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Broad relays hormone signals to regulate stem cell differentiation in *Drosophila* midgut during metamorphosis

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SUMMARY

Like the mammalian intestine, the *Drosophila* adult midgut is constantly replenished by multipotent intestinal stem cells (ISCs). Although it is well known that adult ISCs arise from adult midgut progenitors (AMPs), relatively little is known about the mechanisms that regulate AMP specification. Here, we demonstrate that Broad (Br)-mediated hormone signaling regulates AMP specification. Br is highly expressed in AMPs temporally during the larva-pupa transition stage, and *br* loss of function blocks AMP differentiation. Furthermore, Br is required for AMPs to develop into functional ISCs. Conversely, *br* overexpression drives AMPs toward premature differentiation. In addition, we found that Br and Notch (N) signaling function in parallel pathways to regulate AMP differentiation. Our results reveal a molecular mechanism whereby Br-mediated hormone signaling directly regulates stem cells to generate adult cells during metamorphosis.

KEY WORDS: *Drosophila*, Adult midgut progenitors, Intestinal stem cells, Hormone, Broad, Differentiation

INTRODUCTION

Adult stem cells maintain tissue homeostasis by continuously replenishing damaged and aged cells in many organisms. It is challenging to address how adult stem cells arise within the tissues of a mammalian system because it requires precise genetic manipulation of stem cells in their native microenvironments and in vivo lineage-labeling techniques. Because *Drosophila* intestinal stem cells (ISCs) in the midgut are an easy genetically tractable system with simple cell lineage, they are a model system for understanding how stem cells arise.

Like the vertebrate intestine, the adult *Drosophila* posterior midgut epithelium is maintained by multipotent ISCs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). In the early embryo stage, the midgut epithelium is formed from the endoderm and contains three distinct cell populations: differentiated epithelial cells [larval enterocytes (ECs)], endocrine cells [larval enteroendocrine cells (EEs)] and undifferentiated adult midgut progenitors (AMPs) (Takashima et al., 2011a). The adult midgut cells, including ISCs, arise from AMPs that remain undifferentiated until the early pupal stage (Jiang and Edgar, 2009; Jiang and Edgar, 2011; Mathur et al., 2010; Micchelli, 2012; Micchelli et al., 2011; Takashima et al., 2011a). AMPs divide approximately seven to ten times at the larval stage. During the first two larval instars (L1 and L2), AMPs divide and spread out, forming islets throughout the midgut. In the third larval instar (L3), AMPs continue to divide within these islets; the division products stay in contact forming AMP clusters called midgut imaginal islands (Jiang and Edgar, 2009; Mathur et al., 2010) (Fig. 1A). The AMP cluster is surrounded by one or more peripheral cells (PCs), which may function as a 'transient niche' to prevent AMP differentiation before the onset of metamorphosis (Mathur et al., 2010). In addition, Notch (N) signaling determines AMP fate: high levels of N activity are required for larval ECs and they limit EE cells, whereas low levels of N activity lead to increased AMP numbers and EEs in the midgut at the expense of differentiated larval ECs (Hartenstein et al., 1992; Takashima et al., 2011a).

At the onset of metamorphosis, the AMP islands start to release AMPs and merge with each other to form a continuous epithelial layer; some AMPs differentiate into ECs positive for Pdm1 (Nubbin – FlyBase). At the same time, PCs spread out and form a transient pupal epithelium surrounding the degenerating larval midgut (Mathur et al., 2010; Takashima et al., 2011a; Takashima et al., 2011b). As metamorphosis continues, most AMPs differentiate into Pdm-positive ECs, while a few AMPs remain undifferentiated (Fig. 1A). These undifferentiated AMPs further divide and develop into ISCs of the adult midgut.

The AMP differentiation occurs during metamorphosis. The entry to metamorphosis from *Drosophila* larvae is coordinately controlled by two lipophilic hormones. At the end of L3, the juvenile hormone (JH) declines and a strong pulse of 20-hydroxyecdysone (20E), the immediate downstream product of ecdysone (E), triggers the larval-pupal transition (King-Jones and Thummel, 2005; Riddiford, 2008; Thummel, 1996). Although the detailed molecular mechanisms of JH and 20E action remain elusive, accumulated evidence suggests that their effects converge on a key transcriptional regulator, Broad (Br, also called Broad-Complex or BR-C). The expression of *br* is directly induced by 20E but inhibited by JH (Huang et al., 2011; Konopova and Jindra, 2008; Suzuki et al., 2008; Zhou et al., 1998; Zhou and Riddiford, 2002). Flies with a loss-of-function *br* mutation develop normally to the end of L3, but they do not enter the pupal stage (Kiss et al., 1988; Restifo and White, 1991). The ectopic expression of *br* during early L2 results in premature puparium (pupal case) formation (Zhou et al., 2004).

Although it is well known that adult midgut cells develop from AMPs, relatively little is known about the mechanisms that regulate AMP specification. Here, we demonstrate that Br-mediated hormone signaling regulates AMP differentiation. Br, which is highly enriched in AMPs, regulates AMP cell-fate specification: *br* loss of function blocks the differentiation of AMPs, whereas *br* overexpression causes premature differentiation

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of AMPs. In addition, Br-mediated hormone signaling is required for AMPs to develop into functional ISCs. We also show that Br and N signaling function in parallel pathways to regulate AMP differentiation, and that the expression of Br in AMPs is regulated by the neuroendocrine organ, the ring gland. Our results reveal a molecular mechanism in which Br-mediated hormone signaling directly regulates progenitors/stem cells to generate adult cells during metamorphosis.

MATERIALS AND METHODS

Fly strains

The following fly strains were used: *esg-Gal4* (from Shigeo Hayashi, Riken); *Su(H)GBE-lacZ* (from Sarah Bray, University of Cambridge); Pswitch lines 5961 (Pswitch^{AMP}) and 5966 (Pswitch^{PC}) (from Haig Keshishian, Yale University); *UAS-br Z1*, *Z2*, *Z3*, *Z4*, *Aug21-Gal4* and *phm-Gal4* (from Lynn Riddiford, Janelia Farm); stocks for the positively marked mosaic lineage (PMML) technique (from Ting Xie, Stowers Institute); *UAS-N*$^{\Delta 34a}$ and *N*^{55e11} (from Ken Irvine, Rutgers); *UAS-N*$^{DN(2X)}$ (from Mark Fortini, NCI-Frederick); *npr1*^{3} (from Linda Restifo, University of Arizona, and Carl Thummel, University of Utah). *Su(H)GBE-Gal4* was generated in our laboratory (Zeng et al., 2010). *br-Gal4* (*GMR69B10*, stock number 46596), *usp*^{2}, *UAS-2XEYFP*, *tub-Gal80*^{ts}, and fly lines used for mosaic analysis with a repressible cell marker (MARCM) clones (*FRT*^{19A},*tub-Gal80*, *hs-flp* and *act>y*$^{+}$*>Gal4, UAS-GFP*) were obtained from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University.

Four Br isoforms (*Z1-4*) (Zhou et al., 1998) were used. The overexpression of *Z1-3* caused AMPs and PCs to prematurely differentiate into Pdm1-positive EC-like cells, whereas overexpression of *Z4* did not affect the timing of AMP/PC differentiation. These results are consistent with a recent report that found that only the *Z1-3* isoforms are expressed in larvae (Huang et al., 2011). We only showed data of overexpressing *br* Z3 in the text.

The following RNAi stocks were obtained from BDSC (Ni et al., 2009) and the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007): *UAS-br*^{IR} (B27272 and V104648), *UAS-EcR*^{IR} (B9326), *UAS-usp*^{IR} (B27258), *UAS-met*^{IR} (B26205), *UAS-Kr-h1*^{IR} (V107935), *UAS-gce*^{IR} (B26323), *UAS-E74*^{IR} (B29353), *UAS-E75*^{IR} (B26717), *UAS-dpp*^{IR} (B25782), *UAS-E75*^{IR} (B26717) and *UAS-tkv*^{IR} (B31040).

