Hindsight/RREB-1 functions in both the specification and differentiation of stem cells in the adult midgut of *Drosophila*

Brittany L. Baechler, Cameron McKnight, Porsha C. Pruchnicki, Nicole A. Biro and Bruce H. Reed*

**ABSTRACT**

The adult *Drosophila* midgut is established during the larval/pupal transition from undifferentiated cells known as adult midgut precursors (AMPs). Four fundamental cell types are found in the adult midgut epithelium: undifferentiated intestinal stem cells (ISCs) and their committed daughter cells, enteroblasts (EBs), plus enterocytes (ECs) and enteroendocrine cells (EEs). Using the *Drosophila* posterior midgut as a model, we have studied the function of the transcription factor Hindsight (Hnt)/RREB-1 and its relationship to the Notch and Egfr signaling pathways. We show that *hnt* is required for EC differentiation in the context of ISC-to-EC differentiation, but not in the context of AMP-to-EC differentiation. In addition, we show that *hnt* is required for the establishment of viable or functional ISCs. Overall, our studies introduce *hnt* as a key factor in the regulation of both the developing and the mature adult midgut. We suggest that the nature of these contextual differences can be explained through the interaction of *hnt* with multiple signaling pathways.

**KEY WORDS:** Hindsight/RREB-1, Intestinal stem cells, Enterocyte, Differentiation

**INTRODUCTION**

Until relatively recently, it was a commonly held view that differentiated somatic tissues of *Drosophila* adults are static and non-proliferative. Lineage tracing techniques, however, have demonstrated the existence of stem cells within several tissues including intestinal stem cells (ISCs) within the posterior midgut (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Singh et al., 2007). ISC regulation is remarkably conserved between mammals and *Drosophila* (Casali and Batlle, 2009; Hartenstein et al., 2012). Asymmetric divisions typically produce one undifferentiated, migrate through the newly formed midgut to become situated along the basal surface of the epithelium (Jiang and Edgar, 2009; Takashima et al., 2011). During the third larval instar, AMPs are found as clusters in which 1-3 AMPs differentiate in a Notch-dependent manner to form a specialized cell type, the peripheral cell (PC), which enwraps the remaining eight or more undifferentiated AMPs at this stage (Mathur et al., 2010; Takashima et al., 2011).

During the larval/pupal transition, the differentiated larval enterocytes (ECs) and enteroendocrine cells (EEs) are eliminated by autophagic cell death (Denton et al., 2009). Most AMPs differentiate to form the adult ECs, while some remaining AMPs become ISCs (Takashima et al., 2011). The mechanism of AMP-to-ISC specification is not understood. AMP-to-EC differentiation, however, can occur through a pathway parallel to Notch signaling (see below) that requires ecdysone signaling and the transcriptional regulator Broad. Ecdysone signaling and Broad are also implicated in the differentiation of fully functional ISCs from AMPs (Zeng and Hou, 2012).

The *hindsight* (*hnt*) gene encodes a nuclear protein containing 14 C2H2-type Zinc fingers. The expression of *hnt* during development is complex and dynamic (Yip et al., 1997). In general, despite a wealth of information, a detailed understanding of how Hnt functions to regulate cellular and developmental processes has remained elusive (Oliva et al., 2015; Pickup et al., 2009; Sun and Deng, 2007; Wilk et al., 2000).

Ras responsive element binding protein-1 (RREB-1), the mammalian homologue of Hnt, can act as either a transcriptional repressor or activator, depending on the context and target gene (Liu et al., 2009; Thiagalingam et al., 1996). Recent studies suggest that Hnt and RREB-1 are functionally conserved (Ming et al., 2013). In humans, RREB-1 has been linked to pancreatic, thyroid, and colorectal cancer (Kent et al., 2013; Zhang et al., 2003).

