were cultured at 25 °C under a 12-h light/dark cycle. Developmental stage and lethality were scored at 6-h intervals, starting at 84 hAH.

**qPCR.** Total RNA was extracted from whole larvae or the ring glands (RG) using the QIAGEN RNeasy Mini kit and the QIAGEN RNeasy Micro kit, respectively. RNA was reverse-transcribed using SuperScript III (Invitrogen), and the resultant cDNA was used as a template for qPCR using Quantifast SYBR Green PCR kit (QIAGEN). The amount of target RNA was normalized to an endogenous control, *ribosomal protein 49* (*rp49*), and then the relative expression level was calculated. The primer sets used for qPCR are summarized in Table S2.

**In Situ Hybridization.** Antisense and sense RNA probes were synthesized from the templates using an in vitro transcription kit (Roche, 11175025910) from the T3 and T7 promoters, respectively. RNA probes for *Octβ3R* transcripts were generated from an iPCR Collection clone obtained from the *Drosophila* Genomics Resource Center (clone ID = IP08282). Templates for other RNA probes were amplified by nested PCR from cDNA generated from third-instar larvae. The primer pairs used for PCR are shown in Tables S2 and S3. In situ hybridization of sectioned and whole-mount tissue was performed as previously described (1).

**Antibody Preparation.** An antibody against Neverland protein was raised in guinea pigs. A mixture of two synthetic peptides (NH<sub>2</sub>-QTELPWDLVPMGEIDDC-COOH and NH<sub>2</sub>-CFYSSNSKI-YSEATNIGW-COOH), which correspond to residues 207–223 and 413–429 of Neverland amino acid sequence (AB232987), respectively, was used for immunization.

**Immunostaining.** Larvae were dissected in PBS and fixed for 20 min in 4% (wt/vol) paraformaldehyde (PFA) in PBS. Tissues were washed three times for 15 min each in 0.1% Triton X-100 (Sigma) in PBS (PBTw), washed in 1% Triton X-100 in PBS for 15 min, blocked with 10% (vol/vol) goat serum in PBTw for 30 min, and then incubated at 4 °C overnight with primary antibodies diluted in blocking solution. Next, tissues were washed with PBTw three times for 10 min each and incubated at 4 °C overnight with the secondary antibodies in PBTw. The tissues were washed three times for 10 min each with PBTw and then incubated at room temperature for 15 min with To-Pro (Molecular Probes, T3605) diluted at a 1:1,500 in PBTw. After washing three times for 10 min each with PBTw, the tissues were mounted in Vectashield mounting medium (Roche). Confocal images were taken with a Leica TCS-SP5 microscope.

The following primary antibodies were used at the indicated dilutions: anti-diphosphorylated-ERK (Sigma, mouse monoclonal, M8159), 1:250; anti-GFP (Molecular Probes, A11122), 1:1,000; anti-Neverland, 1:1,000; anti-Shroud 1:1,000 (2); anti-Phantom (3), 1:200; anti-Disembodied (3), 1:200; and anti-Shadow (4), 1:200. Anti-Phantom, anti-Disembodied, and anti-Shadow were gifts from Michael B. O'Connor, University of Minnesota, Minneapolis.

Antibody detection was carried out using Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies (Molecular Probes).

**Tyramine and Octopamine Staining.** Tyramine staining was performed as previously described with minor modifications (5). Larvae were put on ice for 1 h before being prefixed with an opened cuticle for 5 min in 0.65% glutaraldehyde in 0.1 M sodium cacodylate buffer with 1% sodium metabisulfite (SMB). Next, the tissues were fixed at room temperature for 1 h, rinsed four times with Tris-HCl SMB (0.05 M Tris-HCl and 0.45% SMB), treated with 0.3% sodium borohydride in Tris-HCl SMB for 20 min, and then rinsed four times with Tris-HCl SMB and two times with Tris-HCl SMB TX (Tris-HCl SMB containing 0.3% Triton X-100). The tissues were blocked for 2 h with 10% (vol/vol) goat serum in Tris-HCl SMB TX, and then incubated at 4 °C for 48 h with the primary anti-tyramine antibodies (Chemicon International, rabbit polyclonal, AB124) diluted 1:250 in blocking solution. Subsequently, the tissues were rinsed six times with Tris-HCl TX and then incubated at 4 °C for 24 h with secondary antibodies in Tris-HCl TX. After washing five times with Tris-HCl TX, the tissues were incubated at room temperature for 15 min with TO-PRO-3 (Molecular Probes) diluted 1:1,500 in Tris-HCl TX. After washing three times with Tris-HCl TX, stained tissues were mounted in Vectashield mounting medium.

