Egfr Signaling Is a Major Regulator of Ecdysone Biosynthesis in the Drosophila Prothoracic Gland

Highlights
- Egfr signaling regulates steroid hormone ecdysone biosynthesis in the PG
- Halloween gene expression and ecdysone vesicle secretion relies on Egfr pathway
- Egf ligands Vn and Spi activate Egfr signaling in the PG in an autocrine manner
- Egfr signaling and ecdysone act in a positive feedback loop circuit in the PG

Authors
Josefa Cruz, David Martín, Xavier Franch-Marro

Correspondence
david.martin@ibe.upf-csic.es (D.M.), xavier.franch@ibe.upf-csic.es (X.F.-M.)

In Brief
Levels of the steroid hormones dictate the final size of insects by triggering metamorphosis. Steroid synthesis is thought to be mainly induced by Ptth/torso signaling in the prothoracic gland. Cruz et al. now provide evidence that Egfr signaling, rather than Ptth/torso, is the major contributor of steroid biosynthesis in Drosophila.
Egfr Signaling Is a Major Regulator of Ecdysone Biosynthesis in the Drosophila Prothoracic Gland

Josefa Cruz,1 David Martín,1,∗ and Xavier Franch-Marro1,2,∗

1Institute of Evolutionary Biology (IBE, CSIC-Universitat Pompeu Fabra), Passeig de la Barceloneta 37, 08003 Barcelona, Catalonia, Spain
2Lead Contact
∗Correspondence: david.martin@ibe.upf-csic.es (D.M.), xavier.franch@ibe.upf-csic.es (X.F.-M.)
https://doi.org/10.1016/j.cub.2020.01.092

SUMMARY

Understanding the mechanisms that determine final body size of animals is a central question in biology. In animals with determinate growth, such as mammals or insects, the size at which the immature organism transforms into the adult defines the final body size, as adult individuals do not grow [1]. In Drosophila, the growth period ends when the immature larva undergoes the metamorphic transition to develop the mature adult [2]. This metamorphic transition is triggered by a sharp increase of the steroid ecdysone, synthesized in the prothoracic gland (PG), that occurs at the end of the third instar larvae (L3) [3–6]. It is widely accepted that ecdysone biosynthesis in Drosophila is mainly induced by the activation of tyrosine kinase (RTK) Torso by the pro-thoracicotropic hormone (Ptth) produced in two pairs of neurosecretory cells that project their axons onto the PG [7, 8]. However, the fact that neither Ptth nor torso-null mutant animals arrest larval development but only present a delay in the larva-pupa transition [9–11] mandates for a reconsideration of the conventional model. Here, we show that Egfr signaling, rather than Ptth/torso, is the major contributor of ecdysone biosynthesis in Drosophila. We found that Egfr signaling is activated in the PG in an autocrine mode by the EGF ligands spitz and vein, which in turn are regulated by the levels of ecdysone. This regulatory positive feedback loop ensures the production of ecdysone to trigger metamorphosis by a progressive Egfr-dependent activation of MAPK/ERK pathway, thus determining the animal final body size.

RESULTS AND DISCUSSION

In contrast to the developmental delay phenotype observed in larvae with reduced Ptth or torso, we found that specific depletion of Drosophila homolog transducers ras (ras85D), Raf onco-gene (Raf), and ERK, the core components of the MAPK/ERK pathway, in the prothoracic gland (PG) using the pphmGal4 driver (pphm>) induced developmental arrest at L3 (Figures 1A, 1B, and S1). This result suggests that additional RTKs might play important roles in ecdysone production. To study this possibility, we knocked down all known Drosophila RTKs in the PG and found that only depletion of Egfr phenocopied L3 arrested development observed in pphm > ras85D larvae (Figures 1A and 1B). Likewise, overexpression in the PG of a dominant-negative form of Egfr (EgfrDN) or depletion of the transcription factor pointed (pnt), the principal nuclear mediator of the Egfr signaling pathway [12, 13], also resulted in arrested L3 larvae (Figures 1A and 1B). Same results were obtained upon inactivation of Egfr or different components of the MAPK/ERK pathway using an alternative PG specific driver, amncG551Gal4 [14] (Figure S1A). Consistent with the observed phenotypes, overexpression of a constitutively activated form of either Egfr (EgfracT) or Pnt (PntP2Vp16) in the PG induced premature pupariation and reduced pupal size (Figures 1B and 1C). These results are in agreement with a previous report showing that overexpression of a constitutively activated form of Ras (RasV12) in the PG produced the same phenotype [14]. Furthermore, overexpression of RasV12 in Egfr-depleted larvae rescued the developmental arrest phenotype and forced premature pupation (Figures 1B and 1C). These results strongly suggest that Egfr signaling in the PG is required for the synthesis of the ecdysone pulse that triggers metamorphosis. Confirming this hypothesis, ecdysone titers in larvae depleted of either Egfr or pnt in the PG were dramatically reduced (Figure 1D). Accordingly, Hr3 and Hr4 expression, two direct target genes of the hormone that have been used as readouts for ecdysone levels [7, 8, 15], was completely abolished in pphm > EgfracT and pphm > pntRNAi L3 larvae compared to control animals (Figure 1E). Moreover, addition of the active form of ecdysone, 20-hydroxyecdysone (20E), to the food rescued the developmental arrest phenotype induced by inactivation of Egfr signaling in the PG (Figure 1F). Altogether, these results indicate that Egfr signaling in the PG endocrine cells is required for the production of the ecdysone pulse that triggers pupariation and fixes adult body size.