In *Drosophila*, ISCs are marked by the expression of *esg* and can divide either symmetrically or asymmetrically (de Navascues et al., 2012). Asymmetric divisions typically produce one daughter cell that retains the ISC identity and a second committed daughter cell known as an enteroblast (EB) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). EBS also express *esg* and differentiate without dividing to become either ECs or EEs; the former undergo endoreduplication and become polyploid, while the latter remain diploid (Strand and Michelli, 2013; Zeng et al., 2013a). ISCs express the Notch ligand Delta, and EBS that receive a higher level of Notch activation differentiate as ECs, whereas EBS receiving a lower level of Notch activation differentiate as EEs (Ohlstein and Spradling, 2007). Reduced Notch signaling results in uncontrolled ISC division, decreased EC differentiation, and an increased number of EE-like cells (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007), while overexpression of activated Notch promotes EC differentiation (Michelli and Perrimon, 2007).
RESULTS

**hnt is expressed in the adult intestinal epithelium**

The Notch signaling pathway is required for the normal maintenance and regeneration of the adult midgut (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Interestingly, *hnt* has been identified as a Notch-responsive gene and its expression has been shown to be Notch-dependent in some contexts (Krejci et al., 2009; Sun and Deng, 2007; Terriente-Felix et al., 2013).

The Egfr/Ras/MAPK signaling pathway (hereafter the Egfr pathway) is required for ISC proliferation (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang and Edgar, 2009). Over-activation of Egfr signaling results in increased ISC proliferation and midgut hyperplasia (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang and Edgar, 2009). While Egfr signaling promotes ISC proliferation, it does not influence subsequent differentiation events (Biteau and Jasper, 2011).

An additional pathway regulating ISC proliferation is the JAK/STAT pathway. Activation of this pathway in ISCs leads to increased ISC proliferation while reduced JAK/STAT signaling leads to an accumulation of EB-like cells, suggesting that this pathway is required for the competence of EB cells to undergo EC or EE differentiation (Beebe et al., 2010).

As summarized in several recent reviews, the list of signaling pathways and genes regulating midgut development, homeostasis, and regeneration has become extensive (Buchon et al., 2014; Kux and Pitsouli, 2014; Naszai et al., 2015; Tipping and Perrimon, 2014; Zeng et al., 2013a). Several studies have recently expanded our understanding of both EC and EE differentiation. Regarding the former, the expression of esg has been found to suppress EC differentiation through the repression of EC-specific genes such as *Pdm1* (Korzelius et al., 2014). Additionally, BMP signaling (Dpp/Gbb) as well as the chromatin remodeling proteins Brahma and Osa have been shown to be required for proper EC differentiation (Jin et al., 2013; Zeng et al., 2013b; Zhou et al., 2015). EE differentiation is promoted by the proneural genes *asense* and *scute*, with the transcriptional regulation of *asense* being Osa-dependent (Bardin et al., 2010; Zeng et al., 2013b). More recently, robo/slit signaling has been found to regulate a negative feedback mechanism that limits EE regeneration (Biteau and Jasper, 2014).

This study represents the first detailed analysis of the expression and function of *hnt* in the adult midgut. We report that ISCs/EBs express *hnt* and that this expression is increased in differentiated ECs and is absent from EEs. We find *hnt* expression in ISCs/EBs to be independent of Notch signaling and Egfr-dependent. In addition, we show that *hnt* overexpression induced in ISCs/EBs results in EC differentiation and we conclude that *hnt* can promote, but is not sufficient, for EC differentiation. Qualitative and quantitative mosaic analysis of loss-of-function alleles demonstrates a requirement for *hnt* in ISC-to-EC differentiation, but not in AMP-to-EC differentiation. An additional and novel finding of our study is the requirement for *hnt* in the establishment of viable or functional ISCs. Overall, our work ascertains that the transcriptional regulator Hnt/RREB-1 is an important component of the developing and homeostatic adult midgut where it functions in both the specification and subsequent differentiation of ISCs.

