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The Ecdysone receptor controls the post-critical weight switch to nutrition-independent differentiation in *Drosophila* wing imaginal discs

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In holometabolous insects, a species-specific size, known as critical weight, needs to be reached for metamorphosis to be initiated in the absence of further nutritional input. Previously, we found that reaching critical weight depends on the insulin-dependent growth of the prothoracic glands (PGs) in *Drosophila* larvae. Because the PGs produce the molting hormone ecdysone, we hypothesized that ecdysone signaling switches the larva to a nutrition-independent mode of development post-critical weight. Wing discs from pre-critical weight larvae [5 hours after third instar ecdysis (AL3E)] fed on sucrose alone showed suppressed Wingless (WG), Cut (CT) and Senseless (SENS) expression. Post-critical weight, a sucrose-only diet no longer suppressed the expression of these proteins. Feeding larvae that exhibit enhanced insulin signaling in their PGs at 5 hours AL3E on sucrose alone produced wing discs with precocious WG, CT and SENS expression. In addition, knocking down the Ecdysone receptor (EcR) selectively in the discs also promoted premature WG, CUT and SENS expression in the wing discs of sucrose-fed pre-critical weight larvae. EcR is involved in gene activation when ecdysone is present, and gene repression in its absence. Thus, knocking down EcR derepresses genes that are normally repressed by unliganded EcR, thereby allowing wing patterning to progress. In addition, knocking down EcR in the wing discs caused precocious expression of the ecdysone-responsive gene *broad*. These results suggest that post-critical weight, EcR signaling switches wing discs to a nutrition-independent mode of development via derepression.

KEY WORDS: Ecdysone receptor, Critical weight, Size control, Wing imaginal disc patterning, *Drosophila*

INTRODUCTION

Attaining species-specific adult size depends on mechanisms that both monitor size during growth and set the length of the growth period in a size-dependent manner. In insects, size-dependent events occur at various stages throughout development. They control the timing of larval molts and establish when the larva is of sufficient size to enter metamorphosis (Nijhout, 1975; Nijhout and Williams, 1974b). We have focused on the mechanism that determines when the larva is of sufficient size to initiate metamorphosis in the absence of nutrition, an event called critical weight.

In *Drosophila* and the tobacco hornworm, *Manduca sexta*, the switch from the growth phase of development to the differentiative phase occurs as a result of surpassing critical weight in the last instar (Beadle et al., 1938; Nijhout, 1975; Nijhout, 2003; Nijhout and Williams, 1974a; Nijhout and Williams, 1974b). Critical weight corresponds to a developmental switch in the way the larva responds to starvation; before reaching critical weight, most larvae die when starved and those that survive will significantly delay the onset of metamorphosis. Larvae that are starved after critical weight has been reached will metamorphose without delay (Mirth and Riddiford, 2007; Nijhout, 2003). We and others have shown that the insulin-dependent growth of the prothoracic glands (PGs), the glands that synthesize the molting hormone ecdysone, determines when critical weight is reached in *Drosophila* (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Overexpressing a positive regulator of insulin signaling, Dp110 [phosphatidylinositol 3-kinase (PI3K); PI3K92E – FlyBase], in the PGs causes larvae to achieve critical weight earlier and at smaller body sizes, and to metamorphose early into miniature adults.

Since the primary function of the PGs is to produce the molting hormone ecdysone, ecdysone is a likely candidate to be involved in signaling to the rest of the body that critical weight has been reached (Mirth and Riddiford, 2007). In the early third instar, ecdysone titers slowly increase, reaching a peak at ~9 hours after third instar ecdysis (AL3E) (Warren et al., 2006), approximately the same time that critical weight is attained. Furthermore, enhanced insulin signaling in the PGs is known to increase the transcription of *phantom* (*phm*) and *disembodied* (*dib*), the products of which are both ecdysteroid biosynthetic enzymes (Caldwell et al., 2005; Colombani et al., 2005). Recently, Walkiewicz and Stern found that enhancing insulin signaling specifically in the insulin-producing cells in the brain increases the expression of *dib* and of the ecdysone response gene *E74B* (*Eip74EF*) (Walkiewicz and Stern, 2009). These data infer a very simple mechanism for size-dependent development at critical weight: if the larval body and PGs grow in parallel in response to insulin signaling in the early third instar, and the result of increased insulin signaling in the PGs is increased ecdysone production by this gland, then critical weight is the size at which the PGs produce sufficient ecdysone to sustain growth and patterning in imaginal tissues even in the absence of nutrition (Mirth and Riddiford, 2007).

Ecdysone and its more active metabolite 20-hydroxyecdysone (20E) bind to the ecdysone receptor complex, a heterodimer between Ecdysone receptor (EcR) (Koelle et al., 1991) and Ultraspiracle (USP) (Yao et al., 1992). The EcR-USP complex is responsible for the coordination of the ecdysone signaling cascade that is necessary for molting and metamorphosis by controlling the transcription of ecdysone-responsive genes in three different ways: repression, derepression and activation (Cherbas et al., 2003). Repression occurs when the unliganded EcR-USP complex binds to target gene promoters and prevents transcription (Brown et al., 2006; Schubiger

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et al., 2005; Schubiger and Truman, 2000). Once EcR binds to 20E, genes that were repressed by unliganded EcR-USP become transcriptionally active. Thus, suppressing the expression of either EcR or USP, by RNAi or in *usp*$^{-}$ clones, eliminates repression by the unliganded EcR-USP complex, thereby allowing developmental programs to proceed. The binding of 20E to EcR also positively regulates the transcription of genes by recruiting transcriptional activators and co-factors to the promoter; this is known as activation (Cherbas et al., 2003; Cherbas and Cherbas, 1996; Hu et al., 2003).

In this study, we use the differentiative state of the wing imaginal disc as an assay for whether ecdysone is the signal for sufficient size at critical weight. In *Drosophila*, the majority of the adult body is built from imaginal discs and imaginal histoblasts, which initiate their final differentiation at the onset of metamorphosis (Fristrom and Fristrom, 1993). Consequently, coordinating the development of the imaginal discs might be an important function of critical weight. Imaginal discs are also known to regulate developmental timing; delaying the development of these tissues delays the development of the entire animal by altering critical weight and the onset of metamorphosis (Poodry and Woods, 1990; Simpson et al., 1980; Stieper et al., 2008). Therefore, the imaginal discs are likely to show differences in their development in response to nutrition pre- and post-critical weight, and these differences might be under the control of ecdysone.