MARCM clone assay

To induce MARCM clones of *FRT*^{19A} *sn*^{3} w^{1118}, *FRT*^{19A}*-npr1*^{3}, *FRT*^{19A}*-usp*^{2} and *FRT*^{19A}*-N*^{55e11}, we generated the following flies: *FRT*^{19A} *mutant/FRT*^{19A}*, tub-Gal80*, *hs-flp*; *act>y*$^{+}$*>Gal4, UAS-GFP/+*. Larvae were heat shocked at 37°C for 45 minutes between late L1 and early L2 and were analyzed at the early pupae stage or in adult female flies. For the adult ISC MARCM clone, adult female flies were heat shocked twice at 37°C for 45 minutes and were analyzed in 5 days.

Gal4/Gal80-UAS-mediated gene overexpression or depletion

Male UAS-br or UAS-RNAi transgenic flies were crossed with esg-Gal4, UAS-2XEYFP/esg-Gal4, UAS-2XEYFP; tub-Gal80^{ts}/tub-Gal80^{ts} or Su(H)GBE-Gal4, UAS-mCD8-GFP/Su(H)GBE-Gal4, UAS-mCD8-GFP; tub-Gal80^{ts}/tub-Gal80^{ts} virgin female flies at 18°C. The progenies were transferred to 29°C during early L2 and dissected during late L3 and the early pupal stage.

Developmental staging

Embryos were collected for 1 hour and larvae were staged according to the hours after egg laying (AEL), anterior spiracle morphology, and AMP morphology as described earlier (Bodenstein et al., 1994; Gilboa and Lehmann, 2004; Mathur et al., 2010). Newly formed white prepupae were collected as 0 hours after puparium formation (APF) and were staged in a Petri dish lined with moist Whatman filter paper.

Pswitch analyses

Control and experimental Pswitch^{AMP} (5961) and Pswitch^{PC} (5966) larvae were transferred at the appropriate developmental stages to vials with fly food containing RU-486 (10 μg/ml) (Sigma) and were grown at 29°C to induce GAL4 expression, as described previously (Mathur et al., 2010).

Histology and image capture

The intestines were dissected in PBS and fixed in PBS containing 4% formaldehyde for 30 minutes. After three 5-minutes rinses with PBT (PBS + 0.1% Triton X-100), the samples were blocked with PBT containing 5% normal goat serum overnight at 4°C. The samples were incubated with primary antibody at room temperature for 2 hours. The tissues were then incubated with the fluorescence-conjugated secondary antibody for 1 hour at room temperature. Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). We used the following antibodies: rabbit polyclonal anti-β-Gal (1:1000; Cappel); rabbit polyclonal anti-Arm (1:1000; Santa Cruz); mouse anti-Br core (1:20; DSHB); mouse anti-EcR common (1:20; DSHB); mouse anti-Dl (1:20; DSHB); mouse monoclonal anti-Pros (1:50; DSHB); rabbit polyclonal anti-Pdm1 (1:1000, a gift from X. Yang); and chicken polyclonal anti-GFP (1:3000; Abcam). Secondary antibodies used were goat anti-mouse, anti-chicken, and goat anti-rabbit IgG conjugated to Alexa 488 or Alexa 568 (1:400; Invitrogen). Images were captured with a Zeiss LSM 510 confocal system and processed with LSM Image Browser and Adobe Illustrator.

Quantification and statistical analysis

To quantify the percentage of Pdm1-positive AMP in Fig. 2 and supplementary material Fig. S2, the pdm1-positive AMPs and total GFP-positive AMPs were counted in 1×10^{3} μm^{2} area of the field. In Fig. 4, only the clones that contain at least three cells were counted in the midgut because we could not ascertain whether the single individual cells in the *br* or *usp* mutant MARCM clone arise from single clone or multiple clones; in Fig. 5, the nucleus diameter was measured using LSM5 Image Browser (Zeiss). All the data were analyzed using Student's *t*-test.

RESULTS

Br is highly enriched in AMPs temporally

AMP differentiation occurs at the early stage of metamorphosis. During this stage, the E titer peaks. In addition, *br* is an E-inducible transcription factor that is involved in pupal commitment at the onset of metamorphosis (Zhou et al., 1998; Zhou and Riddiford, 2002). To explore the potential function of *br* and E on AMPs, we examined the expression of Br and the E receptor (EcR) through their respective antibodies. We observed that Br was highly enriched in the AMP islands, including *Su(H)GBE-Gal4, UAS-mCD8-GFP*-labeled PCs starting from late L3, with no detectable Br expression before late L3 (Fig. 1B,C). We also detected weak expression of Br in ECs (Fig. 1C,D). The expression of Br in AMPs remained prominent APF (Fig. 1D,E) and faded by 36 hours APF. Br expression was undetectable in the adult midgut (supplementary material Fig. S1C,C′). One *Gal4* line driven by a *br* promoter fragment, *GMR69B10* (Pfeiffer et al., 2008), directs *UAS-mCD8-GFP* expression in a pattern that closely resembles the temporal and spatial expression pattern of the Br protein (Fig. 1F). In contrast to the high temporal expression of Br in AMPs, the EcR protein was present during all the larval, pupal and adult developmental stages, and was present at high levels in the nuclei of all cell types in the larval and pupal midgut, including polyploid ECs and AMP clusters (supplementary material Fig. S1A-B′).

Br and E signaling are required for AMP differentiation

To investigate the function of Br and E on AMP fate regulation, we depleted Br and EcR and its obligate co-receptor, Ultraspiracle (Usp), in AMPs by expressing Br and EcR RNAi in larval AMPs using the *esg-Gal4, UAS-GFP/+; tub-Gal80*^{ts}*/+* driver (referred to as *esg*^{ts}) (Jiang et al., 2009). At 2 hours APF at 29°C, we observed that AMPs were released from

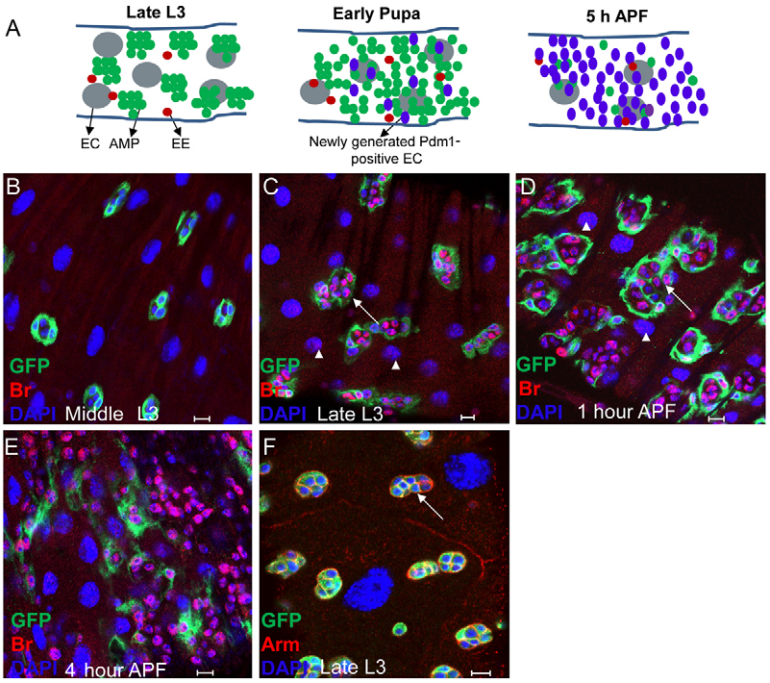

Fig. 1. Br is highly expressed in AMPs. (**A**) A schematic diagram of AMP development. The adult AMPs divide symmetrically to increase their numbers, and they remain dispersed as individual cells throughout the midgut during the first two instars (L1 and L2). Then, each dispersed AMP further divides symmetrically for several rounds to form AMP clusters (islands) at the third instar stage (L3). At about 1-1.5 hours APF at 29°C or 3-4 hours at 25°C, AMPs are released from islands and some of the AMPs start differentiating into ECs. As the pupa develops, most AMPs differentiate into enterocytes (ECs) and only a few AMPs remain undifferentiated. These undifferentiated AMPs further divide to increase their numbers and develop into adult ISCs. (**B-E**) *Su(H)GBE-Gal4, UAS-mCD8-GFP* flies were grown at 25°C and stained at middle L3 (B), late L3 (C), 1 hour APF (D) and 4 hours APF (E). Br was highly expressed in AMPs (arrow) at the late larval and pupa stages, but was also weakly expressed in ECs (arrowhead). GFP, green; Br, nuclear red; DAPI, nuclear blue. (**F**) GMR69B10, a *br* promoter reporter transgenic line, drove UAS-mCD8-GFP in the endogenous Br expression pattern. GFP, green; Arm, red; DAPI, nuclear blue. Arrows point to AMP clusters. Scale bars: 10 μm.