Since Egfr signaling is involved in cell proliferation and survival [9–11, 16–19], we analyzed whether the above-described phenotype was due to compromised viability of PG cells. Although reduced activation of Egfr signaling diminished cell size, PG cell number and viability were not affected (Figures S1B–S1D). Interestingly, ecdysone synthesis has been recently shown to correlate with endocyte progression and therefore cell size of PG cells [12, 13, 20]. PG cells undergo three rounds of endoreplication during larval development resulting in chromatin values (C values) of 32–64 C by late L3 (Figure S1E). Remarkably, we observed a clear reduction in the C value of PG cells of pphm > EgfracT larvae at 120 h AEL, with most cells

This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
at 8–16 C (Figures S1E and S1F), indicating that Egfr activation is also required to promote polyploidy in the PG cells.

This result raised the possibility that Egfr signaling regulates ecdysone production by determining the size of the PG. To analyze this hypothesis, we examined the effect of Egfr signaling in ecdysone production. Steroidogenesis in the PG cells depends on the timely expression of ecdysone biosynthesis enzyme-encoding genes that mediate the conversion of cholesterol to ecdysone (Figure 1G) [14, 21]. To analyze whether Egfr signaling controls ecdysone synthesis by regulating the expression of these genes, we performed qRT-PCR in early (72 h after egg laying [AEL]), mid (96 h AEL), and late (120 h AEL) phm > EgfrRNAi and phm > pntRNAi larvae. Whereas expression of the six ecdysone biosynthetic genes increased gradually from mid to late L3 in control larvae, correlating with the production of the high-level ecdysone pulse that triggers metamorphosis [1, 22], inactivation of the Egfr pathway in the PG resulted in a dramatic reduction in the expression levels of neverland (nvd), spook (spo), shroud (sro), and phantom (phm) in late L3 larvae (Figure 1G). In contrast, the expression of disembodied (dib) and shadow (sad) was not significantly reduced in Egfr-depleted larvae, which suggests that compromising Egfr signaling in the PG does not result in a general reduction in the transcriptional activity by its minor C value, as previously shown [20], but rather by a specific transcriptional effect (Figure 1G). Further confirming this point, the overexpression of CycE in Egfr-depleted PGs was unable to restore normal expression of ecdysteroid biosynthetic genes nor induced proper pupariation of these animals (Figures S1F–S1H), indicating that Egfr signaling is required for proper expression of ecdysone enzyme-encoding genes independently of promoting polyploidy of PG cells.

As the levels of circulating ecdysone are influenced by the rates of hormone production and release, we next studied whether Egfr signaling also regulates ecdysone secretion. Recently, it has been shown that ecdysone secretion from the PG cells is mediated by a vesicular regulated transport mechanism [23]. After its synthesis, ecdysone is loaded through an ATP-binding cassette (ABC) transporter, Aet, into Syt1-positive secretory vesicles that fuse to the cytoplasmic membrane for release of the hormone in a calcium-dependent signaling [23].
To analyze the role of Egfr signaling in this process, we visualized secretory vesicles in PG cells of phm > EgfrRNAi and phm > pntRNAi L3 larvae by expressing eGFP-tagged Syt1 (Syt-GFP) in these glands [24, 25]. Whereas Syt-GFP vesicles accumulate at the plasma membrane with a small number of vesicles in the cytoplasm in wild-type L3 larval PGs, a dramatic accumulation of Syt-GFP vesicles in the cytoplasm was observed in PGs with reduced Egfr signaling (Figures 2 and S2A). Similar results were obtained when we analyzed the subcellular localization of the ecdysone transporter Atet-GFP [23] (Figures 2 and S2A). Consistently, overexpression of rasV12 in PGs of phm > EgfrRNAi larvae restored the subcellular localization of both Syt and Atet-GFP (Figure 2). Furthermore, mRNA levels of several genes involved in vesicle-mediated release of ecdysone [23], including Syt and Atet, were dramatically downregulated in the PG of phm > EgfrRNAi and phm > pntRNAi larvae (Figure S2B). Therefore, the results show that Egfr signaling is also required for the vesicle-mediated release of ecdysone from PG cells. Interestingly, direct effects of Egfr signaling on the endocytic machinery have been already described in Drosophila tracheal cells as well as in human cells [26–33].

The next question was to determine which of the EGF ligands were responsible for the Egfr pathway activation in the PG. In Drosophila, Gurken (Gur), Spitz (Spi), Keren (Krn), and Vein (Vn) serve as ligands for Egfr [34]. Expression analysis of the four ligands revealed that only vn and spi were expressed in the PGs involved in vesicle-mediated release of ecdysone [23].