The wing discs were chosen as their development has been described in detail (Baker, 2007; Cohen, 1993). There are a number of well-defined patterning genes, the expression patterns of which change in the early third instar larva. In particular, we have made use of the Wingless (WG), Cut (CT) and Senseless (SENS) protein expression patterns. WG, an ortholog of vertebrate WNTs, is at the top of a patterning hierarchy that distinguishes the notum from the wing region and defines the dorsal/ventral boundary in the wing (Williams et al., 1993). CT is a homeodomain transcription factor that is expressed in external sensory organs (Jack et al., 1991). SENS is a zinc-finger transcription factor involved in the early stages of sensory organ specification (Nolo et al., 2000). In this investigation, we found that the WG, CT and SENS expression patterns are nutrition dependent before, but not after, critical weight has been reached. Furthermore, enhancing insulin signaling in the PGs or knocking down *EcR* transcripts specifically in the wing discs resulted in precocious maturation of WG, CT and SENS expression under starvation conditions.

MATERIALS AND METHODS

Fly stocks

Two GAL4 lines were used to manipulate insulin signaling in the PGs*: phm GAL4, UAS mCD8::GFP/TM6B Tb* (a gift from Dr Michael O'Connor, University of Minnesota) and *P0206, UAS mCD8::GFP* (FlyView). In addition, we used two imaginal disc-specific GAL4 lines, C765 (Nellen et al., 1996) and MS1096. These lines were crossed to one of nine stocks: *w; UAS EcR RNAi*, *w; UAS EcR B1*^{w650}, *w; UAS broad (br) Z1*$^{18-13}$, *w; UAS br Z2*$^{11-4}$, *w; UAS br Z3*$^{131-6}$, *w; UAS br Z4*$^{37-6}$, *yw; UAS Pten (III)*, *hs flp; UAS Dp110*, or *yw flp; UAS-InR*$^{29.4}$ *(III)* (the latter three are gifts from Bruce Edgar, Fred Hutchinson Cancer Research Center). The w^{1118} fly line was used as a control.

Standardizing larval conditions and larval collections

Eggs were distributed onto food plates at a density of 200 eggs/plate as described (Mirth et al., 2005). Larvae that ecdysed from L2 to L3 over a 2-hour period were transferred onto new food plates and allowed to feed until they reached the appropriate age. Larvae were reared at 25°C under a constant light cycle.

Sucrose feeding and starvation protocols

To assess the effects of rearing larvae on sucrose-only media, 10-20 larvae were dissected for staged controls, 10-20 larvae were taken from the food and transferred to 20% sucrose solution in water (sucrose-fed treatment) and 10-20 larvae were left to feed on cornmeal-molasses medium (standard food). After 24 hours, the sucrose-fed and standard food-fed larvae were dissected and processed for immunocytochemistry. Critical weight experiments were performed in a similar manner, except that starved larvae were transferred onto a 35×10 mm Petri dish containing a medium of 2% agar in water.

Immunocytochemistry

Larvae were dissected and fixed in 4% formaldehyde in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0) for 30 minutes at room temperature. After fixation, the tissue was washed in PBT (PBS containing 1% Triton X-100), blocked for 30 minutes in 2% normal donkey serum in PBT, and incubated in a primary antibody solution overnight at 4°C. The following primary antibodies were diluted into 2% normal donkey serum in PBT: mouse anti-WG (4D4c, diluted 1:100) with guinea pig anti-SENS [a gift from Dr Hugo Bellen (Nolo et al., 2000), 1:3000], mouse anti-CT (2B10, 1:100), mouse anti-Broad-core (25E9.D7, 1:100), mouse anti-Armadillo (N2 7A1 Armadillo, 1:100), mouse anti-Tubulin (E7, 1:100), mouse anti-Patched (Apa 1, 1:40) and mouse anti-Engrailed/Invected (4D9, 1:20). The WG, CT, Broad-core, Armadillo, Tubulin, Patched and Engrailed/Invected antibodies were obtained from the Developmental Studies Hybridoma Bank. After washing again in PBT, the tissue was incubated in secondary antibody solution overnight at 4°C. If Oregon Green Phalloidin (Invitrogen) was used, it was added to the secondary antibody solution. Wing discs were finally rinsed with PBT and mounted on poly-L-lysine-coated coverslips using Fluoromount-G (SouthernBiotech). Samples were imaged using a Zeiss LSM 510 or a Zeiss LSM 710 confocal microscope and processed using ImageJ and Adobe Photoshop.

RESULTS

Wingless, Cut and Senseless expression in the early to mid-third instar larva

To determine the normal developmental expression pattern of WG, CT and SENS in early to mid-third instar (L3) wing imaginal discs, we examined their expression in the wing discs of w^{1118} larvae during the first 35 hours after the molt from second to third instar. At 0 and 5 hours AL3E, WG was expressed in a stripe in the presumptive wing margin (Fig. 1A,B). At 10 hours AL3E, a ring of WG appeared around the wing pouch (Fig. 1C). A faint patch of WG expression was visible in the notum at 15 hours AL3E (Fig. 1D). This patch of WG was more clearly resolved by 25 hours AL3E (Fig. 1F).

The earliest wing discs (0-15 hours AL3E) expressed CT only in the notum (Fig. 1I-L). At 20 hours AL3E, in addition to the expression in the notum, CT first became apparent in the wing margin (Fig. 1M). The pattern in the wing margin became brighter over the next 15 hours of development (Fig. 1N-P).

SENS was not expressed in the youngest wing discs (0-10 hours AL3E, Fig. 1Q-S). At 15 hours AL3E, SENS expression began to appear in a single bristle sensory organ precursor (SOP) in the notum (Fig. 1T). Five hours later, SENS was expressed in the SOP of the wing hinge chordotonal organ (Fig. 1U) and in a few more bristle SOPs in the wing and notal regions. By 30 hours AL3E, SENS was upregulated in two stripes of triple-row SOPs at the wing margin and in a number of newly differentiated bristle SOPs in the wing and notum (Fig. 1W).

The effects of nutrition on Wingless, Cut and Senseless expression in pre- and post-critical weight larvae fed on sucrose alone

We then determined whether the development of mature WG, CT and SENS expression patterns was dependent on nutrition in pre- and post-critical weight larvae. Critical weight is normally reached between 9 and 12 hours AL3E (Mirth et al., 2005; Shingleton et al., 2005; Stieper et al., 2008). To test the developmental effect of nutrition on the differentiation of the disc, we fed pre- and post-