the islands and that 52.2% of AMPs (*n*=26 fields from 11 guts) differentiated into Pdm1-positive ECs in the wild-type control flies (Fig. 2A,A′,G) (Mathur et al., 2010; Takashima et al., 2011a). However, in *br*-RNAi flies in which Br was efficiently depleted (supplementary material Fig. S1D,D′), the differentiation of AMPs was blocked, so that at 2 hours APF at 29°C, all the AMPs still stayed in contact in islands and almost all were Pdm1-negative (only 2.0% of AMPs expressed Pdm1, *n*=21 fields from nine guts; Fig. 2B,B′,G). Knockdown of one of the two 20E receptors, *EcR* (Fig. 2C,C′) or *usp* (Fig. 2D,D′), by RNAi also significantly delayed AMP differentiation, and only 0.5% (*n*=31 fields from 12 guts) of *EcR*-RNAi AMPs and 0.3% of *usp*-RNAi (*n*=26 fields from ten guts) AMPs were Pdm1-positive (Fig. 2G). We also knocked down other E early response genes, *E74* and *E75*, with RNAi using esg^{ts} (Baehrecke, 2000; Thummel, 1996). Like the wild-type control flies (50.8% of AMPs expressed Pdm1, *n*=24 fields from nine guts; supplementary material Fig. S2A,A′D), AMPs were released from islands, 50.6% (*n*=25 fields from nine guts; supplementary material Fig. S2B,B′D) and 49.6% (*n*=21 fields from ten guts; supplementary material Fig. S2C,C′,D) of AMPs differentiated into Pdm1-positive ECs in *E75* and *E74 RNAi* flies at 2 hours APF at 29°C, respectively. These data suggest that *E74* or *E75* are not required for AMP differentiation and that *br* specifically mediates the effects of E on AMP differentiation.

To further follow AMP development in these RNAi flies, we checked the wild-type control and *br*, *EcR* and *usp*-RNAi AMPs at 5 hours APF at 29°C. Although AMPs had been released from the islands in these RNAi flies, most of them (only 15.2% of *br*-RNAi, 14.01% of *EcR*-RNAi and 13.1% of *usp*-RNAi AMPs were Pdm1-positive; *n*=26 fields from seven guts, *n*=25 fields from 11 guts and *n*=21 fields from eight guts, respectively) were still Pdm1-negative, compared with 89.0% (*n*=26 from nine guts) of AMPs that were Pdm1-positive in the wild-type control (Fig. 2G). Interestingly, most of these Pdm1-negative cells expressed the AMP marker Delta (Dl) (Fig. 2F,F′) (Mathur et al., 2010), indicating that these cells were AMPs. By contrast, very few Dl-labeled AMPs remained and most AMPs differentiated into Pdm1-positive ECs in the wild-type control flies (Fig. 2E,E′).

Br-mediated E signaling cell-autonomously regulates AMP differentiation

To further examine the function of *br* and E signaling in AMPs, we generated $npr1^3$ (a null allele of *br*) (Restifo and White, 1991) and usp^2 (a null allele of *usp*) (Henrich et al., 1994) clones, using the MARCM technique (Lee and Luo, 1999). Clones marked with green fluorescent protein (GFP) were induced at the larval stage between late L1 and early L2 with heat shock at 37°C for 45 minutes, and were analyzed at an early pupal stage (Fig. 3A). Clones marked homozygous for wild type (Fig. 3B,B′), $npr1^3$ (Fig. 3C,C′) and usp^2 (Fig. 3D,D′) were generated in AMPs and identified with GFP expression. We found that 90.2% of AMPs in the GFP-marked wild-type clones differentiated into Pdm1-positive ECs at 5 hours APF (Fig. 3B,B′,E, *n*=34 clones). However, the cells in the GFP-marked clones of $npr1^3$ (Fig. 3C,C′,E) and usp^2 (Fig. 3D,D′E) still remained in contact and most of them were Pdm1-negative (only 3.1% of the cells in the $npr1^3$ clone and 2.4% of the cells in the usp^2 clone were Pdm1-positive, *n*=23 and 27 clones, respectively; Fig. 3E). In addition, the nuclei of GFP-labeled mutant *br* and *usp* cells were smaller than those of neighboring non-GFP Pdm1-positive ECs, indicating that these GFP-labeled cells were AMPs because AMPs are diploid and have smaller nuclei, while differentiated ECs are polyploid and have larger nuclei (Jiang and Edgar, 2009; Mathur et al., 2010). These results suggest that Br and E signaling cell-autonomously regulate cell-fate determination and AMP differentiation during metamorphosis.

Consistent with previous reports that E signaling regulates AMP proliferation (Micchelli et al., 2011), we also found that the clone sizes of $npr1^3$ and usp^2 were smaller than those of the wild-type control. Compared with an average of 8.2 cells (*n*=34 clones) in wild-type clones, there were on average only 6.1 cells (*n*=23, $P<0.05$) and 5.9 cells (*n*=27, $P<0.05$) in $npr1^3$ and usp^2 clones, respectively (Fig. 3F).

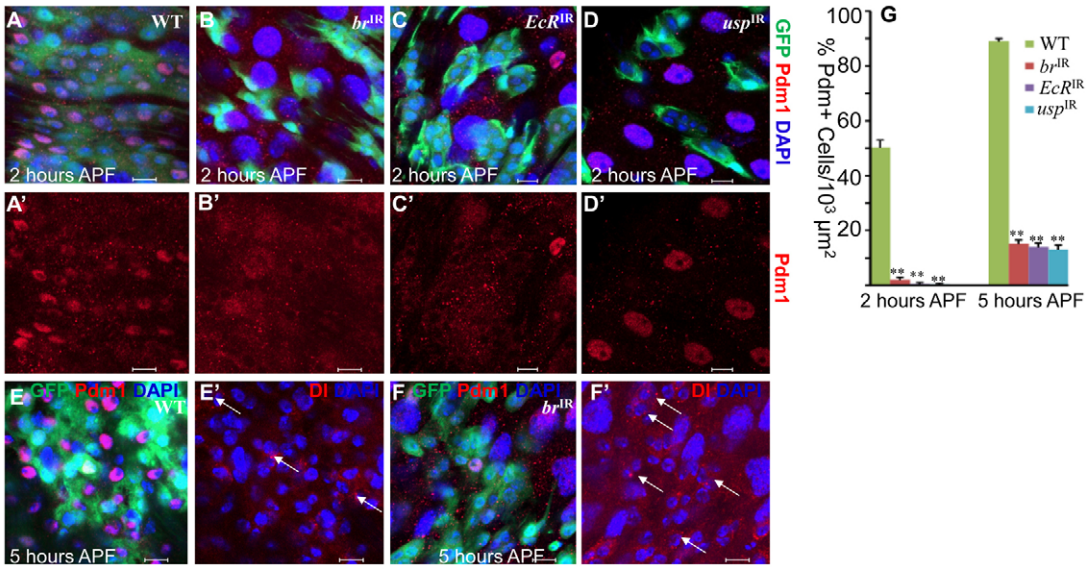

Fig. 2. Br and E signaling are required for AMP differentiation. (**A**,**A′**) In wild-type flies, most of the GFP-labeled AMPs had been released from islands and differentiated into Pdm1-positive ECs at 2 hours APF at 29°C. (**B-D′**) In *br*, *EcR* and *usp-RNAi* flies, AMP differentiation was blocked and the GFP-labeled AMPs still stayed in contact with the islands and were Pdm1-negative at 2 hours APF at 29°C. (**E-F′**) At 5 hours APF at 29°C, wild-type flies (E,E′) retained very few Delta (Dl)-positive AMPs (arrow), which are interspersed among Pdm1-positive ECs while in *br-RNAi* flies (F,F′). Although AMPs had been released from the islands, most AMPs were still Dl-positive (arrow) and Pdm1-negative. (**G**) The percentage of Pdm1-positive ECs in GFP-labeled cells in wild-type control and *br*, *EcR*, and *usp-RNAi* flies at 2 hours APF and 5 hours APF, respectively. Data were represented as mean±s.e.m. (**P<0.01). Wild-type control, *UAS-br*^{IR}, *UAS-EcR*^{IR} and *UAS-usp*^{IR} were driven by *esg*^{ts} from L2 and stained using the indicated antibodies. GFP, green; Pdm1, nuclear red; Dl, cytoplasmic red; DAPI, nuclear blue. Scale bars: 10 μm.