Figure 2. Egfr Signaling Regulates Ecdysone Vesicle Localization
(A) PG image of control, EgfrRNAi, pntRNAi, and EgfrRNAi; rasV12 larva at 96 h AEL overexpressing either Syt-GFP (phm22 > Syt-GFP) or YPet-Atet (phm22 > YPet-Atet). Magnified view of the PG cells shows the aggregation of small vesicle-like structures along the membrane (arrowheads). Note that compared to the control, in EgfrRNAi and pntRNAi PG cells vesicles accumulate in the cytoplasm. Overexpression of rasV12 rescues the vesicles aggregation in the cytoplasm induced by depletion of EgfrRNAi. The scale bars represent 100 μm in top panels and 25 μm in magnified views. See also Figure S2 for related results.
revealed that
ure 3A). A temporal expression pattern of staged L3 PGs re-
See also Figure S3 for related results. Average values of 3 independent datasets are shown with standard errors. Statistical significance was calculated using t test (*p < 0.05 and **p
without 20E and processed for qRT-PCR measurements of vn, spi, and Hr4. Hr4 was used as a positive control to the activation of the ecdysone signaling. Average values of 3 independent datasets are shown with standard errors. Statistical significance was calculated using t test (p < 0.05 and **p ≤ 0.005).
See also Figure S3 for related results.

(Figures 3A and S3A–S3D). Consistently, the intramembrane protease rhomboid (rho), which is necessary for the proteolytic activation of Spi [35], was also expressed in the PG cells (Figure 3A). A temporal expression pattern of staged L3 PGs revealed that rho expression progressively increased during the last larval stage, while the expression of spi and vn increased sequentially, with vn upregulated at mid L3 and spi at late L3 (Figure 3B). Consistent with the expression of the ligands, mRNA levels of Egfr also showed a clear upregulation by late L3 (Figures 3B and S3B). Likewise, a specific expression of PntP2 isoform was also observed in the PG of late L3 larvae (Figures S3F and S3G). Altogether, these results suggest that Vn and Spi might activate Egfr signaling in an autocrine manner to induce ecdysone production.

To determine the functional relevance of each ligand, we knocked down vn, spi, or both simultaneously in the PG. As in the case of phm > EgfrRNAi, depletion of spi, vn, or both ligands at the same time caused developmental arrest in L3, although Spi appeared to have a minor effect as around 40% of phm > spiRNAi larvae underwent delayed pupariation (Figures 3C and 3D). The attenuated effect of spi-depleted animals was probably due to a weaker effect of the spiRNAi lines as depletion of the Spi-processing protease rho in the PG resulted in all phm > rhoRNAi animals arresting development at L3 (Figures 3C and 3D). Importantly, ecdysteroid levels in mid and late L3 were significantly reduced in animals depleted of either vn or spi (Figure 3E). Consistent with their role in controlling ecdysone production, overexpression of either Vn or an active-cleaved form of Spi in the PG induced precocious pupariation and smaller pupae (Figures 3C and 3D). Altogether, these findings show that spi and vn act in an autocrine manner as Egfr ligands in the PG to induce ecdysone biosynthesis during the last larval stage. In fact, the correlation between vn and spi expression with the occurrence of increasing levels of ecdysteroids points to a possible positive-feedback loop regulation with 20E inducing vn and spi expression. Consistent with this possibility, vn and spi mRNA levels were reduced in PGs of ecdysteroid deficient larvae that were generated by depleting spo (phm > spoRNAi) or by overexpressing a dominant-negative form of the ecdysone receptor (phm > EcRDN) (Figure 3F). Moreover, we cultured staged PGs for 6 h ex vivo in presence or absence of 20E and found that vn and spi mRNA levels were significantly upregulated in the presence of the hormone (Figure 3G). Altogether, these observations demonstrate that ecdysone exerts a positive-feedback effect on PG cells amplifying its own synthesis by inducing the expression of vn and spi. This result is consistent with a previous proposed

Figure 3. The Egf Ligands Vn and Spi Activate Egfr Signaling in the PG

(A) RNA in situ hybridization shows the expression of vn, spi, and rho specifically in the PG cells. Note the absence of signal in the CA (black arrowheads) and CC (white arrowheads).

(B) mRNA levels of Egfr, vn, spi, and rho of control larvae at 72, 96, and 120 h AEL, measured by qRT-PCR.

(C) Overexpression of vnRNAi, spiRNAi, both vnRNAi and spiRNAi, and rhoRNAi in the PG induces arrested development. In contrast, expression of either vn or spi in the PG results in accelerated pupariation and smaller pupae.

(D) Percentages of pupariated of control, UASvn, UASSpi, animals reared at 18°C until early L3 and then switched to 29°C and control, rhoRNAi, vnRNAi, spiRNAi and vnRNAi; spiRNAi animals reared at 18°C until L2 and then switched to 29°C, shown at indicated stages. Numbers of animals analyzed are indicated in parenthesis.

(E) ELISA measurements of whole-body ecdysteroid levels in control, vnRNAi, and spiRNAi larvae at 96 and 120 h AEL. Average values of 3 independent datasets are shown with standard errors. Statistical significance was calculated using t test (*p < 0.05 and **p ≤ 0.005).

(F) Expression of vn and spi of dissected ring glands (RGs) in control, pmh > EcRDN, and pmh > spoRNAi animals measured by qRT-PCR. Average values of three independent datasets are shown with standard errors. Asterisks indicate differences statistically significant at *p ≤ 0.05 and **p ≤ 0.005 (t test).

(G) vn and spi are activated by 20E in the ring gland. Ring glands dissected from third instar larvae at 96 and 120 AEL were cultured with and without 20E and processed for qRT-PCR measurements of vn, spi, and Hr4. Hr4 was used as a positive control to the activation of the ecdysone signaling.
model of ecdysone regulation in an autonomous mechanism by a positive feedback and biogenic amines [36, 37]. Thus, we proposed a model in which increasing levels of ecdysone promote the expression of vn and spi in the PG cells, which, in turn, increases Egfr signaling in this gland in an autocrine manner to further promote the production of ecdysone. Interestingly, it has been already shown that expression of Spi and Vn in midgut cells of Drosophila depends on ecdysone activity during metamorphosis [38]. In addition, in vertebrates, other hormones have been postulated to control Egfr activity, such as Trophin-releasing hormone [38]. In addition, in vertebrates, other hormones have been postulated to control Egfr activity, such as Trophin-releasing hormone [38].