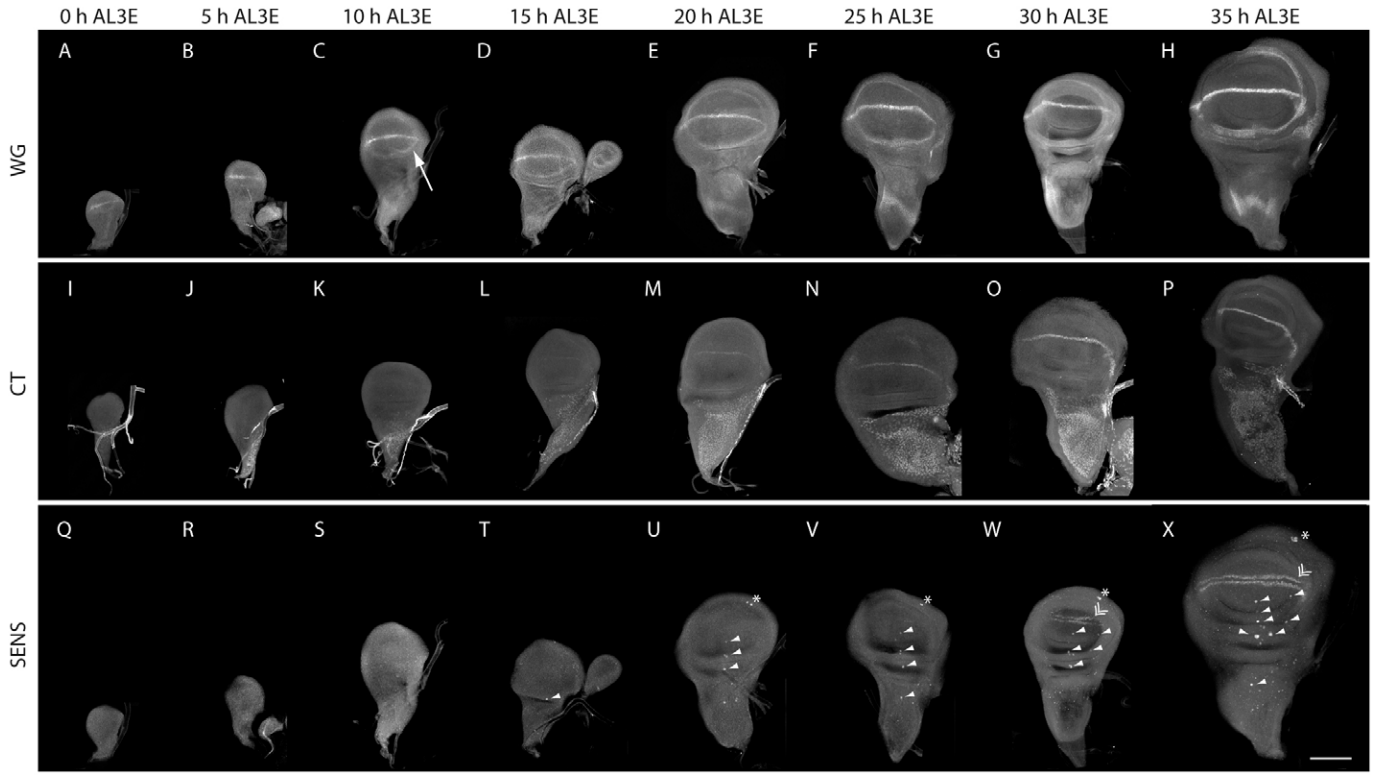

Fig. 1. The development of the Wingless, Cut and Senseless expression patterns in wing discs of early third instar w^{1118} *Drosophila* larvae. Expression of (**A-H**) Wingless (WG), (**I-P**) Cut (CT) and (**Q-X**) Senseless (SENS) in wing discs at 0 (A,I,Q), 5 (B,J,R), 10 (C,K,S), 15 (D,L,T), 20 (E,M,U), 25 (F,N,V) 30 (G,O,W) and 35 (H,P,X) hours (h) after third instar ecdysis (AL3E). In C, the arrow points to the ring of WG expression around the wing pouch. The asterisks in U-X mark the precursor of the wing hinge chordotonal organ, and the small arrowheads indicate sensory bristle precursors. The double arrowhead in W and X points to the sensory bristle precursors in the triple row of the wing margin. Scale bar: 100 μm.

critical weight larvae on a 20% sucrose solution. This feeding regime provided energy in the form of sucrose, but did not contain the amino acids, lipids, vitamins or minerals present in the normal diet. Larvae fed on sucrose alone can survive for over a week, but are malnourished and require amino acids for continued growth (Britton and Edgar, 1998).

At 5 hours AL3E, wing discs expressed WG in a stripe in the wing margin (Fig. 1B and Fig. 2A). SENS was not expressed in discs at this time (Fig. 1J and Fig. 2B). When these pre-critical weight larvae were fed on 20% sucrose for 25 hours (5-30 hours AL3E), WG expression was reduced in the wing margin (Fig. 2D) and neither CT nor SENS appeared (Fig. 2E,F). If pre-critical weight larvae (from 5-30 hours AL3E) were starved completely on 2% agar medium, they showed similarly low WG expression and the absence of SENS expression (see Fig. S1C,D in the supplementary material). In larvae fed on standard food until 30 hours AL3E, WG was strongly expressed throughout the wing margin, around the wing pouch and in the notum. CT was expressed in the notum and wing margin in these discs, and SENS was expressed in the bristle SOPs of the notum and wing, in the SOPs of the triple row in the wing margin and in the SOPs of the hinge chordotonal organ (Fig. 2G-I).

When post-critical weight larvae (after 14 hours AL3E) were fed on sucrose alone, their wing discs underwent substantial growth and differentiation. Twenty four hours later, WG was upregulated in the wing margin and around the wing pouch, CT was expressed strongly in the wing margin and notum and SENS was upregulated in the SOPs of the notum, the wing pouch, the wing margin and of the hinge chordotonal organ (Fig. 2M-O). Although these discs were smaller than those of the standard food controls (Fig. 2P-R), they were similar in the development of their WG, CT and SENS expression patterns. Similar results were found for post-critical weight larvae (15-39 hours AL3E) that were starved on 2% agar medium alone (see Fig. S1I,J in the supplementary material).

To assess whether the effects on the expression of WG, SENS and CUT in pre-critical weight larvae that were fed only sucrose were due to overall reductions in protein translation, we examined the expression of two anterior/posterior axis patterning gene products, using an antibody that recognizes both Engrailed and Invected proteins (EN) and an antibody against Patched (PTC), and three housekeeping gene products, Actin, Armadillo and Tubulin. There were no changes observed in the expression of PTC or EN from pre-critical weight or post-critical weight larvae fed on sucrose alone when compared with the fed controls (see Fig. S2G-L in the supplementary material). The level of expression of the three housekeeping gene products, Armadillo (*Drosophila* β-catenin) (see Fig. S3A-F in the supplementary material), Actin (via phalloidin staining; see Fig. S3G-L in the supplementary material) and Tubulin (see Fig. S3M-R in the supplementary material) was similar in the discs of larvae fed on sucrose and those from fed controls.