Octopamine staining was performed as described previously. Anti-octopamine antibody (Jena Bioscience, mouse monoclonal, ABD-029) was used at a 1:500 dilution.

Confocal images were collected using a Leica TCS-SP5 microscope. The confocal settings were the same for all images of the PG stained with the same antibody. To compare the expression of octopamine or tyramine in the PG of control and knockdown larvae, the gain for image acquisition was set such that the signal intensities in the brain lobe adjacent to the PG were at the same level.

**Temperature-Shift Experiments.** Larvae hatched at 25 °C were either cultured at 18 °C or at 28 °C. Following temperature upshift (18 °C to 28 °C) and downshift (28 °C to 18 °C) at the representative stages, the number of *phm* > *Octβ3R*<sup>RNAi-1</sup> + *dicer2*+*tub-Gal80*<sup>S</sup> animals developed into prepupae/pupae or arrested at the larval stage was counted until 200 hAH. The speed of development was accelerated at 28 °C, whereas it was decelerated at 18 °C, relative to the speed at 25 °C. We found that 50% of control larvae (*phm* > *dicer2*+*tub-Gal80*<sup>S</sup>) developed to prepupae until 81.4 hAH at 28 °C [number of animals examined (*n*) = 66], 93.0 hAH at 25 °C (*n* = 80), and 186.0 hAH at 18 °C (*n* = 86). According to the ratios of larval developmental time at different temperature [81.4 (28 °C)/93.0 (25 °C) and 186.0 (18 °C)/93.0 (25 °C)], the timescales at 28 °C and 18 °C were converted to the time scale at 25 °C.

**Starvation Experiment.** Transgenic animals expressing *PH-GFP* were used to visualize I1ps signaling activity in the PG. *Oregon R* animals were used for immunostaining of dpERK and tyramine. Larvae cultured in a standard *Drosophila* medium were transferred onto filter paper soaked in distilled water at 48 and 60 hAH, and immunostaining was performed at 60 and 66 hAH, and 66 hAH, respectively. Larvae cultured continuously in a standard *Drosophila* medium were used as a control.