Thus far, the results above show that MAPK/ERK pathway is a central regulatory element in the control of ecdysone biosynthesis in the PG, with Egfr signaling chiefly contributing to its activity. However, since Ptth/torso signaling operates through the same MAPK/ERK pathway we investigated the relative contribution of this signaling pathway in the overall activity of the PG. The fact that inactivation of Egfr signaling in the PG did not affect the mRNA expression levels of either Ptth or torso (Figure S4A) points to a minor contribution of Ptth/torso signaling in the overall MAPK/ERK activity. To analyze this possibility, we compared the levels of dpERK, a readout of MAPK/ERK activity [35], in PGs of phm > EgfrRNAi and phm > torsoRNAi larvae. As Figure 4A shows, a dramatic reduction of dpERK levels was observed in PGs of phm > EgfrRNAi larvae. Importantly, dpERK levels were also reduced in phm > torsoRNAi PGs, although to a significant lesser extent when compared to phm > EgfrRNAi larvae (Figure 4A). Similar results were observed when nuclear accumulation of dpERK was analyzed in both larvae (Figure 4B). consistently, the level of activity of the MAPK/ERK pathway in phm > pntRNAi and phm > torsoRNAi larvae correlated very well with expression of the biosynthetic enzyme phm and the ecdysone-responsive genes Hr3, Hr4, and Broad-Complex (BrC) (Figure 4C), although the levels of ecdysone were significantly reduced in both cases.
(Figure 4D). The different level of activation of dpERK by Egfr and Pth/torso signaling was also consistent with the respective accumulation of Syt-GFP and Ate1-GFP vesicles at the cytoplasm (Figures 4E and 4B) and the reduction of the C value of PG cells (Figures 4F and S4C). Finally, it is important to note that the level of activity of the MAPK/ERK pathway correlated with the respective phenotypes upon inactivation of each pathway, with **phm** > **EgfrRNAi** larvae arresting development at L3 and **phm** > **torsoRNAi** larvae presenting only a delay in the pupariation time (Figure 4G). In line with this, whereas over-activation of Egfr pathway in the PG of **phm** > **torsoRNAi** larvae induced a significant advancement in pupariation (Figure 4G), the expression of a constitutively activated form of Torso (**torsoD4021** mutants) in PGs with depleted **Egfr (EgfrRNAi; torsoD4021)** was not able to induce precocious pupariation (Figure 4G).

Overall, these results show that the Egfr signaling pathway plays the main role in the biosynthesis of ecdysone by activating the MAPK/ERK pathway in the PG during mid-late L3, whereas Pth/torso signaling acts synergistically only to increase the MAPK/ERK pathway activity thus accelerating developmental timing. In this regard, it is possible that the different strength of MAPK/ERK activation by the two signaling pathways might underlie this distinct requirement of each pathway. Furthermore, temporal expression of the Egfr and Torso ligands may also contribute to the difference strengths of MAPK/ERK activation, as EGF ligands **vn** and **spi** are highly expressed during L3, whereas **Pth** is only upregulated at a specific developmental stage, the wandering stage [8]. Taken together, these data suggest a model in which the increasing circulating levels of ecdysone during the last larval stage are induced by a progressive Egfr dependent activation of MAPK/ERK in the PG, whereas Pth/torso signaling further regulates ecdysone production by integrating different environmental signals such as nutritional status, crowding conditions, and light [11]. It is important to note that, in addition to the Egfr and Pth/torso pathways, ecdysone biosynthesis is also regulated by the insulin/insulin-like growth factor signaling (IIS)/target of Rapamycin (TOR) signaling pathway [14, 41–43]. However, in contrast to the major role of Egfr controlling ecdysteroid levels during mid-late L3, including the strong ecdysosterone pulse that triggers pupariation, the main effect of IIS/TOR pathway is to control the production of the small ecdysosterone peak that is associated to the nutrition-dependen
critical weight checkpoint that occurs at the very early L3 [44]. Thus, decreasing the IIS/TOR activity in the PG delays the critical weight checkpoint, slowing development and delaying pupariation, while increasing IIS/TOR activity in the gland induces precocious critical weight and accelerates the onset of metamorphosis [14, 41–43]. Nevertheless, it is conceivable that the increasing levels of ecdysone at the critical weight checkpoint might initiate the expression of the Egfr ligands, that in turn activates the ecdysone production during mid-late L3.

Finally, since no role of Pth/torso signaling has been characterized in hemimetabolous insects, we postulate that Egfr signaling might be the ancestral ecdysone biosynthesis regulator, whereas Pth/torso signaling has probably been co-opted in holometabolous insects during evolution to fine-tune the timing of pupariation in response to changing environmental cues. Consistent with this view, depletion of **Gb-Egfr** in the hemimetabolous insect *Gryllus bimaculatus*, where no Pth/torso has been described, results in arrested development by the last nymphal instar [45]. Therefore, this double regulation in holometabolous insects might provide developmental timing plasticity contributing to an appropriated adaptation to a time-limited food supply.
Antagonistic actions of ecdysone and insulins determine final size in Drosophila. Science 310, 667–670.