Br-mediated E signal is required for AMP to develop into functional adult ISC

We generated *br*- and *usp*-mutant ISC clones in the adult midgut (supplementary material Fig. S3A). Consistent with the fact that *br* expression was not detected in the adult midgut, *br* was not required for adult ISC differentiation. *br*-mutant adult ISC clones (supplementary material Fig. S3C) were similar to wild-type control clones (supplementary material Fig. S3B), in which there were Dl-labeled ISCs (arrow), differentiated polyploid ECs (star) and Prospero (Pros)-labeled EEs (arrowhead). In addition, *usp* is not required for adult ISC differentiation (supplementary material Fig. S3D).

We traced GFP-marked wild-type-, *br*- and *usp*-mutant AMP clones induced at the larval stage to adult flies (Fig. 4A). The GFP-marked AMPs developed into both ISCs and their differentiated progenies in wild-type adult flies (average 17.75 clones that contain at least three cells/midgut, n=24 guts; Fig. 4B,B′,E), which is consistent with previous studies (Jiang and Edgar, 2009; Micchelli et al., 2011). By contrast, cells derived from the GFP-marked AMPs of *npr1*3 (on average only 0.09 clones that contain at least three cells/midgut, n=31 guts; Fig. 4C,C′,E) and *usp*2 (on average only 0.08 clones that contain at least three cells/midgut, n=25 guts; Fig. 4D,D′,E) developed into individual ISC-like cells in the adult

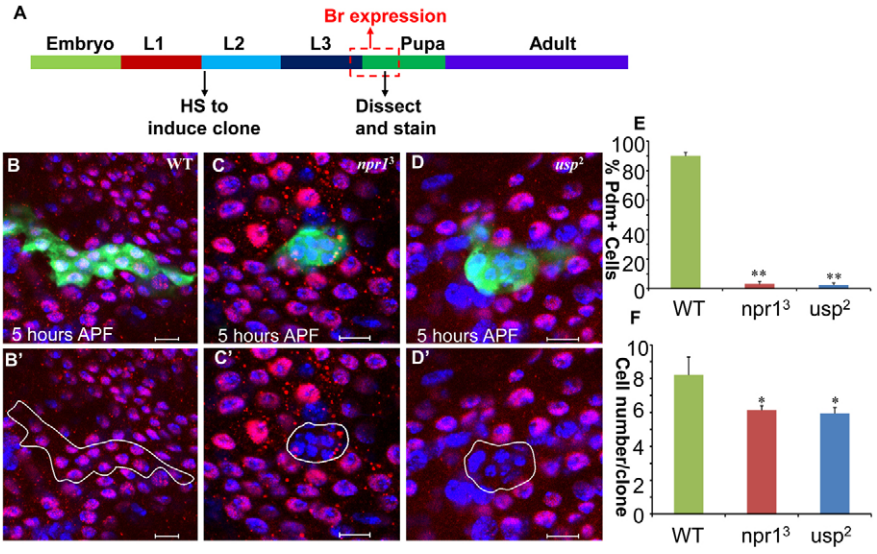

Fig. 3. *br* and *usp* mutations cell-autonomously block AMP differentiation. (**A**) A schematic diagram showing the timing (between late L1 and early L2) of heat shock (HS) to induce MARCM clones during the early larval stage and the time (5 hours APF) of clone analysis at pupariation. (**B-D′**) In wild-type (B,B′) MARCM clones (circled area), most AMPs developed into Pdm1-positive ECs in 5 hours APF, while in *br*- (C,C′) and *usp*- (D,D′) mutant MARCM clones (circled area), the GFP-labeled cells did not express EC marker Pdm1 and had smaller nuclei than neighboring none-GFP Pdm1-positive cells. (**E**) The percentage of Pdm1-positive cells in wild-type, *br* and *usp* MARCM clones at 5 hours APF. (**F**) Analysis of the cell number of wild-type, *br* and *usp* MARCM clones at 5 hours APF. Data were represented as mean±s.e.m. (**P<0.01; *P<0.05). GFP, green; Pdm1, nuclear red; DAPI, nuclear blue. Scale bars: 10 μm.

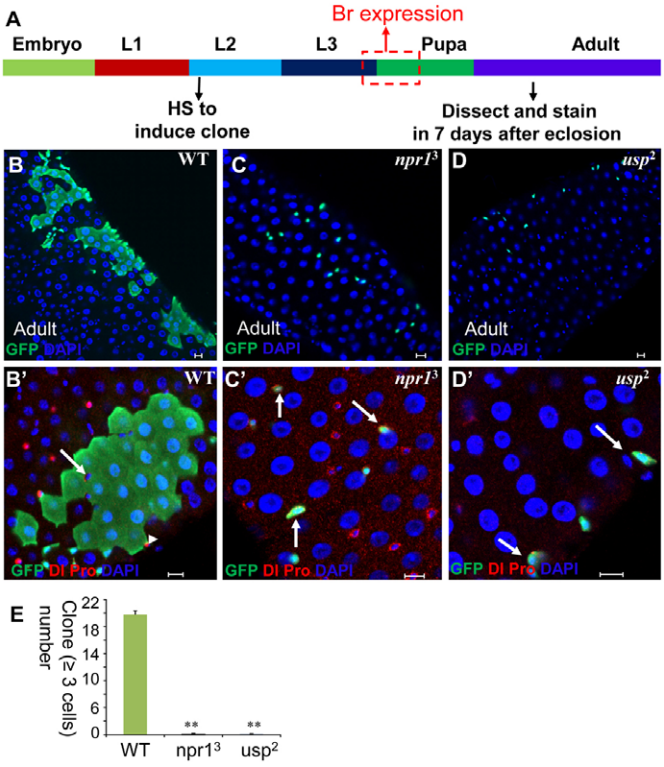

Fig. 4. *br*- and *usp*-mutant AMPs can develop into adult ISC-like cells. (**A**) A schematic diagram of the experimental design for the lineage tracing using MARCM induced by heat shock (HS). The clone was induced between L1 and L2, and flies were dissected 7 days after eclosion at the adult stage. (**B-D**) Low magnification view of FRT19A-WT (B), FRT19A-*npr1*^{3} (C) and FRT19A-*usp*^{2} (D) MARCM clone induced at the larval stage in the adult midgut. (**B′-D′**) The view at higher magnification. Compared with the wild-type GFP-labeled AMPs (B,B′), which developed into adult ISC lineages containing multiple differentiated EC (large nuclei) and EE (arrowhead) cells as well as Dl-positive ISCs (arrow), GFP-labeled *br*- (C,C′) and *usp*- (D,D′) mutant AMPs developed into individual Dl-positive ISC-like cells that may not divide and/or differentiate in the adult midgut. (**E**) The numbers of clones that contain at least three cells in FRT19A-WT, FRT19A-*npr1*^{3} and FRT19A-*usp*^{2} flies. Data were represented as mean±s.e.m. (**P<0.01). GFP, green; Dl, cytoplasmic red; Pros, nuclear red; DAPI, nuclear blue. Scale bars: 10 μm.

midgut. These ISC-like cells were Dl positive, but might have lost the ability to divide and/or differentiate. These results suggest that the *br* and E signaling is required for AMPs to develop into functional adult ISCs.