Enhancing insulin signaling in the prothoracic gland caused discs to differentiate prematurely when larvae are fed on sucrose alone

When insulin signaling is activated in the cells of the PG, larvae reach critical weight prematurely (Mirth et al., 2005). We therefore tested whether wing discs from larvae with insulin signaling

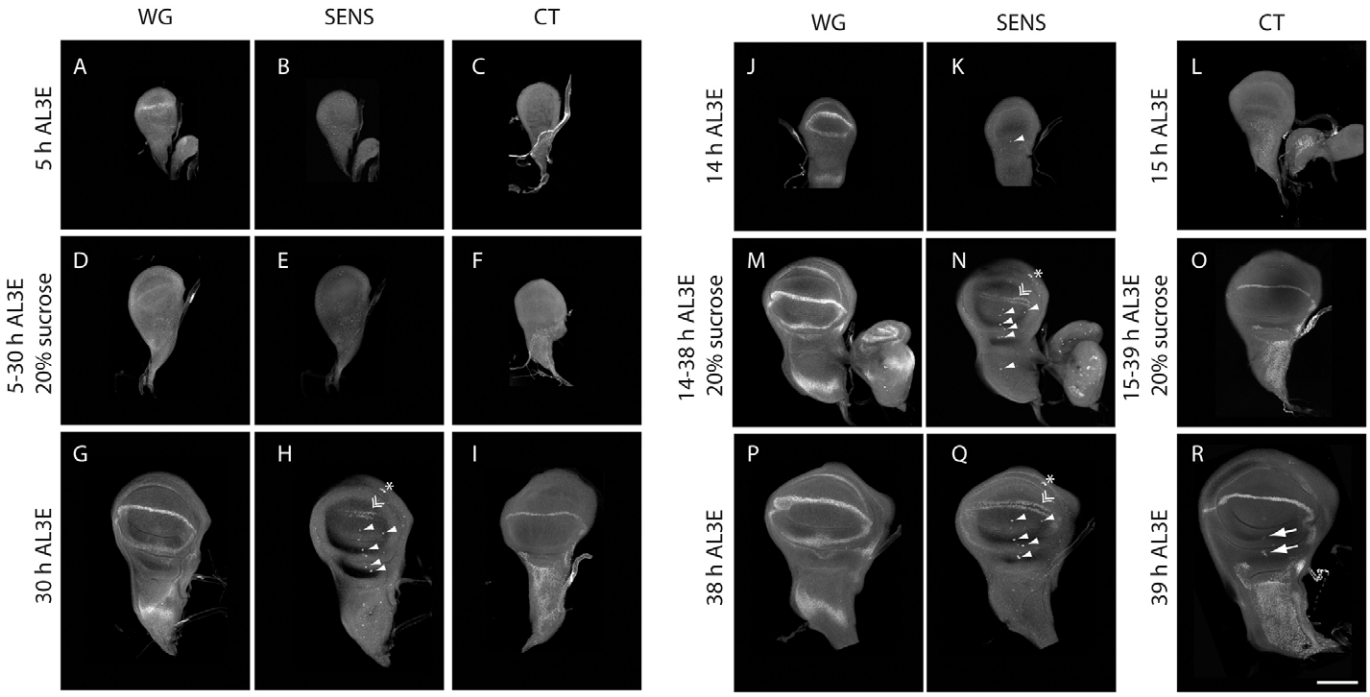

Fig. 2. Patterning in the imaginal discs differs in pre- and post-critical weight w^{1118} larvae fed on sucrose alone. The expression of (**A**,**D**,**G**,**J**,**M**,**P**) WG, (**B**,**E**,**H**,**K**,**N**,**Q**) SENS and (**C**,**F**,**I**,**L**,**O**,**R**) CT in w^{1118} larvae. Wing discs are from (A-C) larvae at 5 hours (h) after third instar ecdysis (AL3E), (D-F) larvae fed on sucrose alone between 5 and 30 hours AL3E, (G-I) larvae at 30 hours AL3E, (J,K) larvae at 14 hours AL3E, (L) larvae dissected at 15 hours AL3E, (M,N) larvae fed on sucrose alone from 14-38 hours AL3E, (O) larvae fed on sucrose alone from 15-39 hours AL3E, (P,Q) larvae staged to 38 hours AL3E, and (R) larvae at 39 hours AL3E. The asterisks in H, N and Q are the sensory organ precursors (SOPs) of the wing hinge chordotonal organ. The double arrowheads in H, N and Q mark the SOPs in the triple row at the wing margin. Other SOPs in the wing pouch and notum are marked by arrowheads (H,N,Q). In R, the arrows mark CT expression in some of the SOPs of the notum and wing pouch. Scale bar: 100 μm.

activated in their PGs would differentiate prematurely on a sucrose-only diet. To activate insulin signaling in the PGs, we used the P0206 GAL4 driver, which is expressed in the ring gland and corpora allata, to overexpress UAS Insulin receptor (InR) (P0206>InR). Overexpressing wild-type InR is known to activate the insulin signaling pathway in imaginal discs and other tissues (Britton et al., 2002; Brogiolo et al., 2001). At 5 hours AL3E, both control larvae (P0206>GFP) and P0206>InR larvae showed WG expression in the presumptive wing margin. CT expression was restricted to the notum and SENS was not expressed (Fig. 3A-C,J-L).

When P0206>InR larvae were fed for 24 hours on sucrose alone between 5 and 29 hours AL3E, the patterning of their wing discs progressed dramatically (Fig. 3M-O) relative to P0206>GFP controls (Fig. 3D-F). Their wing discs showed strong WG expression in the wing margin and in the notum (Fig. 3M). CT was expressed both in the notum and in the wing margin (Fig. 3N), and SENS was expressed in a number of bristle SOPs in the notum and wing pouch, in the hinge chordotonal organ, and prominently in the triple row of bristle SOPs in the wing margin (Fig. 3O). We found similar results when the Phm GAL4 driver, which is expressed in the PGs alone, was used to overexpress Dp110. Phm>Dp110 larvae showed precocious WG and SENS expression in their wing discs when fed on 20% sucrose alone from 5-29 hours AL3E (see Fig. S4 in the supplementary material).

The reverse was seen when insulin signaling in the PGs was suppressed using Phosphatase and tensin homolog (PTEN). P0206>PTEN larvae fed on sucrose alone from 15-39 hours AL3E did not show any differentiation of WG and SENS expression in their wing discs (see Fig. S5 in the supplementary material). Even when fed on a normal diet, P0206>PTEN larvae showed a dramatic delay in the patterning of their wing discs at 39 hours AL3E when compared with control (P0206>GFP) larvae. Although P0206>PTEN larvae were abnormally large, their wing discs were considerably smaller than control discs (compare P0206>GFP with P0206>PTEN discs in Fig. S5 in the supplementary material). This is presumably due to delayed development and delayed attainment of critical weight in the P0206>PTEN larvae.