43. Mirth, C., Truman, J.W., and Riddiford, L.M. (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. Curr. Biol. 15, 1796–1807.

44. Koyama, T., Rodrigues, M.A., Athanasiadis, A., Shingleton, A.W., and Mirth, C.K. (2014). Nutritional control of body size through FoxO-Ultraspireacle mediated ecdysone biosynthesis. eLife 3, https://doi.org/10.7554/eLife.03091.

45. Dabour, N., Bando, T., Nakamura, T., Miyawaki, K., Mito, T., Ohuchi, H., and Noji, S. (2011). Cricket body size is altered by systemic RNAi against insulin signaling components and epidermal growth factor receptor. Dev. Growth Differ. 53, 857–869.

46. Avet-Rochex, A., Kaul, A.K., Gatt, A.P., McNeill, H., and Bateman, J.M. (2012). Concerted control of gliogenesis by InR/TOR and FGF signalling in the Drosophila post-embryonic brain. Development 139, 2763–2772.

47. Jiang, H., and Edgar, B.A. (2009). EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors. Development 136, 483–493.

48. O’Keefe, L., Dougan, S.T., Gabay, L., Raz, E., Shilo, B.Z., and DiNardo, S. (1997). Spitz and Wingless, emanating from distinct borders, cooperate to establish cell fate across the Engrailed domain in the Drosophila epidermis. Development 124, 4837–4845.

49. Queenan, A.M., Ghabrial, A., and Schüpbach, T. (1997). Ectopic activation of torpedo/Egfr, a Drosophila receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development 124, 3871–3880.

50. Halfon, M.S., Carmena, A., Gisselbrecht, S., Sackerson, C.M., Jiménez, F., Baylies, M.K., and Michelson, A.M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. Cell 103, 63–74.

51. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

52. McGuire, S.E., Mao, Z., and Davis, R.L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci. STKE 2004, pl6.

53. Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98, 81–85.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-α-Tubulin antibody, Mouse monoclonal clone DM1A, purified from hybridoma cell culture | Sigma-Aldrich | Cat# T6199; RRID:AB_477583 |
| Monoclonal anti-MAP Kinase, activated (Diphosphorylated Erk-1/2) antibody produced in mouse | Sigma-Aldrich | Cat# M8159; RRID:AB_477245 |
| Goat anti-mouse immunoglobulins/HRP polyclonal | Dako | Cat# P0447; RRID:AB_2617137 |
| anti-PntP2 antibody produced in rat | [46] | N/A |
| anti-β-Galactosidase antibody produced in mouse | Developmental Studies Hybridoma Bank | Cat# 40-1a; RRID:AB_528100 |
| Alexa Fluor 555 goat anti-rat IgG (H+L) | Molecular probes -Invitrogen | Cat# A-21434; RRID:AB_141733 |
| Alexa Fluor 555 goat anti-mouse IgG (H+L) | Molecular probes -Invitrogen | Cat# A-21422; RRID:AB_141822 |
| Anti-digoxin-AP Fab fragments | Roche | Cat# 11093274910; RRID:AB_514497 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| [l-Ecdysone, 2][l,3][l,14±,20][l,22,25-Hexahydroxy-7-cholesten-6-one, Ecdysterone, Insect moulting hormone, Polydine A] | Sigma-Aldrich | Cat#: H5142 |
| NBT/BCIP Stock Solution | Roche | Cat#: 11681451001 |
| Formaldehyde | Sigma-Aldrich | Cat#: 1635 |
| Ethanol | Carlo Erba reagents | Cat#: 4146072 |
| Ribonucleic acid transfer from baker’s yeast | Sigma-Aldrich | Cat#: R5636 |
| Deionized formamide | Fluka | Cat#: 47670 |
| Deoxyribonucleic acid sodium salt from salmon testes | Sigma-Aldrich | Cat#: D6186 |
| Heparin sodium salt from porcine intestinal mucosa | Sigma-Aldrich | Cat#: H3393 |
| Bovine Serum Albumin | Sigma-Aldrich | Cat#: A2153 |
| Vectashield medium with DAPI | Vector Laboratories | Cat#: H1200 |
| Triton X-100 | United States Biochemical | Cat#: 22686 |
| Tween-20 | Sigma-Aldrich | Cat#: P9416 |
| RO1 Dlase | Promega | Cat#: M198A |
| iTaq Universal SYBR Green Supermix | BioRad | Cat#: 1725121 |
| Laemmli Sample Buffer | Bio-Rad | Cat#: 1610747 |
| 2-mercaptoethanol | Sigma-Aldrich | Cat#: M3148 |
| Schneider’s medium | GIBCO | Cat#: 21720024 |
| Heat-inactivated fetal bovine serum | GIBCO | Cat#: 16140063 |
| SuperSignal West Pico Chemiluminescent Substrate | ThermoScientific | Cat#: 34080 |
| Methanol | Carlo Erba reagents | Cat#: 414814 |
| **Critical Commercial Assays** |        |            |
| In Situ Cell Death Detection Kit, TMR red | Roche Applied Science | Cat#: 12156792910 |
| DIG RNA Labeling Mix | Roche | Cat#: 1127703910 |
| 20-Hydroxiedysone ELISA kit | Bertin Bioreagents | Cat#: A05120 |
| GenEluteMammalian Total RNA miniprepKit | Sigma-Aldrich | Cat#: RTN70 |
| Transcriptor First Strand cDNA Synthesis kit | Roche | Cat#: 04379012001 |
| **Experimental Models: Organisms/Strains** |        |            |
| UAS CyE-1 | Bloomington Drosophila Stock Center | RRID: BDSC_30725 |
| UAS-ras85D* RNAi HM6501294 | Bloomington Drosophila Stock Center | RRID: BDSC_34619 |
| UAS-rasDN RNAi | Bloomington Drosophila Stock Center | RRID: BDSC_4845 |
| UAS-Raf RNAi HM038564 | Bloomington Drosophila Stock Center | RRID: BDSC_55679 |
| UAS-Raf RNAi JF01483 | Bloomington Drosophila Stock Center | RRID: BDSC_31038 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xavier Franch-Marro (xavier.franch@ibe.upf-csic.es). This study did not generate new unique reagents