br overexpression drives premature AMP differentiation

We then analyzed the function of Br in the larval midgut by overexpressing the *br* gene in AMPs. We first expressed *UAS-br* in the larval AMPs and PCs using the *esg*^{ts} driver (referred to as *esg*^{ts}>*br*). Male *UAS-br* and Oregon R (as the wild-type control) flies were crossed with *esg*^{ts} female virgins. The larvae were cultured at 18°C until L2, and then were shifted to the restrictive temperature (29°C). The larvae were dissected and examined at late L3. In late L3 wild-type control larvae, the *esg*^{ts}>*GFP* marked both AMPs and PCs, which were Pdm1-negative, in the AMP/PC islands (none of guts had Pdm1-positive AMPs and PCs, *n*=57 guts; Fig. 5A). By contrast, *br* overexpression resulted in the premature differentiation of AMPs and PCs into Pdm1-positive EC-like cells (100%, *n*=44 guts; Fig. 5B). Similarly, the overexpression of *br* in AMPs and PCs using the *Pswitch*^{AMP} *UAS-GFP* driver (Mathur et al., 2010) also resulted in the premature differentiation of AMPs and PCs into Pdm1-positive EC-like cells 100%, *n*=41 guts; Fig. 5D), compared with the wild-type control (none of the guts had Pdm1-positve AMPs and PCs, *n*=30; Fig. 5C). We then specifically expressed *UAS-br* in PCs using the PC-specific driver *Su(H)GBE-Gal4, UAS-mCD8-GFP/+; tub-Gal80*^{ts}/+ (referred to as *Su(H)*^{ts}>*br*) (Zeng et al., 2010) (*n*=49 guts; Fig. 5F) or the *Pswitch*^{PC}, *UAS-GFP* (Mathur et al., 2010) (*n*=52 guts; Fig. 5H) drivers. Interestingly, compared with the wild-type controls (none of the guts had Pdm1-positive PCs for both controls, *n*=23 and 28, respectively; Fig. 5E,G), *br* overexpression in PCs also resulted in premature differentiation into Pdm1-positive cells (the PCs in 95.9% of *Su(H)*^{ts}>*br* and the PCs in 100% of *Pswitch*^{PC}>*br* guts were both Pdm1-positive; Fig. 5F,H). We also noted that *br* overexpression in these AMPs and/or PCs resulted in larger nuclei than in the wild-type controls (Fig. 5K), indicating that these AMPs and PCs were developing into polyploid EC-like cells.

To rule out the possibility that Br functioned outside of the AMP/PC islands to indirectly regulate their differentiation, we generated GFP-marked mosaic clones that were either wild type (0 clones were pdm1-positive, *n*=24 guts; Fig. 5I) or overexpressed *br* (Fig. 5J), using the PMML labeling technique (Kirilly et al., 2005). The overexpression of *br* in GFP-marked PC PMML clones also caused their premature differentiation into Pdm1-positive EC-like cells (100% clones were pdm1-positive, *n*=19 guts; Fig. 5J). In summary, *br* overexpression in AMPs/PCs resulted in the premature differentiation of AMPs/PCs into Pdm1-positive EC-like cells.

We also performed selective ectopic overexpression of *br* in adult ISCs using *esg*^{ts}. Interestingly, ectopic overexpression of *br* drove all ISCs to differentiate into EC-like cells (supplementary material Fig. S4). Thus, *br* may use a general mechanism to drive both AMPs and ISCs to differentiate into EC-like cells.

Br and N signaling function in parallel to regulate AMP differentiation

In the larva and pupa, N pathway activation triggers AMPs to differentiate into ECs (Takashima et al., 2011a). We further examined the function of the N signal transduction pathway in AMP differentiation and generated N^{55e11} (a null allele of *N*) (Rauskolb and Irvine, 1999) MARCM clones (*n*=29 clones; supplementary material Fig. S5A,A′). Loss-of-function *N* cell-autonomously blocked the differentiation of AMPs into ECs during metamorphosis because all of the N-mutant AMPs in the GFP-marked clones were Pdm1-negative at 5 hours APF. However, unlike the *br*-mutant AMPs, which developed into ISC-like cells in the adult stage (Fig. 4C,C′), all cells derived from the GFP-marked N-null AMPs induced at the larval stage between late L1 and early L2 developed into Pros-positive EEs in the adult midgut (*n*=21 guts; supplementary material Fig. S5B,B′). Blocking N signaling by overexpressing a dominant-negative form of N (*esg*^{ts}>N^{DN}) induced AMP expansion (*n*=44 guts; Fig. 6A) but did not inhibit Br expression (100% guts were Br positive, *n*=47 guts; Fig. 6F). Overexpression of an activated form of N in AMPs (*esg*^{ts}>$N^{\Delta 34a}$) resulted in precocious Pdm1 expression (91.6% GFP-positive cells were Pdm1 positive, *n*=43 guts; Fig. 6C) and premature AMP differentiation into EC-like cells (Fig. 6C), but did not result in precocious Br expression (*n*=45 guts; Fig. 6E).

Both N signaling and Br are required for AMP differentiation into EC. To further examine the genetic relationship between the Br and

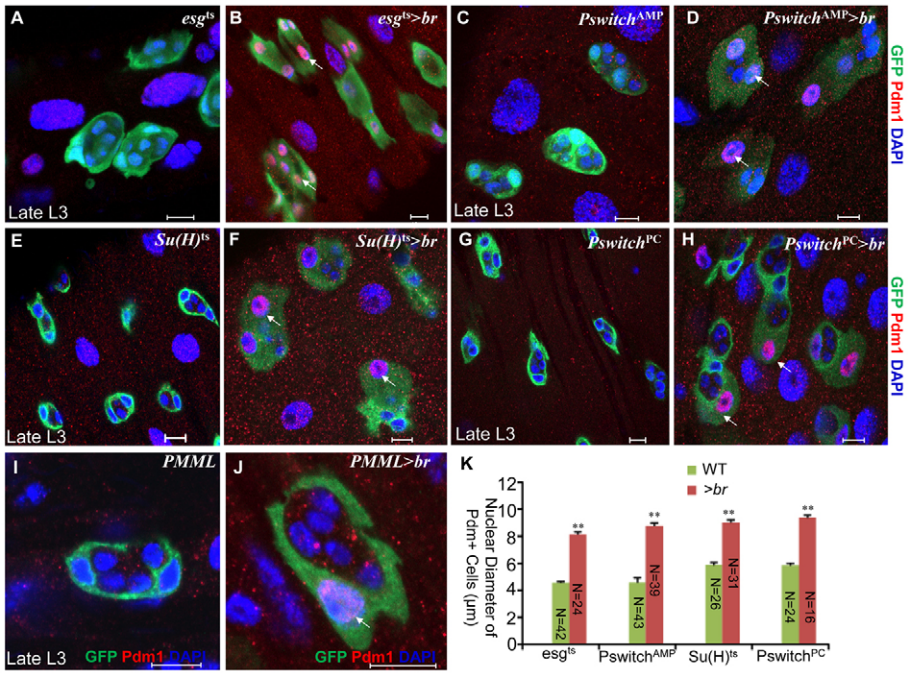

Fig. 5. *br* overexpression in AMPs drives premature differentiation of AMPs. (**A-D**) A wild-type control (A,C) or *UAS-br* (B,D) was driven by esg^{ts} (A,B) or $Pswitch^{AMP}$, *UAS-GFP* (C,D) in AMPs and PCs. *br* overexpression caused premature differentiation of AMPs and PCs into Pdm1-positive EC-like cells. (**E-H**) A wild-type control (E,G) or *UAS-br* (F,H) was driven by PC-specific driver $Su(H)^{ts}$ (E,F) or $Pswitch^{PC}$, *UAS-mCD8-GFP* (G,H). *br* overexpression in PC cells caused PC differentiation into Pdm1-positive EC-like cells. (**I**) The wild-type PMML PC clone. (**J**) The PMML PC clone that overexpressed *br*. (**K**) The nuclear size of wild-type control and Pdm1-positive cells caused by *br* overexpression. Data were represented as mean±s.e.m. (**P<0.01). GFP, green; Pdm1, nuclear red; DAPI, nuclear blue. All larvae were stained at the late L3 stage. Arrows point to Pdm1-positive differentiated cells. Scale bars: 10 μm.

N signal transduction pathways in regulating AMP differentiation, we expressed the dominant-negative forms of *N* and *br* ($esg^{ts}>N^{DN}+br$; n=38 guts; Fig. 6B) or the constitutively activated forms of *N* and br^{IR} ($esg^{ts}>N^{\Delta 34a}+br^{IR}$; n=47 guts; Fig. 6D) in AMPs. Surprisingly, the expression of either $N^{DN}+br$ or $N^{\Delta 34a}+br^{IR}$ still led to the premature differentiation of AMPs into Pdm1-positive EC-like cells (Fig. 6B,D), indicating that the activation of either the Br or N pathways can drive AMP differentiation.