Signaling through EcR is responsible for switching wing imaginal discs to a post-critical weight mode of development

Because the primary function of the PGs is to produce ecdysone, and because ecdysone titers peak around the time of critical weight (Warren et al., 2006), we reasoned that ecdysone might play a role in switching the wing discs to nutrition-insensitive maturation post-critical weight. To modify ecdysone signaling specifically in the imaginal discs, we used a disc-specific GAL4 driver, C765 GAL4, to overexpress either UAS EcR RNAi (C765>EcRi) or a dominant-negative EcR transgene, UAS EcR $B1^{W650}$ (C765>EcR^{DN}).

The UAS EcRi construct has been shown to suppress EcR expression in the wing discs (Schubiger et al., 2005). This causes the derepression of genes repressed by unliganded EcR, thereby allowing development to progress. To suppress both the activation and derepression functions of EcR signaling, we used a dominant-negative form of EcR that bears a mutation in a crucial residue involved in ligand binding (Cherbas et al., 2003). Because EcR^{DN}

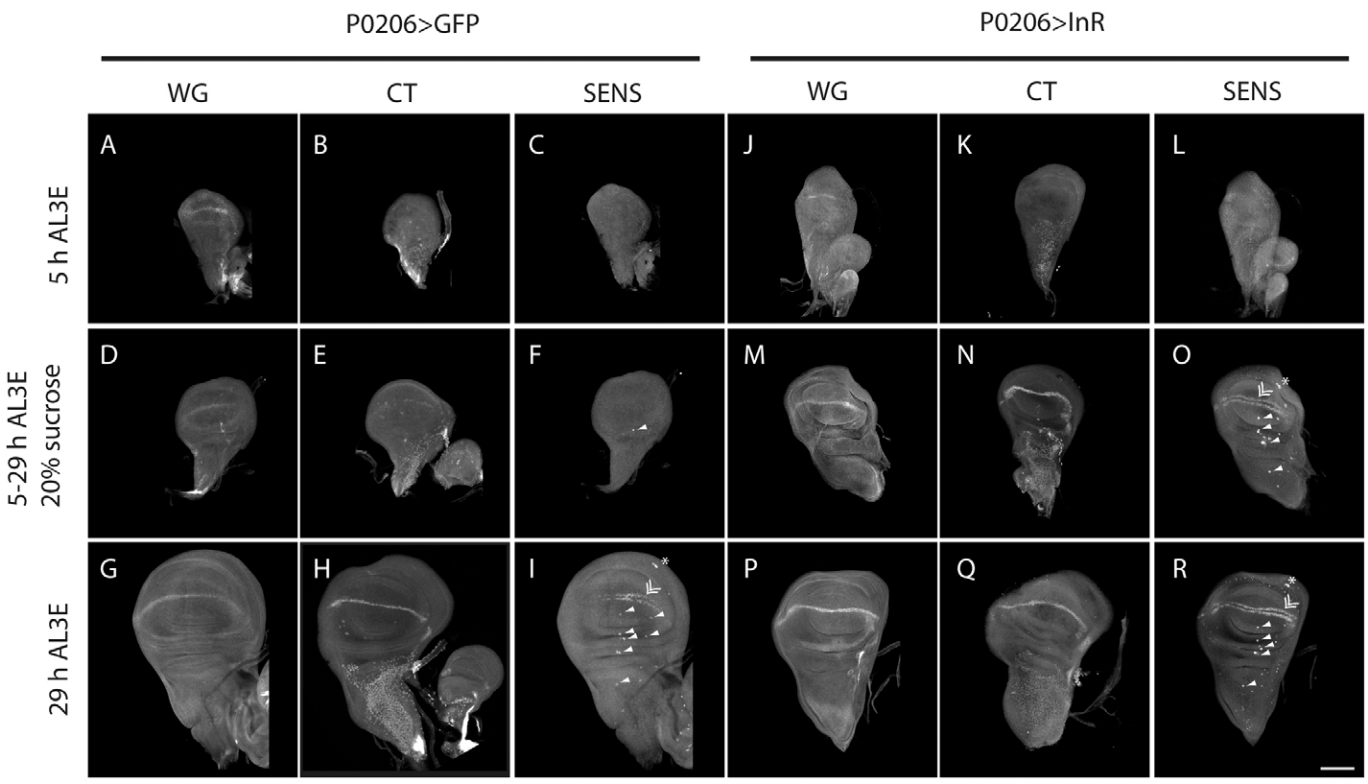

Fig. 3. Enhancing insulin signaling in the ring gland, using the P0206 ring gland GAL4 driver, changes the developmental response of wing discs to starvation. Wing discs from (**A-I**) P0206>GFP control larvae and (**J-R**) P0206>InR larvae. Wing discs are from (A-C,J-L) larvae staged to 5 hours (h) after third instar ecdysis (AL3E), (D-F,M-O) larvae fed on sucrose alone from 5-29 hours AL3E, and (G-I,P-R) larvae at 29 hours AL3E. Shown is expression of (A,D,G,J,M,P) WG, (B,E,H,K,N,Q) CT and (C,F,I,L,O,R) SENS. The asterisks in I, O and R mark the SOPs of the wing chordotonal organ. The double arrowheads (I,O,R) point to the SOPs of the triple row. SOPs in the wing pouch and notum are indicated with arrowheads (I,O,R). Scale bar: 100 μm.

cannot bind its ligand ecdysone, it both inhibits the activation function and prevents the derepression function of EcR-USP. UAS EcR^{DN} has been effectively used to suppress EcR activation and derepression in the Tv neurons (Brown et al., 2006) and wing discs (Schubiger et al., 2005).

At 5 hours AL3E, wing discs from control (C765) and C765>EcRi larvae expressed WG in the presumptive wing margin, but did not express SENS (Fig. 4A,E). Wing discs from C765>EcR^{DN} larvae at 5 hours AL3E showed little or no WG expression in the wing margin (Fig. 4C). If either pre-critical weight (5 hours AL3E) control (Fig. 4I,J) or C765>EcR^{DN} (Fig. 4K,L) larvae were fed on sucrose alone for 24 hours, WG was expressed at low levels in the discs and SENS was not expressed. By contrast, when C765>EcRi larvae were fed on sucrose between 5 and 29 hours AL3E, WG was upregulated in the presumptive wing margin, around the wing pouch and in the notum, and SENS was expressed in the bristle SOPs of the notum and wing pouch, in the hinge chordotonal organ, and in the triple row in the wing margin (Fig. 4M,N). The expression of CT was also premature in the discs of C765>EcRi larvae fed 20% sucrose from 5-29 hours AL3E (see Fig. S6 in the supplementary material). A second GAL4 line, MS1096 GAL4, which drives expression in the dorsal wing discs, was used to confirm the C765>EcRi results. MS1096>EcRi larvae showed premature differentiation of the WG and SENS patterns when pre-critical weight larvae were fed on sucrose alone (Fig. 4O,P). Because this driver is expressed primarily in the dorsal wing pouch, MS1096>EcRi larvae only expressed SENS in a single row of cells at the wing margin (Fig. 4P′).