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila Stocks

All fly stocks were reared on standard flour/agar Drosophila media at 25°C. To overexpress UAS transgenes specifically in the PG either phmGal4 or amnc651Gal4, kindly provided by M. O’Connor and S. Wadell respectively, were used. Conditional activation of

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| UAS-ERKRNAi JF01366 | Bloomington Drosophila Stock Center | RRID: BDSC_31387 |
| UAS-EgfrRNAi JF01368 | Bloomington Drosophila Stock Center | RRID: BDSC_25781 |
| UAS-EgfrRNAi JF001696 | Bloomington Drosophila Stock Center | RRID: BDSC_31183 |
| UAS-EgfrRNAi JF01083 | Bloomington Drosophila Stock Center | RRID: BDSC_31525 |
| UAS-EgfrRNAi JF02883 | Bloomington Drosophila Stock Center | RRID: BDSC_36770 |
| UASpntRNAi HMS01452 | Bloomington Drosophila Stock Center | RRID: BDSC_35038 |
| UAS-rhoRNAi HMS02264 | Bloomington Drosophila Stock Center | RRID: BDSC_41699 |
| UAS-rhoRNAi JF03106 | Bloomington Drosophila Stock Center | RRID: BDSC_28690 |
| UAS-spi RNAi HM03251 | Bloomington Drosophila Stock Center | RRID: BDSC_51496 |
| UAS-spi RNAi | Bloomington Drosophila Stock Center | RRID: BDSC_63134 |
| Egfr-lacZ515S | Bloomington Drosophila Stock Center | RRID: BDSC_2079 |
| Egfr-lacZ | Bloomington Drosophila Stock Center | RRID: BDSC_61765 |
| pnt1277 | Bloomington Drosophila Stock Center | RRID: BDSC_837 |
| UAS-spRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_103817 |
| UAS-spRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_3922 |
| UAS-spRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_3920 |
| UAS-vnRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_109437 |
| UAS-vnRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_50358 |
| UAS-torsoRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_36280 |
| UASpntRNAi | Vienna Drosophila RNAi Center | RRID: VDRC_105390 |
| TorsoD4021 | J. Casanova | N/A |
| UAS-rasV12 | [47] | N/A |
| UAS-vn | [48] | N/A |
| UAS-EgfrPN | [49] | N/A |
| UAS-Egfract | [50] | N/A |
| UAS-pntP2VP16 | [51] | N/A |
| phm>YPetAtet | [23] | N/A |
| phm>SytGFP | [23] | N/A |
| phmGal4 | Bloomington Drosophila Stock Center | RRID: BDSC_80577 |
| amnc651Gal4 | Bloomington Drosophila Stock Center | 3605 |

Oligonucleotides

See Table S1

Software and Algorithms

Fiji [52] RRID: SCR 002285
Photoshop CS4 Adobe Inc. https://www.adobe.com/
iCycler iQ Real Time PCR Detection System Bio-Rad https://www.bio-rad.com/
GraphPad Prism v4.00 software GraphPad Software Inc. RRID:SCR_002798
Microsoft Excel Microsoft 02992-000-000010

e2 Current Biology 30, 1547–1554.e1–e4, April 20, 2020
either RNAi or gene expression was achieved using the Gal4/Gal80\textsuperscript{ts} System [53]. In these experiments, crosses were kept at 18 °C until L2 or L3 molt for conditional induction of RNAi or overexpression, respectively, when larvae were shifted to 29 °C and analyzed at indicated time points. The Drosophila Stock Center at Bloomington, Indiana provided UAS-CyE-1 (#30725), UAS-rasSSD\textsuperscript{RNAi} HMS01294 (#34619), UAS-rasDN (#4845), UAS-ras HMC03854 (#55679), UAS-ras JF01483 (#31038), UAS-ERK JF01366 (#31387), UAS-Egfr\textsuperscript{RNAi} JF01368 (#25781), UAS-Egfr\textsuperscript{RNAi} JF001696 (#31183), UAS-Egfr\textsuperscript{RNAi} JF01085 (#31525), UAS-Egfr\textsuperscript{RNAi} JF02283 (#36770), UAS-pnt\textsuperscript{RNAi} HMS01452 (#35038), UAS-rho\textsuperscript{RNAi} HMS02264 (#41699), UAS-rho\textsuperscript{RNAi} JF03106 (#28690), UAS-spo\textsuperscript{RNAi} HM03251 (#51496), UAS-sSpi (#63134), Egr-lacZ (#2079, #10385 and #61765) and pnt\textsuperscript{RNAi} (#103817, #3922 and #3920), UAS-vp\textsuperscript{RNAi} (#109437 and #50358), UAS-torso\textsuperscript{RNAi} (#36280), UASpnt\textsuperscript{RNAi} (#105390) were obtained from Vienna Drosophila RNAi Center. Torsc\textsuperscript{D Stap} was a kind gift from J. Casanova. UAS-ras\textsuperscript{V12} was obtained from G. Rubin. UAS-vn was obtained from B. Edgar. UAS-Egfr\textsuperscript{DN} was obtained from B. Shilo, UAS-Egfr\textsuperscript{act} was obtained from T. Schüpbach. UAS-pnt\textsuperscript{P2V16} was obtained from A. Michelson. phm\textsuperscript{>}YPetAtet and phm\textsuperscript{>}SytGFP were kindly provided by N. Yamana.