**ISC expression of hnt is not dependent on Notch signaling but is dependent on Egfr signaling**

To address the expression of *hnt* in the context of reduced Notch signaling, anti-Hnt immunostaining was performed on midguts in which Notch was depleted by expression of *UAS-Notch-RNAi* using the *esg*-technique. Consistent with previous findings (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006), we found that midguts with reduced Notch signaling display an overproliferation of two cell types which we observed as small GFP-positive, Hnt-positive cells, as well as small GFP-negative, Hnt-positive cells (Fig. 2A). Our above finding that Hnt is not expressed in differentiated EEs supports the view that the small GFP-negative, Hnt-positive cells represent an intermediate cell type that is neither a fully differentiated EE nor an ISC. In the GFP-positive overproliferating ISC-like cells, however, we found no indication of reduced *hnt* expression. Taken together, these observations support the interpretation that ISCs do not express *hnt* in a Notch-dependent manner.

The mammalian homologue of *hnt* is RREB-1, and several lines of evidence suggest that RREB-1 functions in the regulation of Egfr signaling (Kent et al., 2013, 2014; Thiaigalingam et al., 1996). Moreover, *Egfr* and *hnt* mutants share a number of phenotypes, including premature degeneration and death of the extra-embryonic tissue known as the amnioserosa (Frank and Rushlow, 1996; Shen et al., 2013). For these reasons we examined the expression of *hnt* in the context of reduced Egfr signaling. Midguts depleted of Egfr by expression of *UAS-Egfr-RNAi* were sparsely populated by GFP-positive cells (ISCs/EBs), and these were uniformly Hnt-negative (Fig. 2B). Thus, while we found no dependence on Notch signaling, we find that *hnt* expression in ISCs/EBs is dependent on Egfr signaling.

**Increased hnt expression forces ISC to EC differentiation**

Our initial observations suggested that *hnt* expression may increase in association with EC specification, and decrease during EE differentiation. We were initially interested in determining if *hnt* is a target of Notch signaling in this context, we performed anti-Hnt immunostaining of adult midguts. Immunostaining of *esg*-midguts following shift to permissive conditions (see Materials and Methods) allowed unambiguous identification of ISCs/EBs as Hnt-positive. Large, polyploid, GFP-negative cells corresponding to ECs were also Hnt-positive (Fig. 1A). In general, the intensity of anti-Hnt signal in the *esg*-marked ISCs/EBs was less than the signal observed in ECs. In addition, small GFP-negative cells were sometimes observed to be weakly Hnt-positive (data not shown), possibly representing EEs. Unfortunately, anti-Hnt and the EE marker anti-Prospero (Pros) are both mouse monoclonal antibodies, which precluded a double immunolabeling experiment. To circumvent this problem we used a GFP enhancer trap line, *yet1*, which we here report as a new EE marker. Anti-Pros immunostaining of *yet1* adult midguts confirmed that *yet1* expression and Pros co-localize (Fig. 1B). Anti-Armadillo (Arm) immunostaining also confirmed that *yet1*-expressing cells are single small cells associated with reduced Arm, consistent with EE morphology (Fig. S1). Anti-Hnt immunostaining of the *yet1* line subsequently established that strong GFP-positive cells are Hnt-negative (Fig. 1C), while weakly GFP-positive cells are sometimes weakly Hnt-positive (arrows, Fig. 1C). We conclude that strong *yet1* expression marks differentiated EEs and that this cell type does not express *hnt*. These observations also suggest that *hnt* is down-regulated in EEs that are specified to become EEs.
overexpression in ISCs/EBs (using the esg$^{ts}$ technique) could bias specification towards the EC fate. What we found, however, was a striking loss of all ISCs/EBs. As early as 14 h post shift, ISCs/EBs with high levels of Hnt and a slight increase in size were observed (Fig. 3A). At 4 days post shift no small GFP-positive cells remained, and large weakly GFP-positive cells were observed (Fig. 3B). At 14 days post shift no GFP-positive cells remained in the midgut (Fig. 3C), with the exception of the gastric region stem cells (data not shown). Since esg expression is lost in differentiating EEs and ECs, it remained possible that hnt overexpression did not result in ISC loss through EC differentiation, but that the observed ISC loss is the result of ISC/EB delamination or death. To address this possibility, we repeated hnt overexpression experiments using the esgF/O technique to facilitate ISC lineage tracing (see Materials and Methods). Using esgF/O to express UAS-GFP-hnt, most GFP-positive cells appeared as large differentiated ECs that were integrated into the midgut epithelium at 3 days post shift. The lack of co-localization of Pros and GFP also confirmed that hnt-overexpressing cells do not differentiate as EEs (Fig. 3D). Control esg$^{ts}$ midguts at 5 days post shift were typically found to contain clusters of GFP-positive cells consisting of several large cells as well as one or two small cells (Fig. 3E). At the same time point (5 days post shift), hnt overexpression using hnt$^{EP55}$ resulted in esg$^{ts}$ lineages of only one or two cells that were Pdm1-positive, consistent with EC differentiation (Fig. 3F). Overall, these results show that increased hnt expression can force ISC-to-EC differentiation.