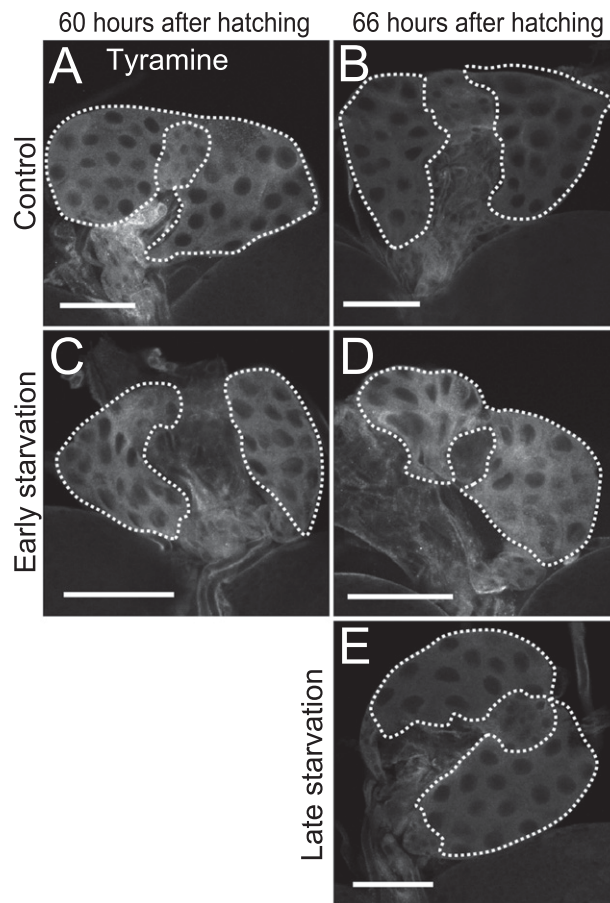




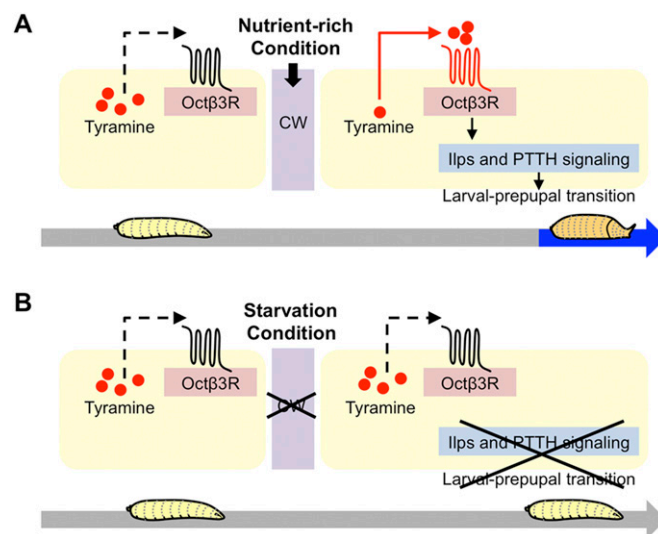








**Fig. S7.** Tyramine accumulates in the PG of larvae starved before the CW. Distribution of tyramine in control larvae (*A* and *B*), early starvation larvae (*C* and *D*), and late starvation larvae (*E*) (Fig. S6A). Immunostaining was performed using antibodies against tyramine at representative stages. Staining level of tyramine at 72 hAH (Fig. 3G) was comparable to that observed at 66 hAH (*B*). The PGs are outlined by dashed lines. (Scale bars, 50 μm.)



**Fig. S8.** A model for the regulation of metamorphosis by Octβ3R signaling. (*A*) Before the attainment of CW, tyramine is stored in the PG cell, so as not to activate Octβ3R. Once larvae have attained CW by growing beyond the critical period (at around 60 hAH under nutrient-rich conditions), tyramine is secreted from the PG to activate Octβ3R signaling in an autocrine manner, leading to the larval-prepupal transition (90–96 hAH) via the Ilps and PTTH signaling pathways. Our data strongly suggest that Octβ3R signaling must be active just after attainment of CW to execute the larval-prepupal transition. (*B*) When larvae fail to attain CW at around 60 hAH under a starvation condition, tyramine remains unsecreted from the PG; consequently, the Octβ3R, Ilps, and PTTH signaling pathways fail to be activated, resulting in arrest at the larval-prepupal transition.

**Table S1. Genotypes of the flies used in this study**

Abbreviations of fly genotypes*	Genotypes of female parents	Genotypes of male parents
<i>UAS-Octβ3R<sup>RNAi-1</sup></i>	<i>y w</i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup></i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup></i>	<i>y w;; phm-22-Gal4</i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup></i>
<i>UAS-Octβ3R<sup>RNAi-2</sup></i>	<i>y w</i>	<i>y w;; UAS-Octβ3R<sup>RNAi-2</sup></i>
<i>phm &gt; Octβ3R<sup>RNAi-2</sup></i>	<i>y w;; phm-22-Gal4</i>	<i>y w;; UAS-Octβ3R<sup>RNAi-2</sup></i>
<i>phm &gt; dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>y w</i>
<i>phm &gt; Octβ3R<sup>RNAi-2</sup>+dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>y w;; UAS-Octβ3R<sup>RNAi-2</sup></i>
<i>phm &gt; Tdc2<sup>RNAi-1</sup>+dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>w;; UAS-Tdc2<sup>RNAi-1</sup></i>
<i>phm &gt; Tdc2<sup>RNAi-2</sup>+dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>y v; UAS-Tdc2<sup>RNAi-2</sup></i>
<i>phm &gt; Tdc1<sup>RNAi</sup>+dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>y v;; UAS-Tdc1<sup>RNAi</sup></i>
<i>phm &gt; Tbh<sup>RNAi-1</sup>+dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>y w;; UAS-Tbh<sup>RNAi-1</sup></i>
<i>phm &gt; Tbh<sup>RNAi-2</sup>+dicer2</i>	<i>w; UAS-dicer2; phm-22-Gal4</i>	<i>y v;; UAS-Tbh<sup>RNAi-2</sup></i>
<i>phm-22-Gal4+PH-GFP</i>	<i>y w;; phm-22-Gal4</i>	<i>w;; PH-GFP</i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup>+PH-GFP</i>	<i>y w;; phm-22-Gal4</i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup> PH-GFP</i>
<i>phm-22-Gal4</i>	<i>y w;; phm-22-Gal4</i>	<i>w</i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup>+GFP</i>	<i>y w;; phm-22-Gal4 UAS-mCD8::GFP</i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup></i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup>+InR<sup>CA</sup></i>	<i>y w;; phm-22-Gal4</i>	<i>w; UAS-InR<sup>CA</sup>; UAS-Octβ3R<sup>RNAi-1</sup></i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup>+Ras<sup>V12</sup></i>	<i>y w;; phm-22-Gal4</i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup> UAS-Ras<sup>V12</sup></i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup>+Babo<sup>CA</sup></i>	<i>y w;; phm-22-Gal4</i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup> UAS-Babo<sup>CA</sup></i>
<i>phm &gt; dicer2+tub-Gal80<sup>ts</sup></i>	<i>w; UAS-dicer2; phm-22-Gal4 tub-Gal80<sup>ts</sup></i>	<i>w</i>
<i>phm &gt; Octβ3R<sup>RNAi-1</sup>+dicer2+tub-Gal80<sup>ts</sup></i>	<i>w; UAS-dicer2; phm-22-Gal4 tub-Gal80<sup>ts</sup></i>	<i>w;; UAS-Octβ3R<sup>RNAi-1</sup></i>