To directly investigate whether Br regulates the N signal transduction pathway, we examined the expression of *Su(H)GBE-lacZ* (a reporter of N signaling) (Furriols and Bray, 2001) in midguts that overexpressed br^{IR} ($esg^{ts}>br^{IR}$) (Fig. 6G,H). We found that br^{IR} overexpression did not affect *Su(H)GBE-lacZ* expression, compared with the wild-type control (supplementary material Fig. S5C,C′).

Together, these results suggest that the Br and N signal transduction pathways may function in parallel pathways to regulate AMP differentiation.

The neuroendocrine organ, the ring gland, regulates Br expression in AMPs

During insect larva-pupa transition, both 20E and JH titers change to regulate this transition. To investigate the potential role of JH in AMPs, we knocked down JH candidate receptor *Methoprene-tolerant* (*Met*) and found that the knockdown of *Met* by RNAi led to the premature differentiation of AMPs into Pdm1-positive EC-like cells (supplementary material Fig. S6A,A′). Interestingly, knockdown of *Met* and *Krüppel homolog 1* (*Kr-h1*), a JH response gene, resulted in precocious Br expression in AMPs (supplementary material Fig. S6B-C′), but knockdown of *Germ cell-expressed* (*gce*), another JH candidate receptor, did not cause precocious Br expression in AMPs (supplementary material Fig. S6D,D′). This is consistent with previous reports that JH inhibits Br expression through Kr-h1 (Huang et al., 2011; Konopova and Jindra, 2008; Suzuki et al., 2008; Zhou et al., 1998; Zhou and Riddiford, 2002). JH may act in concert with 20E to regulate AMP differentiation by controlling Br expression.

The ring gland is the major organ in which JH and 20E are produced. The decapentaplegic (Dpp) signal transduction pathway in the corpus allatum (CA) of the ring gland and the Torso-Ras/Raf-MAPK and insulin-like receptor (InR)-PI3K-Akt pathways in the prothoracic gland (PG) of the ring gland were reported to regulate JH and 20E production, respectively (Fig. 7E) (Caldwell et al., 2005; Gibbens et al., 2011; Huang et al., 2011; Mirth et al., 2005; Rewitz et al., 2009). Manipulating these signaling pathways in the ring gland may change the Br expression pattern in AMPs through JH or 20E. To test this hypothesis, we knocked down *dpp* and one of its receptors, *thick vein* (*tkv*), by expressing transgenic RNAi lines using a CA-specific driver, *Aug21-Gal4* (Huang et al., 2011). We also overexpressed constitutively activated forms of *Ras* ($UAS\text{-}Ras^{V12}$), *Raf* ($UAS\text{-}Raf^{gof}$) and *PI3K* ($UAS\text{-}PI3K^{CAAX}$) using a PG-specific driver, *phm-Gal4* (Gibbens et al., 2011). Indeed, compared with wild-type controls (supplementary material Fig. S7A,A′C,C′), the knockdown of *dpp* (Fig. 7A,A′) and *tkv* (supplementary material Fig. S7B,B′) in CA or the overexpression of constitutively activated forms of *Ras* (Fig. 7C,C′), *Raf* (supplementary material Fig. S7D,D′) and *PI3K* (Fig. 7D,D′) in PG resulted in precocious Br expression in AMPs. Interestingly, knocking down *dpp* with $Su(H)^{ts}$ in PCs did not result in precocious Br expression in AMPs (Fig. 7B,B′). PCs function as an AMP niche by expressing Dpp to activate the Dpp signal transduction pathway in AMPs and preventing AMP differentiation before the onset of metamorphosis (Mathur et al., 2010).

These results, along with those of previous reports (Gibbens et al., 2011; Huang et al., 2011), suggest that signals from the ring gland and morphogenetic hormones converge on Br expression in AMPs to regulate AMP differentiation during metamorphosis.

DISCUSSION

Although it is well known that adult ISCs arise from AMPs, the mechanisms that regulate AMP specification were unclear. In this study, we demonstrated that Br-mediated hormone signaling plays a key role in regulating AMP differentiation and that Br is required

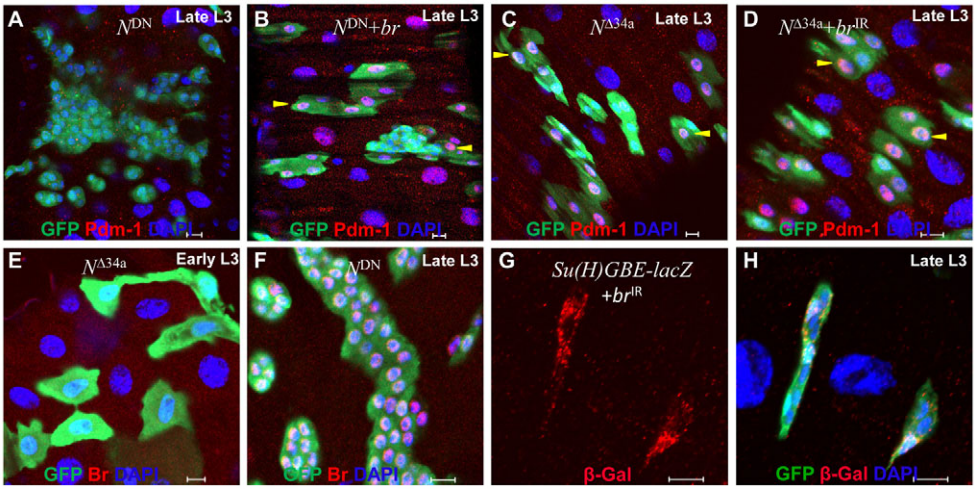

Fig. 6. Br and N regulate AMP differentiation in parallel pathways. (**A-D**) *UAS-N*^{DN} (A), *UAS-N*^{DN}+*UAS-br* (B), *UAS-N*$^{\Delta 34a}$ (C) or *UAS-N*$^{\Delta 34a}$+*UAS-br*^{IR} (D) was driven by *esg*^{ts}. Even though N activity was blocked by *N*^{DN}, *br* overexpression caused AMP premature differentiation (compare B with A). Likewise, even though Br was knocked down, N overactivation caused AMP differentiation (compare C with D). The yellow arrowheads point to Pdm1-positive cells. DAPI, nuclear blue; GFP, green; Pdm1, nuclear red. (**E**,**F**) *UAS-N*$^{\Delta 34a}$ (E) or *UAS-N*^{DN} (F) was driven by *esg*^{ts}. Overexpression of an activated form of N in AMPs resulted in premature AMP differentiation into separated EC-like cells, but did not result in precocious Br expression. Overexpression of a dominant-negative form of N in AMPs induced AMP expansion and blocked AMP differentiation into ECs, but it did not repress Br expression. GFP, green; Br, nuclear red; DAPI, nuclear blue. (**G**,**H**) *UAS-br*^{IR} was driven by *Su(H)GBE-lacZ; esg*^{ts}. The knockdown of *br* did not affect the N activity because the expression of *Su(H)GBE-lacZ*, an N activity reporter, was not affected. GFP, green; β-galactosidase, red; DAPI, nuclear blue. Scale bars: 10 μm.

for AMPs to develop into functional ISCs. Interestingly, our genetic interaction analyses revealed that Br regulates AMP differentiation through a pathway that is parallel to the N signaling pathway. We also provide evidence suggesting that Br expression in AMPs is regulated by multiple signaling pathways in the ring gland, which is a neuroendocrine organ. Our results reveal a molecular mechanism whereby Br-mediated hormone signals directly regulate progenitors/stem cells to generate adult cells during metamorphosis.

