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.

INTRODUCTION

Adult stem cells maintain tissue homeostasis by continuously replenishing damaged and aged cells in many organisms. It is challenging to understand 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; Ohsie 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, are maintained by shifts in the hormone environment from juvenile hormone (JH) to 20E. 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; Suzuki et al., 2008; Zhou et al., 1998; Zhou and Riddiford, 1998). At the onset of metamorphosis, AMPs divide and merge with each other to form a continuous epithelial layer; some AMPs differentiate into ECs positive for Pdm1 (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, 2002; 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.
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\textsuperscript{AMP}) and 5966 (Pswitch\textsuperscript{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-\textsuperscript{N}\textsuperscript{34a} and N\textsuperscript{55e11} (from Ken Irvine, Rutgers); UAS-Suc\textsuperscript{X2N} (from Mark Fortini, NCI-Frederick); npr\textsuperscript{f} (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).

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 \textsuperscript{Z4} isoforms are expressed in larvae (Huang et al., 2011). We only showed data of overexpressing \textsuperscript{br} Z3 in the text.

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

MARC3 clone assay

To induce MARCM clones of FRT\textsuperscript{99A} sn\textsuperscript{3} w1118, FRT\textsuperscript{99A}-npr\textsuperscript{f}, FRT\textsuperscript{99A}-usp\textsuperscript{f} and FRT\textsuperscript{99A}-\textsuperscript{gce} sn\textsuperscript{5611}, we generated the following flies: FRT\textsuperscript{99A} mutant/\textsuperscript{FRT\textsuperscript{99A}} G93b, hs-flp; act>y\textsuperscript{FRT\textsuperscript{99A}} tub-Gal80\textsuperscript{hs-flp} and act>y\textsuperscript{FRT\textsuperscript{99A}} Gal4, UAS-GFP\textsuperscript{+}. Larvae were heat shocked at 37°C for 45 minutes between late L1 and early L2 and were analyzed at the early pupal stage or in adult female flies. For the adult ISC developmental stages, and was present at high levels in the nuclei of all cell types in the larval and pupal midgut, including polyplod ECs and AMP clusters (supplementary material Fig. S1A-B).

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, \textsuperscript{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 \textsuperscript{br} in development, we examined the expression of \textsuperscript{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 protein was undetectable in the adult midgut (supplementary material Fig. S1C,C\textsuperscript{+}). One Gal4 line driven by a \textsuperscript{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 polyplod 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\textsuperscript{+}: tub-Gal80\textsuperscript{+} driver (referred to as esg\textsuperscript{8}) (Jiang et al., 2009). At 2 hours APF at 29°C, we observed that AMPs were released from 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).
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 esgGal4 (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 (DI) (Fig. 2F,F’) (Mathur et al., 2010), indicating that these cells were AMPs. By contrast, very few DI-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 npr13 (a null allele of br) (Restifo and White, 1991) and usp2 (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’,E), npr1 (Fig. 3C,C’,E) and usp2 (Fig. 3D,D’,E) 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 (Fig. 3C,C’,E) and usp2 (Fig. 3D,D’,E) still remained in contact and most of them were Pdm1-negative (only 3.1% of the cells in the npr1 clone and 2.4% of the cells in the usp2 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 (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 npr13 and usp2 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 npr13 and usp2 clones, respectively (Fig. 3F).
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'). By contrast, cells derived from the GFP-marked AMPs of npr1 (on average only 0.09 clones that contain at least three cells/midgut, \( n = 31 \) guts; Fig. 4C,C') and usp2 (on average only 0.08 clones that contain at least three cells/midgut, \( n = 25 \) guts; Fig. 4D,D') developed into individual ISC-like cells in the adult.
AMPs developed into individual Dl-positive ISC-like cells that may not have positive ISCs (arrow), GFP-labeled differentiated EC (large nuclei) and EE (arrowhead) cells as well as Dl-negative PCs, which were Pdm1-negative, in the AMP/PC islands (none of the guts had Pdm1-positive AMPs and PCs, n=57 guts; Fig. 5A). By contrast, br overexpression resulted in the premature differentiation of AMPs/PCs into Pdm1-positive EC-like cells (100%, n=44 guts; Fig. 5B). Similarly, the overexpression of br in AMPs and PCs using the PswitchAMP 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-positive 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-Gal80P+/+ (referred to as Su(H)w>br) (Zeng et al., 2010) (n=49 guts; Fig. 5F) or the PswitchPC, 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)w>br and PCs in 100% of Pswwt>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 esgts. 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 N55e11 (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 (Fig. 4C,C’). The numbers of clones that contain at least three cells in FRT19A-WT, FRT19A-npr1ts and FRT19A-usp7ts 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 esgts driver (referred to as esgts>br). Male UAS-br and Oregon R (as the wild-type control) flies were crossed with esgts 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 esgts>UAS-GFP marked both AMPs and PCs, which were Pdm1-negative, in the AMP/PC islands (none of the 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 PsswitchAMP 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-positive 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-Gal80P+/+ (referred to as Su(H)w>br) (Zeng et al., 2010) (n=49 guts; Fig. 5F) or the PsswitchPC, 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)w>br and PCs in 100% of Pswwt>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.

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We also performed selective ectopic overexpression of br in adult ISCs using esgts. 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 N55e11 (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 (esgts>NDN) 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 (esgts>NAct) 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...
N signal transduction pathways in regulating AMP differentiation, we expressed the dominant-negative forms of N and br (esg^{ts}N^{D+br}; n=38 guts; Fig. 6B) or the constitutively activated forms of N and br^{IR} (esg^{ts}N^{A34A+br^{IR}}; n=47 guts; Fig. 6D) in AMPs. Interestingly, the expression of either N^{D+br} or N^{A34A+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) (Furries 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) 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-Ras^{V12}), Raf (UAS-Raf^{CA}) and PI3K (UAS-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\textsuperscript{DN} (A), UAS-N\textsuperscript{DN}+UAS-br (B), UAS-N\textsuperscript{A\textsubscript{34a}} (C) or UAS-N\textsuperscript{A\textsubscript{34a}}+UAS-br\textsuperscript{m} (D) was driven by esg\textsuperscript{ts}. Even though N activity was blocked by N\textsuperscript{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\textsuperscript{A\textsubscript{34a}} (E) or UAS-N\textsuperscript{DN} (F) was driven by esg\textsuperscript{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\textsuperscript{b} was driven by Suf(H)\textsuperscript{GBE-lacZ}; esg\textsuperscript{ts}. The knockdown of br did not affect the N activity because the expression of Suf(H)\textsuperscript{GBE-lacZ}, an N activity reporter, was not affected. GFP, green; β-galactosidase, red; DAPI, nuclear blue. Scale bars: 10 μm.

The connection between the hormones-Br pathway and the N pathway in regulating AMP differentiation is blocked in midgut degeneration. 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 polyplloid 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...
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/organisms. 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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Fig. 7. Neuroendocrine regulation of Br expression in AMP. (A, A') CA-specific knockdown of dpp (Aug21>dpp(3)) induced precocious Br expression. (B, B') PC-specific knockdown of dpp (Su(H)^ts>dpp(3)) did not induce precocious Br expression. (C, C') Prothoracic gland (PG)-specific expression of a constitutively activated form of Ras (phm>RasV12) 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 \( \mu \)m. (E) The model for AMP differentiation during metamorphosis regulated by Br-mediated hormone signals.