\*The flies used in this study were derived from the female parents mated with the males shown in the right.

**Table S2. Primer sets used for qPCR and in situ hybridization**

Gene name	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
Primer sets used for qPCR		
<i>rp49</i>	ACAAATGGCGCAAGCCCAAGG	ATGTGGCGGGTTCGCGCTTGTT
<i>neverland</i>	GGAAGCGTTGCTGACGACTGTG	TAAAGCCGTCCACTTCTCTCCGA
<i>spookier</i>	TATCTCTTGGGCACACTCGCTG	GCCGAGCTAAATTTCTCCGCTT
<i>shroud</i>	CCACAACATCAAGTCGGAAGGAGC	ACCAGGCGAATGGAATCCGGG
<i>cyp6t3</i>	CATCCGAGGGATTCTCACTG	AATCTCTCGGGATCAAAGACG
<i>phantom</i>	GGATTTCTTTTCGGCGCATGTG	TGCTCAGTATCGAAAAGCCGT
<i>disembodied</i>	TGCCCTCAATCCCTATCTGGTC	ACAGGGTCTTCACCCCATCTC
<i>shadow</i>	CCGCATTCAGCAGTCAGTGG	ACCTGCCGTGTACAAGGAGAG
<i>Tdc1</i>	CTTGGATCCGGTTACCTCA	GAAGAAGTTGGGGTGGTTCC
<i>Tdc2</i>	TTGGGGAGTTCCACTCAGTC	GGCCAGCTTGATATGGTGAC
<i>Tbh</i>	CTCGCAAGAGTTCGGTTTCC*	CTCCGGTAATTGGACGACCT*
	AAATGGAGTGCAGCAAGGAC†	GGTAGATCTCGTGCAGCTTTG†
Primer sets used for nested PCR		
<i>neverland</i>	TGGACAACGAAAAATGGTCA	CCATATCCACTTAGCCGTCAG
<i>spookier</i>	TCCCTATCTCTTGGGCACAC	CTTTGCCCTATGCATGTACG
<i>shroud</i>	CAACTTCTTCGCGCCCTCGG	ACTCGTAGGTGGGCATCGCG
<i>cyp6t3</i>	TACTTCAGGAGCCGTGGGATTCC	GATGGAGGCCGAGTATTGAGATG
<i>phantom</i>	TGGGAAACCAAGAAGCTGAC	GCTCCAGTTCCTCCAAAGTG
<i>disembodied</i>	AGTGGATGGAGTGACCAAGG	ACCCACAGCCTTTCAATCAC
<i>shadow</i>	GAGCAGGTGCAGGTGGTTAC	GCATCTCGTTAAGGCAGCTC
<i>Tdc1</i>	GGGATTCGAAAGTGATCGAG	GTCCCGCAGTATTTCTCAG
<i>Tdc2</i>	CAAATGGTCTGCTGACGAACCTCG	TCGCCACGGCACTCATAGTATTC
<i>Tbh</i>	ATCGGGTAACCGATACGTACACC	CCATTCGATGCTCCGGTAATTGG

\*Indicated by green arrows in Fig. S1D.

†Indicated by yellow arrows in Fig. S1D.