When fed on standard food, WG and SENS expression patterns developed at the same rate in the C765, C765>EcRi and MS1096>EcRi (Fig. 4Q,R,U-X) larvae. C765>EcR^{DN} larvae showed delayed patterning in their discs at 29 hours AL3E with respect to the other genotypes (Fig. 4S,T).

Because the discs of C765>EcRi larvae differentiated precociously when larvae were fed on sucrose alone, we postulated that these larvae might also reach critical weight earlier than normal. Previously, we approximated critical weight by determining the size at which 50% of the larvae pupariate when starved. Subsequent studies by Stieper and co-authors (Stieper et al., 2008) have found that a more accurate measure of critical weight is to determine the minimum size at which starvation no longer delays metamorphosis. C765 and C765>EcRi larvae had critical weights of ~0.85-0.9 mg and they reached this point ~9 hours AL3E (Fig. 5). Therefore, C765>EcRi larvae do not reach critical weight prematurely.

Knocking down EcR in the wing discs causes precocious upregulation of Broad

Schubiger and co-authors found that EcRi clones in the wing discs caused premature differentiation of wing sensilla, with a corresponding upregulation in the ecdysone-responsive transcription factor Broad (BR) (Schubiger et al., 2005). We reasoned that BR expression might be differentially regulated in pre-critical versus post-critical weight discs.

To determine whether BR is involved in nutrition-independent differentiation in the discs, we examined BR expression at 5 and 15 hours AL3E in larvae fed on sucrose alone. All tissue was scanned

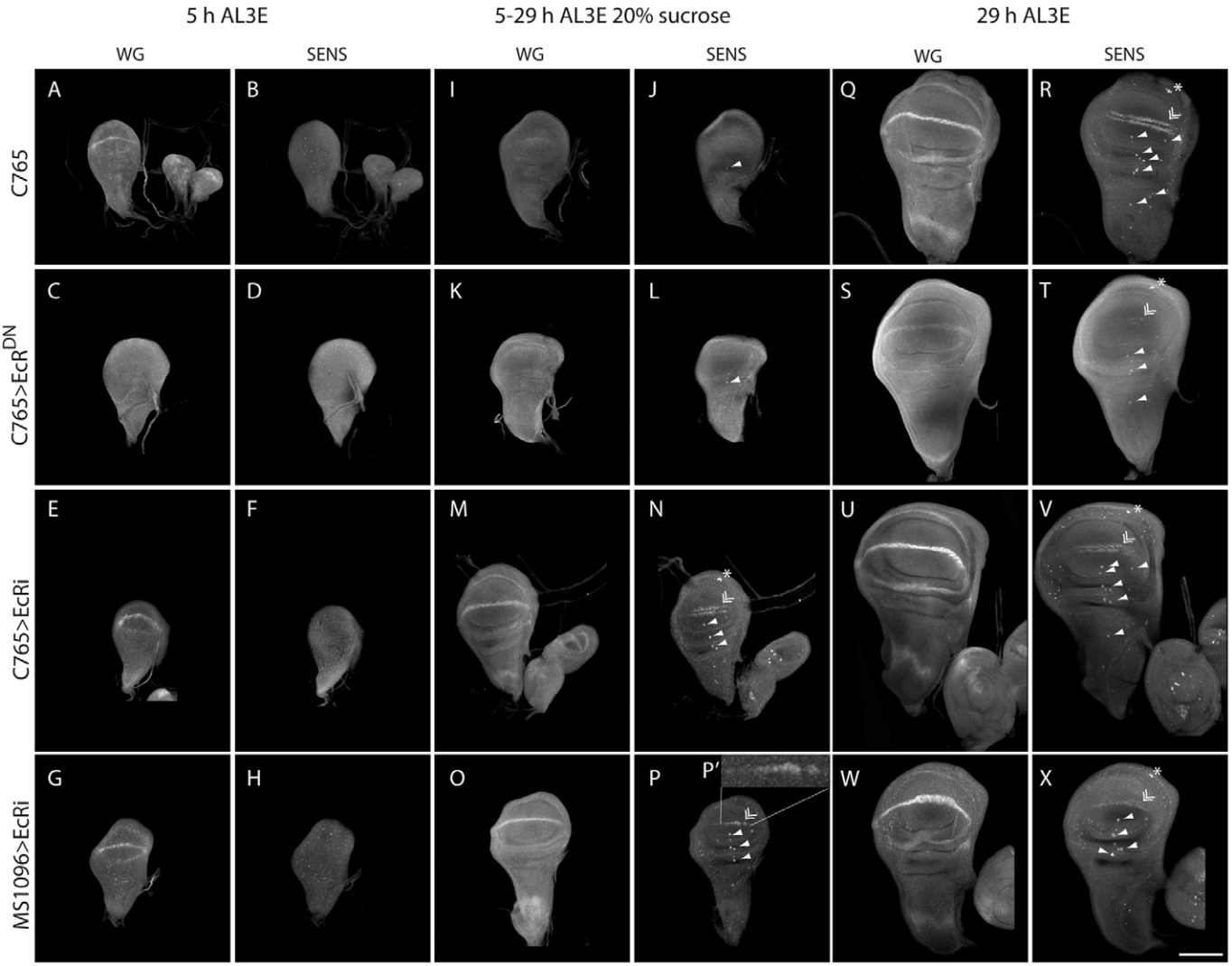

Fig. 4. Suppressing ecdysone receptor expression in the wing discs of starved pre-critical weight larvae promotes premature differentiation of the Wingless and Senseless expression patterns. Wing discs (**A-H**) dissected at 5 hours AL3E, (**I-P**) from larvae fed on sucrose alone from 5-29 hours AL3E, and (**Q-X**) from larvae fed on standard food and dissected at 29 hours AL3E. Four different genotypes were examined: C765 (A,B,I,J,Q,R), C765>EcR^{DN} (C,D,K,L,S,T), C765>EcRi (E,F,M,N,U,V) and MS1096-EcRi (G,H,O,P,W,X). Shown is expression of WG and SENS as labeled. The SOPs of the triple row are marked by double arrowheads (N,P,R,T,V,X). Asterisks mark the SOPs of the hinge chordotonal organ (N,P,R,T,V,X). Arrowheads indicate the SOPs of the wing pouch and notum (N,P,R,T,V,X). (**P′**) This inset shows the expression of SENS in a single row of sensory organ precursors at the margin. Scale bar: 100 μm.

using identical confocal settings to compare levels of BR between samples. At 5 and 15 hours AL3E, BR was expressed at very low levels (Fig. 6A,D). BR remained low in wing discs from larvae fed on sucrose alone from 5-30 hours AL3E (Fig. 6B). However, BR was substantially upregulated in wing discs from post-critical weight larvae (15 hours AL3E) fed on sucrose alone for 24 hours (Fig. 6E), as well as in fed controls at 30 and 39 hours AL3E (Fig. 6C,F).