**The overexpression of hnt as ‘differentiation therapy’ in the fly**

The successful treatment of some forms of cancer involves ‘differentiation therapy’, whereby treatment aims to force malignant cells to resume normal differentiation (Warrell et al., 1991). The adult Drosophila posterior midgut has emerged as a model for mammalian midgut homeostasis and displays remarkable parallels in terms of dysregulation and hyperplasia (Casali and Batlle, 2009; Hartenstein et al., 2010; Wang and Hou, 2010). As previously shown in the literature and repeated in this study, reduced Notch signaling in the Drosophila midgut results in overproliferation of ISC-like cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). The activation of Egfr signaling is also known to result in overproliferation (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang and Edgar, 2009). Similar to differentiation therapy, we tested the ability of hnt overexpression to suppress each overproliferation background. Using the esg$^{ts}$ technique, we found that expression of Egfr$^{AcT}$ resulted in discernable hyperplasia in the posterior midgut region within 24 h of induction (Fig. 4A). Such hyperplasia was not evident in midguts co-expressing UAS-GFP-hnt (Fig. 4B). By 3 days post induction Egfr$^{AcT}$ expression resulted in extensive hyperplasia (Fig. 4C) that was completely suppressed by UAS-GFP-hnt.
co-expression (Fig. 4D). Similarly, the overproliferation of ISC-like cells associated with the expression of Notch-RNAi (Fig. 4E) was suppressed by UAS-GFP-hnt co-expression at 7 days post induction (Fig. 4F). Overall, these results suggest that increased hnt expression can abrogate overproliferation by forcing ISCs to differentiate as ECs.

Recent results have established that esg is required for ISC maintenance (Korzelius et al., 2014), and this prompted us to determine if co-expression of esg could suppress the forced differentiation associated with hnt overexpression. Our main approach for these experiments was to generate marked somatic clones within the ISC population of the adult midgut. Using a FLP/FRT-based twin spot technique, which permits analysis of all cell types, we first generated clones in which daughter cells either inherited two copies or no copy of an X-linked His2Av-GFP transgene marker in the background of a third chromosome carrying His2Av-RFP (see Materials and Methods). Following clone induction in mature adult females, twin spots composed of both small cells (presumptive ISCs/EBs or EEs) and large cells (presumptive ECs) were observed (Fig. 5A). Clones induced in hntXE81 heterozygotes, on the other hand, did not propagate the hntXE81 mutant side of the twin spot (RFP-only) and large hntXE81 mutant cells (presumptive ECs) were never observed (Fig. 5B). These observations suggested that hnt could be required for ISC
maintenance and proliferation or EC differentiation. To distinguish between these two possibilities, a quantitative analysis was performed using a modified MARCM technique. Unlike the above GFP/RFP twin spot analysis, where small hnt mutant cells (RFP-only) were difficult to score, mutant cells in our MARCM-based mosaic analysis were readily observed. This technique (see Material and methods) generates GFP<sup>flu</sup>-labeled hnt mutant clones using either esgGAL4 (esg-MARCM) or NP6293 (NP6293-MARCM). This approach allowed us to evaluate the hnt mutant clones with respect to their ability to express either ISC/EB or EC specific markers. While the esgGAL4 driver is well established as an effective reporter of ISCs/EBs, we have found NP6293 to be an excellent reporter of EC fate throughout the adult midgut (Fig. S3). Using esg-MARCM and clone induction by heat shocking adults produced ample esg<sup>GFP</sup>-positive cells throughout the midguts of both control and hnt<sup>XE81</sup> heterozygotes (Fig. 5C, Fig. 6A). Using NP6293-MARCM under identical conditions resulted in NP6293<sup>GFP</sup>-positive cells in control, but not in hnt<sup>XE81</sup> heterozygotes (Fig. 6B). These results suggest that hnt is not required for ISC/EB maintenance and proliferation, but that there is a requirement for hnt in EC differentiation in the homeostatic adult midgut. Interestingly, we found that hnt mutant cells in the GFP/RFP twin spot analysis were more readily observed in the midguts of older flies (Fig. 5D). Anti-Arm immunostaining confirmed that these hnt<sup>XE81</sup> mutant cells are indeed part of the midgut epithelium. Based on morphology, most hnt<sup>XE81</sup> mutant cells resemble either ISCs or EBs, while some appear to be EEs (Fig. 5E). These observations further support our interpretation that hnt is not required in ISC maintenance or proliferation. In addition, even in the context of the aged midgut, which is prone to hyperplasia (Biteau et al., 2008; Choi et al., 2008), hnt mutant cells are incapable of EC differentiation.