With the decline of JH at the end of L3, a strong 20E pulse triggers the larval-pupal transition (King-Jones and Thummel, 2005; Riddiford, 2008; Thummel, 1996). During metamorphosis, the larval tissues undergo histolysis through programmed cell death, while adult structures grow from adult cells generated by adult progenitor cells (Jiang et al., 1997; Thummel, 1996). Type I programmed cell death (PCD), apoptosis, and type II PCD, autophagy, are both involved in larval tissue histolysis (Liu et al., 2009; Ryoo and Baehrecke, 2010; Yin and Thummel, 2005). In comparison to the extensive studies on hormone-regulated PCD during metamorphosis, there is a major information gap between the direct actions of the hormones and AMP differentiation. In this study, we show that Br is one of the key missing connectors. Br is highly enriched in AMPs immediately before the onset of metamorphosis, and it regulates AMP cell-fate specification. We also demonstrat that neuroendocrine signals regulate Br expression in AMPs via JH and 20E. Previous studies suggested that the N signal transduction pathway plays a major role in regulating AMP differentiation during metamorphosis (Mathur et al., 2010; Takashima et al., 2011a); however, we did not find any direct connection between the hormones-Br pathway and the N pathway in regulating AMP differentiation. These pathways may function in parallel, converging on downstream factors that directly regulate AMP differentiation. A previous study also found that larval midgut degeneration is blocked in *br* mutants (Restifo and White, 1992), suggesting that Br regulates not only adult cell generation but also larval PCD. It will be very interesting to examine in future experiments how Br coordinates larval cell death with adult cell generation during the onset of metamorphosis.

During larval development, AMPs generate at least one PC through asymmetric division, to wrap around the AMPs. PCs function as a niche to maintain undifferentiated AMP by secreting Dpp to activate Dpp signaling in AMPs before the onset of metamorphosis (Mathur et al., 2010). Dpp may regulate AMP differentiation from two directions. In one direction, Dpp signaling regulates JH biosynthesis in CA and indirectly regulates Br expression in AMPs to drive differentiation. In the other direction, Dpp in PCs may directly activate the Dpp signal transduction pathway in AMPs (Mathur et al., 2010), thereby regulating AMP differentiation through a Br-independent pathway.

In the early stages of metamorphosis, AMPs are released from the PC niche and AMP islands start to merge (Mathur et al., 2010). We speculate that because both AMPs and PCs express high levels of Br, Br-mediated hormone signals trigger AMP differentiation to be released from the PCs by controlling both AMPs and PCs at the same time. AMPs with *br* or *usp* loss of function still stay in contact and do not differentiate in 5 hours APF, indicating that hormone signaling controls the dispersion and differentiation of AMPs directly. Furthermore, PCs start to express the differentiation marker Pdm1 and exhibit polyploid nuclei when *br* is overexpressed in PCs at the larval stage. These differentiating PCs may not fulfill normal niche functions to encase the AMPs to prevent their release.

Adult ISCs develop from AMPs, but AMPs and ISCs also share many similarities. First, both express the same specific marker, Dl (Mathur et al., 2010; Ohlstein and Spradling, 2007). AMPs and ISCs can also both give rise to EEs and ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Takashima et al., 2011a). Another similarity is that N signaling controls the fate of AMPs and

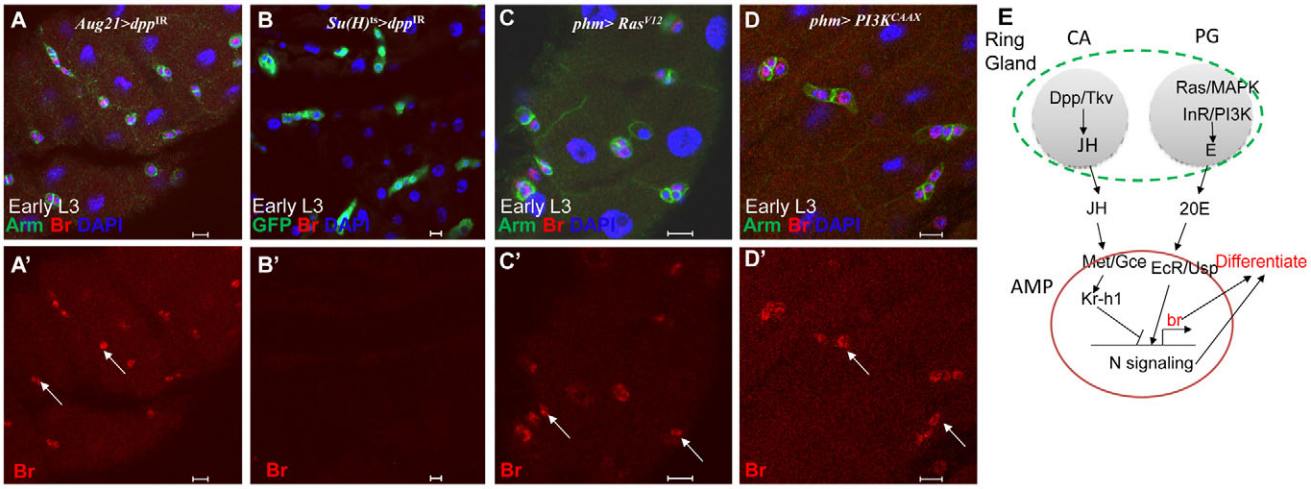

Fig. 7. Neuroendocrine regulation of Br expression in AMP. (**A**,**A′**) CA-specific knockdown of *dpp* (*Aug21>dpp*^{IR}) induced precocious Br expression. (**B**,**B′**) PC-specific knockdown of *dpp* [*Su*(*H*)^{ts}>*dpp*^{IR}] did not induce precocious Br expression. (**C**,**C′**) Prothoracic gland (PG)-specific expression of a constitutively activated form of Ras (*phm>Ras*^{V12}) induced precocious Br expression. (**D**,**D′**) PG-specific expression of a constitutively activated form of PI3K (*phm>PI3K*^{CAAX}) induced precocious Br expression. The arrow indicates precocious Br expression in AMPs. Arm or GFP, green; Br, nuclear red; DAPI, nuclear blue. Scale bars: 10 μm. (**E**) The model for AMP differentiation during metamorphosis regulated by Br-mediated hormone signals.

ISCs. Finally, the epidermal growth factor (EGF) signal from the visceral muscle supports AMP and ISC proliferation (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang and Edgar, 2009; Jiang et al., 2011; Xu et al., 2011). Interestingly, we also found that ectopically expressing *br* in AMPs and ISCs drives their differentiation into Pdm1-positive EC-like cells, which indicates that Br may regulate the differentiation of AMPs and ISCs by a general mechanism. Strikingly, the transient expression of Br in AMPs in the late larval and early pupal stages is essential for AMPs to develop into functional ISCs. AMPs with *br* loss of function develop into ISC-like cells, which may not divide and/or differentiate in the adult stage. The transient expression of Br may be one of the key factors involved in programming AMPs into functional ISCs.

Br is also expressed in imaginal disc cells and histoblast during metamorphosis and it plays a key role in regulating imaginal disc differentiation and histoblast proliferation (Kiss et al., 1988; DiBello et al., 1991; Zhou and Riddiford, 2002). Thus, Br may play a universal role in regulating the differentiation of progenitors, including imaginal cells during metamorphosis.

In mammals (including humans), the passage through puberty has some similarities to insect metamorphosis. In both cases, neuropeptide signaling in response to developmental, environmental and physiological cues regulates the expression of steroid hormones that further trigger the transition process from juveniles into sexually mature adults. The onset of puberty in humans is marked by breast development in girls, testicular enlargement in boys and pubic hair growth in both girls and boys (Carel and Leger, 2008). However, it is unclear how neuropeptide/steroid hormone signaling regulates the biological changes occurring in peripheral tissues. Human puberty, unlike insect metamorphosis, does not involve massive cell death. Rather, it mostly involves developing new tissues/organs. Transcription factors, such as homologues of Br, may relay neuroendocrine/steroid hormone signals to regulate the generation of adult cells from their progenitor/stem cells, leading to pubic hair production from follicle stem cells, spermatogenesis from testis germline stem cells, and breast development from mammary gland stem cells. Future experiments in mammals along these lines, and new details about metamorphosis in insects and amphibians, may provide novel insights into human puberty and the disorders affecting it.