In C765>EcRi larvae fed from 5-29 hours AL3E on sucrose alone, BR was expressed at similar levels as in the fed C765 controls at 29 hours AL3E (Fig. 7C,E). However, C765>EcRi larvae fed on standard food until 29 hours AL3E showed reduced BR expression, possibly indicating that at later stages of development BR requires activation via EcR for its full expression. Likewise, C765>EcR^{DN} larvae showed reduced levels of BR expression in the discs at 29 hours AL3E.

Using MS1096 GAL4 to drive UAS EcRi in the dorsal region of the wing disc resulted in upregulation of BR primarily in the dorsal wing pouch in larvae fed on sucrose alone (Fig. 7K). There was no obvious difference in the relative strength of BR expression across the disc in MS1096>EcRi larvae at 5 and 29 hours AL3E. However, when overexpressed throughout the discs, none of the BR isoforms (Z1-Z4) was sufficient to promote premature differentiation of the WG and SENS expression patterns in pre-critical weight, sucrose-fed larvae (see Fig. S7 in the supplementary material).

DISCUSSION

Previously, our work and that of others had shown that the PGs were involved in regulating the critical weight transition in *Drosophila* (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). This led us to hypothesize that levels of ecdysone might be

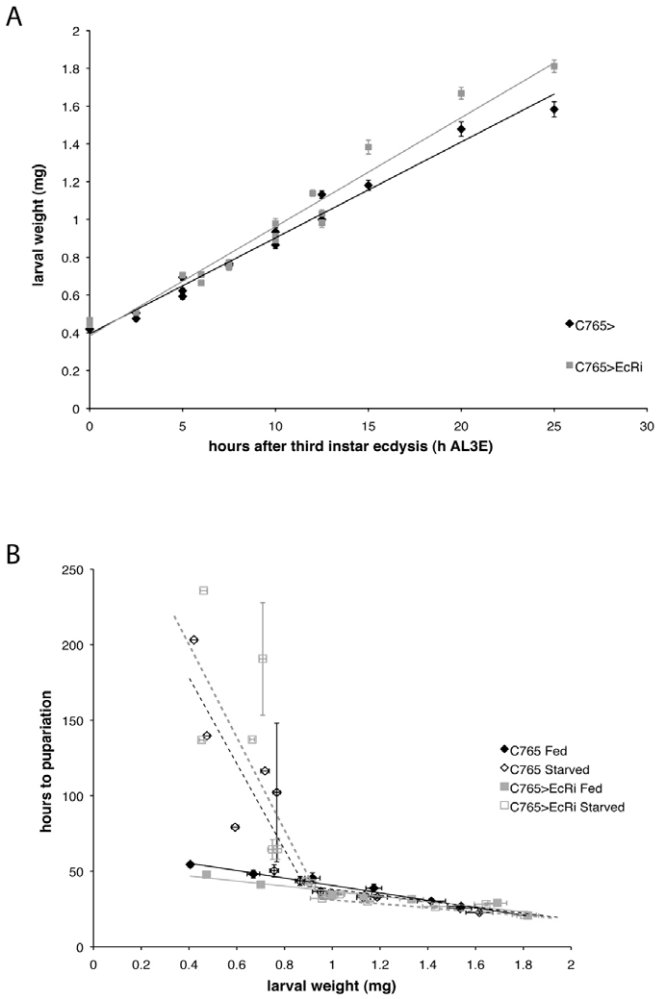

Fig. 5. Larvae in which EcR is suppressed in the wing discs reach critical weight at normal times. (**A**) The growth rate over time of C765 and C765>EcRi larvae. (**B**) The time to pupariation in C765 and C765>EcRi larvae of different size classes when either fed on standard food or starved on 2% agar. Error bars represent s.e.m.

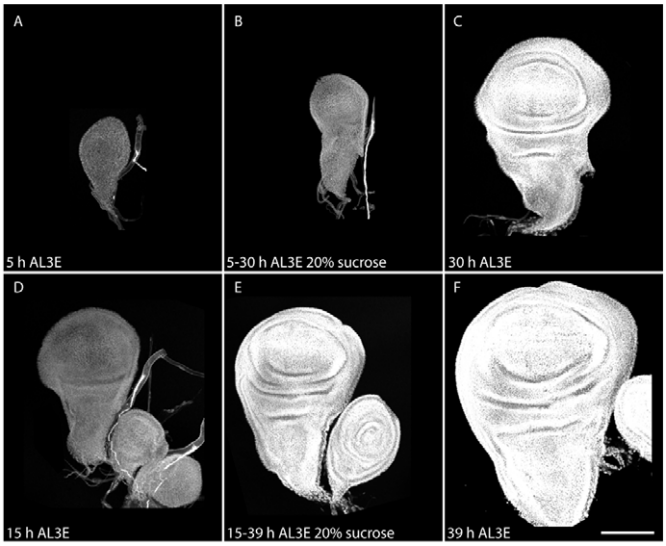

Fig. 6. The ecdysone response gene *broad* is upregulated in discs of w^{1118} larvae post-critical weight. (**A**,**D**) Broad (BR) was expressed at low levels throughout the wing discs at 5 and 15 hours (h) after third instar ecdysis (AL3E). (**B**) BR levels remained low when larvae were fed on sucrose alone from 5-30 hours AL3E. (**E**) Larvae fed on sucrose alone from 15-39 hours AL3E, or (**C**,**F**) larvae staged to 30 and 39 hours AL3E, expressed BR at considerably higher levels than at 5 or 15 hours AL3E. All images were scanned at the same laser power. Scale bar: 100 μm.

important in determining when critical weight has been reached (Mirth and Riddiford, 2007). Here, we have demonstrated that, at least in the imaginal discs, ecdysone signaling is involved in mediating the switch in the developmental response to starvation that occurs at critical weight.

The PGs, ecdysone and critical weight

Our finding that knocking down EcR in the wing discs allows them to pattern in the absence of nutrition offers evidence that ecdysone is acting via EcR to regulate the critical weight transition. Either enhancing insulin signaling in the PGs, which upregulates the ecdysone biosynthetic genes *phm* and *dib* (Caldwell et al., 2005; Colombani et al., 2005), or knocking down EcR in the imaginal discs, results in premature differentiation of WG and SENS expression patterns under sucrose-only conditions. Furthermore, because knocking down EcR in the wing discs, but not overexpressing EcR^{DN}, results in premature differentiation, we conclude that the release of repression mediated by unliganded EcR-USP underlies the switch to nutrition-independent differentiation of the wing disc post-critical weight.