Additional quantitative analysis compared hnt mutant ISC clones of the null allele hnt<sup>XE81</sup> to the hypomorphic allele hnt<sup>108</sup>. In this experiment, induced clones were marked by the expression of UAS-GFP<sup>flu</sup> under the control of both esgGAL4 and NP6293 (see Materials and Methods). Using this technique we measured the percentage of large GFP-positive cells, presumably indicative of differentiated ECs, at various time points after clone induction. Overall, large cells (EC-like) were more frequent in controls, significantly less frequent in hnt<sup>108</sup> clones, and were negligible in hnt<sup>XE81</sup> clones (Fig. S2A-C). Interestingly, while hnt<sup>108</sup> mutant clones contained a small percentage of large GFP-positive cells, anti-Pdm1 immunostaining showed these cells to be Pdm1-negative, suggesting that these large cells are not fully differentiated ECs (Fig. S2D).

Finally, to rule out the possibility that hnt ISC mutant clones result in increased EE differentiation, we measured the average number of Pros-positive cells in midguts harboring control, hnt<sup>XE81</sup>, and hnt<sup>108</sup> mutant ISC clones. We found no significant difference in the frequency of Pros-positive cells among the three genotypes, suggesting that hnt mutant ISC clones do not result in increased EE differentiation (Fig. S2F).

Taken together, both qualitative and quantitative analysis of somatic clones using two different hnt loss-of-function alleles allows us to conclude that differentiated ECs are not found within hnt mutant clones induced in ISCs. Therefore, hnt function is necessary for EC differentiation from the ISC/EB state in the adult midgut.

**hnt is not required for EC differentiation from the AMP state but is required for the establishment of ISCs from AMPs**

Having established that hnt is required for EC differentiation from the ISC/EB state, we wished to determine if hnt is generally required for EC differentiation, or if this requirement is specific to the adult ISC/EB. To address this question we repeated clonal analysis experiments in which clone induction was performed on third instar larvae. In so doing, hnt loss-of-function clones were induced among the population of proliferating AMPs. Most AMPs differentiate directly to ECs in the formation of the adult midgut, without passing through the ISC/EB state (Jiang and Edgar, 2009; Micchelli et al., 2011). Using NP6293-MARCM and clone induction by heat shocking third instar larvae produced ample NP6293<sup>GFP</sup>-positive cells in both control and hnt<sup>XE81</sup> heterozygous (Fig. 7A,B; Fig. 6D).