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Competing interests statement
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References
Baehrecke, E. H. (2000). Steroid regulation of programmed cell death during *Drosophila* development. *Cell Death Differ.* **7**, 1057-1062.
Biteau, B. and Jasper, H. (2011). EGF signaling regulates the proliferation of intestinal stem cells in *Drosophila*. *Development* **138**, 1045-1055.
Bodenstein, D. (1994). The postembryonic development of *Drosophila*. In *Biology of Drosophila* (ed. M. Demerec), pp. 275-367. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Buchon, N., Broderick, N. A., Kuraishi, T. and Lemaitre, B. (2010). *Drosophila* EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. *BMC Biol.* **8**, 152.
Caldwell, P. E., Walkiewicz, M. and Stern, M. (2005). Ras activity in the *Drosophila* prothoracic gland regulates body size and developmental rate via ecdysone release. *Curr. Biol.* **15**, 1785-1795.
Carel, J. C. and Leger, J. (2008). Clinical practice. Precocious puberty. *N. Engl. J. Med.* **358**, 2366-2377.
DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W. and Guild, G. M. (1991). The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics* **129**, 385-397.

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448**, 151-156.
Furriols, M. and Bray, S. (2001). A model Notch response element detects Suppressor of Hairless-dependent molecular switch. *Curr. Biol.* **11**, 60-64.
Gibbens, Y. Y., Warren, J. T., Gilbert, L. I. and O'Connor, M. B. (2011). Neuroendocrine regulation of *Drosophila* metamorphosis requires TGFbeta/Activin signaling. *Development* **138**, 2693-2703.
Gilboa, L. and Lehmann, R. (2004). Repression of primordial germ cell differentiation parallels germ line stem cell maintenance. *Curr. Biol.* **14**, 981-986.
Hartenstein, A. Y., Rugendorff, A., Tepass, U. and Hartenstein, V. (1992). The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. *Development* **116**, 1203-1220.
Henrich, V. C., Szekely, A. A., Kim, S. J., Brown, N. E., Antoniewski, C., Hayden, M. A., Lepesant, J. A. and Gilbert, L. I. (1994). Expression and function of the *ultraspiracle* (*usp*) gene during development of *Drosophila melanogaster*. *Dev. Biol.* **165**, 38-52.
Huang, J., Tian, L., Peng, C., Abdou, M., Wen, D., Wang, Y., Li, S. and Wang, J. (2011). DPP-mediated TGFbeta signaling regulates juvenile hormone biosynthesis by activating the expression of juvenile hormone acid methyltransferase. *Development* **138**, 2283-2291.
Jiang, C., Baehrecke, E. H. and Thummel, C. S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* **124**, 4673-4683.
Jiang, H. and Edgar, B. A. (2009). EGFR signaling regulates the proliferation of *Drosophila* adult midgut progenitors. *Development* **136**, 483-493.
Jiang, H. and Edgar, B. A. (2011). Intestinal stem cells in the adult *Drosophila* midgut. *Exp. Cell Res.* **317**, 2780-2788.
Jiang, H., Patel, P. H., Kohlmaier, A., Grenley, M. O., McEwen, D. G. and Edgar, B. A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the *Drosophila* midgut. *Cell* **137**, 1343-1355.
Jiang, H., Grenley, M. O., Bravo, M. J., Blumhagen, R. Z. and Edgar, B. A. (2011). EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in *Drosophila*. *Cell Stem Cell* **8**, 84-95.
King-Jones, K. and Thummel, C. S. (2005). Nuclear receptors-a perspective from *Drosophila*. *Nat. Rev. Genet.* **6**, 311-323.
Kirilly, D., Spana, E. P., Perrimon, N., Padgett, R. W. and Xie, T. (2005). BMP signaling is required for controlling somatic stem cell self-renewal in the *Drosophila* ovary. *Dev. Cell* **9**, 651-662.
Kiss, I., Beaton, A. H., Tardiff, J., Fristrom, D. and Fristrom, J. W. (1988). Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. *Genetics* **118**, 247-259.
Konopova, B. and Jindra, M. (2008). Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development* **135**, 559-568.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Liu, Y., Sheng, Z., Liu, H., Wen, D., He, Q., Wang, S., Shao, W., Jiang, R. J., An, S., Sun, Y. et al. (2009). Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*. *Development* **136**, 2015-2025.
Mathur, D., Bost, A., Driver, I. and Ohlstein, B. (2010). A transient niche regulates the specification of *Drosophila* intestinal stem cells. *Science* **327**, 210-213.
Micchelli, C. A. (2012). The origin of intestinal stem cells in *Drosophila*. *Dev. Dyn.* **241**, 85-91.
Micchelli, C. A. and Perrimon, N. (2006). Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. *Nature* **439**, 475-479.
Micchelli, C. A., Sudmeier, L., Perrimon, N., Tang, S. and Beehler-Evans, R. (2011). Identification of adult midgut precursors in *Drosophila*. *Gene Expr. Patt.* **11**, 12-21.
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.
Ni, J. Q., Liu, L. P., Binari, R., Hardy, R., Shim, H. S., Cavallaro, A., Booker, M., Pfeiffer, B. D., Markstein, M., Wang, H. et al. (2009). A Drosophila resource of transgenic RNAi lines for neurogenetics. *Genetics* **182**, 1089-1100.
Ohlstein, B. and Spradling, A. (2006). The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. *Nature* **439**, 470-474.
Ohlstein, B. and Spradling, A. (2007). Multipotent *Drosophila* intestinal stem cells specify daughter cell fates by differential notch signaling. *Science* **315**, 988-992.
Pfeiffer, B. D., Jenett, A., Hammonds, A. S., Ngo, T. T., Misra, S., Murphy, C., Scully, A., Carlson, J. W., Wan, K. H., Laverty, T. R. et al. (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 9715-9720.
Rauskolb, C. and Irvine, K. D. (1999). Notch-mediated segmentation and growth control of the *Drosophila* leg. *Dev. Biol.* **210**, 339-350.
Restifo, L. L. and White, K. (1991). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of the central nervous system in *Drosophila*. *Dev. Biol.* **148**, 174-194.
Restifo, L. L. and White, K. (1992). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila* salivary glands, muscle, and gut. *Roux's Arch. Dev. Biol.* **201**, 221-234.
Rewitz, K. F., Yamanaka, N., Gilbert, L. I. and O'Connor, M. B. (2009). The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. *Science* **326**, 1403-1405.
Riddiford, L. M. (2008). Juvenile hormone action: a 2007 perspective. *J. Insect Physiol.* **54**, 895-901.
Ryoo, H. D. and Baehrecke, E. H. (2010). Distinct death mechanisms in *Drosophila* development. *Curr. Opin. Cell Biol.* **22**, 889-895.
Suzuki, Y., Truman, J. W. and Riddiford, L. M. (2008). The role of Broad in the development of Tribolium castaneum: implications for the evolution of the holometabolous insect pupa. *Development* **135**, 569-577.
Takashima, S., Younossi-Hartenstein, A., Ortiz, P. A. and Hartenstein, V. (2011a). A novel tissue in an established model system: the *Drosophila* pupal midgut. *Dev. Genes Evol.* **221**, 69-81.
Takashima, S., Adams, K. L., Ortiz, P. A., Ying, C. T., Moridzadeh, R., Younossi-Hartenstein, A. and Hartenstein, V. (2011b). Development of the *Drosophila* entero-endocrine lineage and its specification by the Notch signaling pathway. *Dev. Biol.* **353**, 161-172.
Thummel, C. S. (1996). Flies on steroids-*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12**, 306-310.
Xu, N., Wang, S. Q., Tan, D., Gao, Y., Lin, G. and Xi, R. (2011). EGFR, Wingless and JAK/STAT signaling cooperatively maintain *Drosophila* intestinal stem cells. *Dev. Biol.* **354**, 31-43.
Yin, V. P. and Thummel, C. S. (2005). Mechanisms of steroid-triggered programmed cell death in *Drosophila*. *Semin. Cell Dev. Biol.* **16**, 237-243.
Zeng, X., Chauhan, C. and Hou, S. X. (2010). Characterization of midgut stem cell- and enteroblast-specific Gal4 lines in *Drosophila*. *Genesis* **48**, 607-611.
Zhou, B., Hiruma, K., Shinoda, T. and Riddiford, L. M. (1998). Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **203**, 233-244.
Zhou, X. and Riddiford, L. M. (2002). Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development* **129**, 2259-2269.
Zhou, X., Zhou, B., Truman, J. W. and Riddiford, L. M. (2004). Overexpression of broad: a new insight into its role in the *Drosophila* prothoracic gland cells. *J. Exp. Biol.* **207**, 1151-1161.