Measurement of growth parameters

Time to pupariation, the time at which half the population had pupated, was calculated by recording the number of pupariated individuals every 12hrs. For measuring imaginal tissue area, tissues were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, mounted in glycerol, imaged by DIC on a Zeiss Axioplan2 microscope, and measured in ImageJ (NIH). The area of staged larvae was imaged, after a 10min treatment in PBS at 80°, on an Olympus DP21 microscope digital camera when viewed from the dorsal aspect, and measured in ImageJ.

Indirect immunofluorescence

Dissected tissues were fixed for 20 minutes in 4% paraformaldehyde, washed in PBS with 0.3% Triton-X100 to permeabilize cells, treated with primary antibodies (overnight at 4°; rabbit anti-cleaved Caspase-3 (Asp175) 1:100, Cell Signaling Technology, MA), and secondary antibodies (4 hrs at room temperature). Cell death detection by TUNEL with TMR red fluorescent probe (Hoffmann-La Roche, Basel, Switzerland) was performed following manufacturer instructions. Labeling buffers were mixed with secondary antibody stain and incubated for 2hrs at 37°.

Ecdysone measurements

Ecdysone levels in third instar larvae were quantified using a competitive enzyme immunoassay (Cayman Chemicals, MI) as described previously (Hackney et al. 2012).

NADPH-diaphorase assay

NOS enzymatic activity was detected by measuring NADPH-diaphorase activity through an adapted method (Elphick 1997). Tissues were fixed for 1hr in 4% paraformaldehyde and then permeabilized in 0.3% Triton X-100 for 20min. Fixed tissues were suspended in NADPH-diaphorase staining solution in the dark for 15min, then washed in PBS, mounted in 80% glycerol, and imaged by DIC.

PCR

Semi-quantitative PCR

RNA was isolated from staged larvae using TRIzol reagent treatment (Invitrogen-Life Technologies, CA) followed by RNeasy cleanup (Qiagen, Limburg, Netherlands) and DNase treatment with the Turbo DNase-kit (Ambion-Life Technologies, CA). RNA yield was quantified by using UV spectroscopy to measure A260. cDNA template for RT-PCR was generated using 1µg sample RNA as a substrate for Roche Transcriptor first strand cDNA synthesis using poly dT primers. Polymerase chain reaction (PCR) was performed with TaKaRa Ex Taq DNA Polymerase (Takara, Otsu, Japan)
in a MJ research PTC-200 DNA Engine Cycler. Conditions for amplification were as follows: 94° for 2 minutes, then 94° for 15 seconds, 60° for 15 seconds, and 72° for 15 seconds for 23 cycles with Tubulin primers or 31 cycles with E75B primers. Amplified products were then identified by electrophoresis on a 3% agarose gel and visualized with SYBR Green (Life Technologies, CA) through epifluorescent analyzer (Fujifilm Intelligent Lightbox LAS-3000). Relative expression differences were measured in ImageJ in relation to tubulin expression. Primers: E75B (Moeller et al. 2015), tubulin (tub-L CTCATAGCCGGCAGTTCG)(tub-R GATAGATACATTACGCATATTGAG).

Quantitative RT-PCR

RNA was isolated and cDNA was generated as described above except for Fig. S7, which used ReliaPrep™ RNA Cell and Tissue Miniprep Systems (Promega) and poly dT primers with random hexamer primers. cDNA was analyzed using a Mastercycle EP Replex real-time PCR system (Eppendorf). Fold change was calculated relative to tubulin expression by the -ΔΔCt method [53]. Isolates were taken from at least three sets of larval stagings to calculate the mean fold change. Two to three independent RNA isolations were assayed within each staging and used to calculate standard error of the mean across stagings. Primers: E74B (Colombani et al. 2015), spookier (spo-L CGGTGATCGAAACAACTCACTGG, spo-R GGATGATTCCCGAGGAGCAG), disembodied (dib-L AGGCTGCTGCGTAATCAG, dib-R TCGATCAGCCTGGAGCAGC).

Ecdysone media

Exogenous application of ecdysteroid was preformed as previously described (Halme et al. 2010). Briefly, larvae were transferred at 80hrs AED (Bx>eiger, Tub>dilp8, Bx>dilp8, hs>NOSnec) or 124hrs AED (phm>NOS) to either 0.6 mg 20-hydroxyecdysone (Sigma) dissolved in 90% ethanol/ml of media, or an equivalent volume of ethanol alone. For ecdysone restriction assays, a defined yeast media was prepared with the erg-6 mutant yeast strain, sucrose, and agar (Bos et al. 1976, Parkin et al. 1986), and larvae were transferred from standard media to erg6Δ or erg6ΔΔ media at 80hrs AED.

Supporting Information Literature Cited