Using esg-MARCM under identical conditions resulted in
numerous esg> GFP-positive cells in control, but not a single esg> GFP-positive cell was observed in hntXE81 heterozygous midguts (Fig. 6C). AMP clone induction by larval heat shock was also repeated using the GFP/RFP twin spot method as described above. Following clone induction in third instar larvae, twin spots composed of both small cells (presumptive ISCs/EBs or EEs) and large cells (presumptive ECs) were observed in the control (Fig. 7C). Clones induced in hntXE81 heterozygotes, on the other hand, did produce large EC-like hntXE81 mutant cells (RFP-only) but were devoid of small hntXE81 mutant cells (Fig. 7D). Overall, the ability of hnt mutant AMPs to differentiate into ECs suggests that hnt is not generally required for EC differentiation, but that hnt is required during the specific differentiation of ECs from the ISC/EB state in the adult midgut. In addition, the complete absence of hnt mutant ISCs in adults following clonal induction in AMPs strongly suggests that hnt is required for the establishment of viable or functional ISCs from AMPs.

**DISCUSSION**

**hnt expression and Notch signaling**

Our results demonstrate that hnt expression is maintained in ISCs depleted of Notch. While this establishes that hnt expression in ISCs is Notch-independent, it does not rule out the possibility that EC differentiation could require Notch-dependent hnt expression. For example, it remains possible that Notch signaling from the ISC to the EB could augment hnt expression in the EB, thereby promoting EC fate. Consistent with this possibility, our anti-Hnt immunostaining results suggest that hnt expression is increased in ECs relative to ISCs. In addition, our finding that hnt is not expressed in mature EEs also suggests that hnt is down-regulated as EBs enter the EE differentiation pathway, possibly reflecting reduced Notch signaling associated with EE differentiation. Taken together, our results do not rule out the possibility that hnt expression is Notch-dependent during ISC-to-EC differentiation.

**Hnt can promote but is not sufficient for ISC-to-EC differentiation**

We report that co-expression of hnt and esg suppresses ISC-to-EC differentiation (compare Fig. 4G with H). Combined expression of NotchIntra and esg was reported to partially suppress ISC-to-EC differentiation associated with the over-activation of Notch signaling (Korzelius et al., 2014). Our co-expression experiment, however, used hntEP55, which expresses at a lower level than UAS-GFP-hnt. This difference in the level of expression was apparent in our esgG experiments where hntEP55 was less effective than UAS-GFP-hnt in promoting ISC-to-EC differentiation (data not shown). It remains possible that co-expression of esg and a higher level of hnt overexpression could force ISC-to-EC differentiation, and that there is a threshold effect with respect to the level of Hnt and EC differentiation. The question as to whether Hnt and Esg compete for target genes, or if expression of either of these genes affects the regulation of the other remains unexplored at this time.

The JAK/STAT signaling pathway has also been implicated in the specification of EC cell fate (Beebe et al., 2010). Although not a main focus of our study, we found that RNAi knockdown of Stat92E had no effect on hnt expression. In addition, we found that co-expression of UAS-GFP-hnt with UAS-Stat92E-RNAi resulted in robust EC differentiation, suggesting that Hnt functions either in parallel or downstream of JAK/STAT signaling in EC differentiation (data not shown).

Overall, our analysis of hnt mutant clones supports the interpretation that hnt is necessary for ISC-to-EC differentiation. A recent genome-wide RNAi-based screen reported that depletion of Hnt using the esgG technique results in ISC-to-EC differentiation.
This context is Egfr-dependent. In the scenario whereby hnt expression is positively regulated by Egfr signaling, we might expect similarities between Egfr and hnt mutant phenotypes. In support of this, we suggest that the phenotype of hnt mutant clones resembles Egfr mutant clones with respect to ISC proliferation and survival, and that these are dramatically different from the effects of reduced Notch signaling (Biteau and Jasper, 2011). The behavior of hnt and Egfr clones generated in the adult ISCs, however, differed with respect to cell differentiation. In mosaic analysis, and when using the MARCM technique, somatic recombination and subsequent mitotic division generate single cells that can be either mutant or wild type, presumably with equal probability. In the case of Egfr mutant clones, many newly formed single-cell clones can result in differentiated ECs or EEs, indicating that Egfr is not required for subsequent differentiation in the cases where the EB daughter cell is mutant (Biteau and Jasper, 2011). By contrast, hnt mutant ISC clones generated in the adult midgut fail to differentiate as ECs. We have established that Hnt is required for ISC-to-EC differentiation, and the above clonal analysis suggests that Egfr is not required for EC differentiation. From this, we speculate that any expression or upregulation of hnt required to promote EC differentiation is most likely independent of Egfr signaling. This interpretation further supports the notion of a separate induction of hnt expression that is independent of Egfr signaling.