**METHODS DETAILS**

**Immunohistochemistry**
For fluorescent imaging, dissected ring glands (RGs) from L3 larva were dissected in 1x phosphate-buffered saline (PBS) and fixed in 4% formaldehyde or 8% for the specific detection of dpERK. RGs were rinsed in PBST and incubated with 0.1 M citrate buffer, 0.5% Triton X-100 for 10 min at ice. As a positive control, RGs were treated with DNase (Promega) for 10 min at 37 °C.

**Briefly,** RGs were fixed in 4% formaldehyde, wash with PBS and permeabilized with 0.1 M citrate buffer, 0.5% Triton X-100 for 10 min at ice. As a positive control, RGs were treated with DNase (Promega) for 10 min at 37 °C.

**For fluorescent imaging,** dissected ring glands (RGs) from L3 larva were dissected in 1x phosphate-buffered saline (PBS) and fixed in 4% formaldehyde or 8% for the specific detection of dpERK. RGs were rinsed in PBST and incubated with corresponding secondary antibody (Molecular Probes, 1:500) during 2 h at r.t. and rinsed with PBST before mounting. The following primary antibodies were used at indicated dilution: monoclonal Anti-MAP Kinase, Activated (dpERK-1&2, 1:250) antibody (Sigma-Aldrich), anti-ßGalactosidase (clone: 401.a, 1:200) from Developmental Studies Hybridoma Bank and anti-PntP2 (1:50) [46]. TUNEL labeling was performed using the TMR-Red In Situ Cell Death Detection Kit (Roche) according to the protocol described in a previous study [54] with minor modifications. Briefly, RGs were fixed in 4% formaldehyde, wash with PBS and permeabilized with 0.1 M citrate buffer, 0.5% Triton X-100 for 10 min on ice. As a positive control, RGs were treated with DNase (Promega) for 10 min at 37 °C. Samples were washed with PBS, 0.3% Triton X-100 before proceed with the TUNEL reaction. RGs were mounted in Vectashield medium with DAPI (Vector Laboratories, H1200). Images were obtained with Leica TCS SP5 confocal microscope and processed with either Fiji or Photoshop CS4 (Adobe).

**In situ Hybridization**
For in situ hybridization, dissected RGs from L3 larva were fixed in 4% formaldehyde. In situ hybridization was performed following the method described in a previous study [55] with minor modifications. Tissues were prehybridized in hybridization buffer (50% formamide, 5x SSC, 50 μg/ul heparin, 0.1% Tween-20, 100 μg/ul sonicated and denatured salmon sperm DNA, 100 μg/ul tRNA from Yeast) (HS) for 9 h at 55 °C and RNA probes were denatured for 10 min in HS at 95 °C and chilled on ice. Probe hybridization was performed 0/N at 55 °C. RGs were washed with a series of 3:2, 1:1 and 2:3 mixture of HS and 2xSSC, 0.1% Tween-20 (2xSSCTw) for 10 min at 55 °C. Tissues were washed again at 55 °C with 2xSSCTw and 0.2xSSCTw, then at r.t. with a series of 3:2 and 2:3 mixture of 0.2xSSCTw/PBSTw (0.1% Tween-20 in PBS) before incubation with anti-digoxigenin-AP antibody (Roche, 1:500), diluted in 1% BSA, 0.3% Triton X-100 in PBS, O/N at 4 °C. After antibody labeling, the tissues were washed with PBS, 0.3% Triton X-100 and with alkaline phosphate buffer (100mM NaCl, 50mM MgCl\textsubscript{2}, 100mM Tris-HCl pH 9.5, 0.1% Tween-20). To develop color reaction, samples were incubated with NBT/BCIP (Roche) solution in dark for 30 min to 1 h. vn, spi, Kn and rho probes were generated from DNA fragments amplified by PCR from genomic DNA with specific primers and digoxigenin-labeled RNA probes were generated by in vitro transcription following the manufacturer’s instructions (DIG RNA Labeling Mix, Roche). Images were obtained with the Zeiss microscope.

**20E Rescue Experiments**
20E (Sigma, H5142) was dissolved in ethanol at 5mg/ml. Standard flour-agar medium was supplemented with 0.35 mg/ml of the hormone or an equal amount of ethanol. For rescue experiments, embryos were collected after L3 molt and reared on 20E-supplemented medium or control medium with ethanol. phm \textsuperscript{>} w\textsuperscript{1118} was used as a control.