In pre-critical weight larvae fed on sucrose alone, WG expression was not only developmentally delayed but also showed suppressed levels of expression. This suppression was not due to an overall reduction in translation because Actin, Armadillo, EN, PTC and Tubulin were all expressed at the same levels in pre-critical weight larvae fed on sucrose alone and in larvae fed on a standard diet. Instead, the fact that WG expression was reduced in P0206>PTEN and C765>EcR^{DN} larvae fed on standard medium implies that ecdysone might be important in maintaining WG expression in pre-critical weight larvae.

The role of imaginal discs in modulating critical weight

Classic studies of disc overgrowth mutants and of disc fragmentation have led many authors to speculate that critical weight is modified by the growth status of the imaginal discs. Injecting fragmented imaginal discs into prewandering larvae causes a substantial delay to metamorphosis (Simpson et al., 1980). Furthermore, the disc overgrowth mutant *lethal (2) giant larvae* also exhibits a significant delay to metamorphosis (Sehnal and Bryant, 1993). More recently, Stieper and co-authors have shown that slowing disc growth alters critical weight (Stieper et al., 2008). Either damaging discs using X-rays, or reducing their growth rate by knocking down the ribosomal RNA gene *Minute* specifically in the discs, delays critical weight and produces overgrowth phenotypes (Poodry and Woods, 1990; Simpson et al., 1980; Stieper et al., 2008). Clearly, imaginal disc growth has at least an inhibitory effect on critical weight.

Although slow growth in the discs can delay critical weight, our results show that premature differentiation in the disc does not cause critical weight to be reached prematurely. C765>EcRi larvae showed precocious differentiation in their discs when starved, yet their critical weight was not altered. Furthermore,

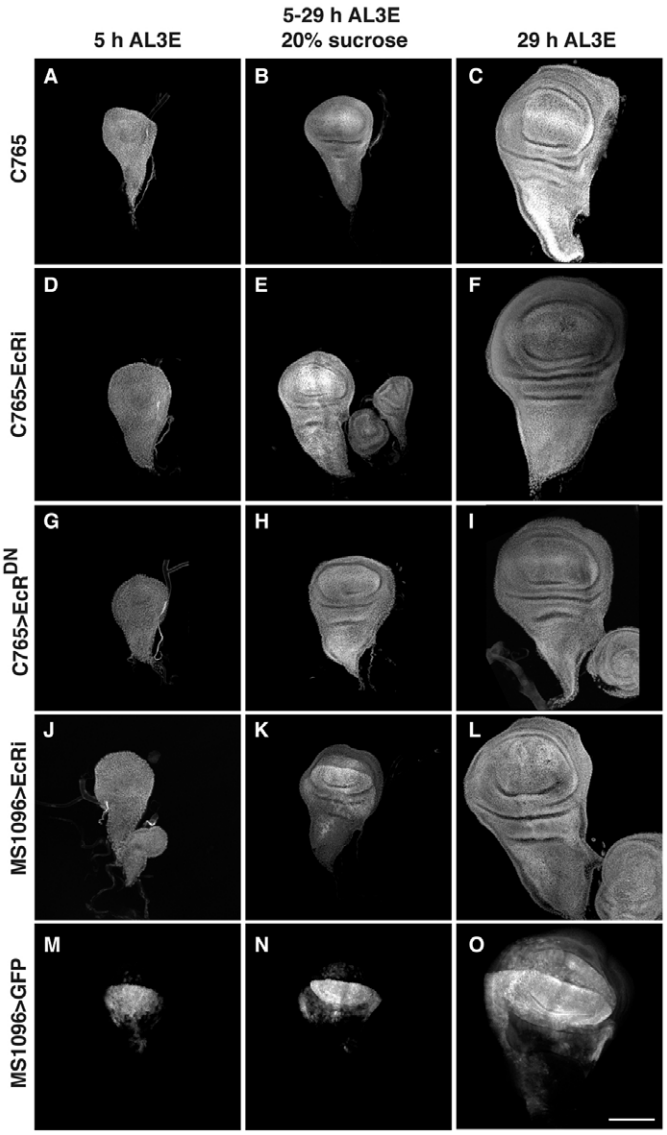

Fig. 7. The regulation of Broad in discs with suppressed EcR signaling. (**A-L**) Expression of Broad in wing imaginal discs from C765 (A-C), C765>EcRi (D-F), C765>EcR^{DN} (G-I), and MS1096>EcRi (J-L) larvae. (**M-O**) GFP expression pattern driven by the MS1096 GAL4 driver. Discs are from (A,D,G,J,M) larvae staged to 5 hours (h) after third instar ecdysis (AL3E), (B,E,H,K,N) larvae fed on sucrose alone from 5-29 hours AL3E, and (C,F,I,L,O) larvae at 29 hours AL3E. Images A-L were scanned at the same laser power. Scale bar: 100 μm.

when discs are completely ablated, metamorphosis occurs on time (Poodry and Woods, 1990; Simpson et al., 1980). Thus, although the discs are able to modulate critical weight, this modulation is only inhibitory.

Interactions between the imaginal disc patterning pathways, the ecdysone pathway and nutrition

Although the ecdysone response pathway has been well described for late-stage larvae and prepupae (Thummel, 1996), less is known about the regulation of ecdysone-responsive genes in the early third instar. *broad* seemed a likely candidate for a gene that could act downstream of EcR to promote post-critical weight development. BR is a zinc-finger transcription factor (DiBello et al., 1991) that is expressed early in the third instar, at least in the fat body (Mugat et al., 2000). In addition, Schubiger et al. had previously found that in wandering-stage wing discs, BR is both upregulated in *usp* mutant clones and necessary for the premature differentiation of wing sensilla in these clones (Schubiger et al., 2005).

In these studies, we found that although BR was upregulated in post-critical weight discs, in C765>EcRi discs, and in the dorsal wing pouch of MS1096>EcRi discs, it was insufficient for stimulating premature differentiation of the WG, CT and SENS expression patterns. Thus, it seems that BR, although necessary for the later stages of imaginal disc patterning, does not function in promoting earlier phases of differentiation during the critical weight transition.

One principal difference between the studies conducted by Schubiger et al. (Schubiger et al., 2005) and the present study is that we only observed premature differentiation in discs from larvae that were malnourished. This implies that pre-critical weight, nutrition modulates ecdysone biosynthesis and that this in turn controls the patterning of gene expression.

Conclusions

Previously, we have shown that insulin-dependent growth in the PGs is involved in regulating the critical weight transition. Here, we have found that attaining critical weight signifies a developmentally important event that allows tissues to continue patterning even in starved or severely malnourished larvae. Furthermore, we have described that ecdysone signaling is the link between the PG-mediated regulation of critical weight and the developmental consequences of reaching critical weight. Ecdysone signaling is important in switching imaginal discs to a post-critical weight developmental program, and this switch is mediated through the derepression of genes repressed by unliganded EcR.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/14/2345/DC1

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