**Raising L3 larvae for timed sample collections**
Larvae were synchronized by allowing flies to lay eggs at 25 °C for 4h on agar plates supplemented with yeast paste. Between 25 to 30 freshly eclosed L1 larvae were collected and transferred to vials and incubated at 18 °C until L2 or L3 molting and then shifted to 29 °C. The time and date of pupariation were scored every 1-5 h during the light cycle and the time in hours. Data from 8-10 vials were put together and ordered by pupariation time and cumulative percentage pupariation and subsequently analyzed in Microsoft Excel.

**RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)**
Total RNA was isolated with the GenElute\textsuperscript{TM} Mammalian Total RNA kit (Sigma), DNase treated (Promega) and reverse transcribed with Transcriptor First Strand cDNA Synthesis kit (Roche, #04379012001). Relative transcripts levels were determined by real-time PCR (qPCR), using iTaq Universal SYBR Green Supermix (Bio-Rad). To standardize the qPCR inputs, a master mix that contained iTaqUniversal SYBR Green PCR Supermix and forward and reverse primers was prepared (final concentration: 100nM/μl). The qPCR experiments were conducted with the same quantity of tissue equivalent input for all treatments and each sample.
was run in duplicate using 2 μl of cDNA per reaction. All the samples were analyzed on the iCycler iQ Real Time PCR Detection System (Bio-Rad). For each standard curve, one reference DNA sample was diluted serially. RNA expression was calculated in relation to the expression of Rpl32.

**Western Blotting**

40 RGs were dissected in cold PBS and transferred to 40 μl Laemmli Sample Buffer (Bio-Rad, 1610747) supplemented with 2-mercaptoethanol. Samples were boiled for 5 minutes, centrifuged at 14,000 g and 20 μl supernatant were loaded in duplicate on a 10% polyacrylamide gel followed by transfer onto a PVDF membrane (Millipore). The membrane was incubated with PBS, 0.05% Tween-20, 5% nonfat dry milk (PBSTwM) O/N at r.t. with gentle agitation, then washed with PBSTw at r.t. Membrane was cut in half, and each samples replicate was incubated with the corresponding primary antibody O/N at 4°C. Mouse anti-alfa-Tubulin DM1A, 1:1000 (Sigma, #T6199) antibody was used as a loading control and mouse anti-phospho-ERK, 1:2000 (Sigma, #M8159) antibody to detect the levels of dpERK. Membranes were washed with 0.05% PBSTw at r.t for 2h. Secondary antibody incubation was performed at r.t. for 2h, then, the membrane was washed with 0.05% PBSTw at r.t prior to signal development with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, #34080). Secondary antibody Goat anti-mouse immunoglobulins/HRP polyclonal, 1:2000 (Dako, #P0447) were used. The blot was scanned on an Odyssey Fc (LI-COR) and Fiji, was used for image processing and protein quantification.

**PG Culture**

RGs from 80h AEL larvae reared at 25°C, were dissected in PBS. Dissected ring glands from 10 animals per each replicate were cultured at 25°C in Schneider’s medium (GIBCO, #21720024) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, #16140063) for one hour, before transferring them to fresh medium supplemented with 5x10^-6M of 20E (Sigma Aldrich, #H5142) dissolved in 10% ethanol, or with the same volume of 10% ethanol. Samples were processed for qRT-PCR after 6 hours in culture.

**Ecdysteroid Measurements**

Fifteen staged third instar larvae were preserved in 500μl of methanol. Samples were homogenized and centrifuged (10cmin at 18c000 × g). The remaining tissue was re-extracted twice in 0.5 mL methanol and the resulting methanol supernatants were dried using a SpeedVac. Samples were resuspended in enzyme immunoassay buffer (0.4cM NaCl, 1cM EDTA, 0.1% bovine serum albumin in 0.1 M phosphate buffer). ELISA was performed according to the manufacturer’s instructions using a commercial ELISA kit (Bertin Bioreagents) that detects ecdysone and 20-hydroxyecdysone with the same affinity. Absorbance was measured at 405 nm on a plate reader, SpectramaxPlus (Molecular Devices, Sunnyvale, CA) using GraphPad Prism v4.00 software (GraphPad Software Inc., San Diego, CA).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**DNA Quantification**

For DNA quantification, DNA staining intensity in the PG cells was obtained from z stacked images of DAPI stained L3 larvae. DNA staining intensity of PG cells was normalized using average DNA staining intensity of the diploid cells of Tr2 tracheal system. Thus, the C value of PG nucleus was set as DNA staining intensity in the tracheal cells of Tr2/DNA staining intensity in PG. Images were obtained with Leica SP5 confocal microscope. A series of 2D images were taken every 0.25 μm slices. Image analysis was performed using Fiji.

**Statistical Analysis**

Experiments were performed with three biological replicates. At qPCR experiments, each biological replicate corresponds to 10 whole larvae, in Figures 1G, S1H, and S4A and 20 RGs in all other experiments. The biological replicates for ecdysteroids measurements in ELISA quantification corresponds to 15 whole larvae. The average and standard error mean were represented. Two-tailed Student’s test was performed to determine which values were significantly different. Asterisks indicate differences statistically significant at p ≤ 0.05 (*), and p ≤ 0.005 (**). The sample size for pupariation time and cumulative pupariation percentage is indicated at the corresponding figure.

**DATA AND CODE AVAILABILITY**

This study did not generate any new computer code or algorithms. Raw images used for quantitative analyses are available form the lead author upon request.