**Hnt is not required for AMP-to-EC differentiation but is required in the establishment of ISCs from AMPS**

We show that Hnt is essential for ISC-to-EC differentiation, but not AMP-to-EC differentiation. As mentioned previously, Broad is known to play an important role in promoting AMP-to-EC differentiation, acting in parallel to the Notch signaling pathway. Additionally, Notch and Broad can effectively compensate for the loss of one another in allowing AMPS to differentiate as ECs (Zeng and Hou, 2012). We speculate that such AMP-to-EC differentiation in hnt mutant AMPS is occurring through the ecdysone/Broad pathway and this possibility merits further investigation.

Interestingly, we find that hnt mutant clones induced in AMPS never result in small, ISC or EE-like cells. This is unlike either the broad or Notch mutants. broad mutant clones induced in AMPS generate non-functional ISCs, which are Delta-positive but fail to proliferate or differentiate (Zeng and Hou, 2012). Notch mutant AMP clones, on the other hand, differentiate as EEs (Zeng and Hou, 2012). The absence of ISC or EE-like cells in hnt mutant clones suggests that hnt may play a primary role in the establishment of the ISCs from the AMP state. It also remains possible, however, that hnt mutant AMPS are preferentially eliminated by programmed cell death specific to the period of the larval-to-pupal transition. Interestingly, recent work in ovarian follicle cell differentiation has suggested that Broad functions together with Hnt in regulating the Notch-dependent mitosis-to-endocycle transition and cell differentiation (Jia et al., 2014). Given that broad mutant AMPS fail to generate fully functional ISCs (Zeng and Hou, 2012), and the complete lack of ISCs in hnt mutant AMP clones, allows us to speculate that, like the follicle cell context, Hnt and Broad may function cooperatively to establish the ISCs of the adult midgut.

**MATERIALS AND METHODS**

*Drosophila* stocks

All cultures were raised on standard *Drosophila* medium at 25°C under a 12 h light/dark cycle, unless otherwise indicated. Unless otherwise stated, stocks were obtained from the Bloomington *Drosophila* Resource Center. Controls were performed using y w 1118 or y w 1118 P[ry +t7.2]=neoFRT
19A stocks. \(P[\text{ry}^{+7.2}] = \text{neoFRT}^{19A}\) is here abbreviated as \(\text{FRT}^{19A}\). The reporter line \(w^{1185}\), \(P[\text{w}^{+}\text{mC}] = \text{UAS-GFP.nls}^{14}\), abbreviated in this report as \(\text{UAS-GFP}^{\text{mth}}\), was used to characterize \(\text{GAL4}^{\text{expression}}\) patterns. The nuclear markers \(\text{His}2A\text{-GF}^{\text{P}}\) and \(\text{His}2A\text{-RF}^{\text{P}}\) are fully described as \(P[\text{w}^{+}\text{mC}] = \text{His}2A\text{-EGFP.C}^{2}\) and \(P[\text{w}^{+}\text{mC}] = \text{His}2A\text{-mRF}^{\text{P}1}\text{II.2}\) or \(P[\text{w}^{+}\text{mC}] = \text{His}2A\text{-mRF}^{\text{P1}}\text{III.1}\), respectively. The \(\text{UAS-Notch-RNAi}^{\text{line}}\) used was \(P[\text{w}^{+}\text{mC}] = \text{UAS-N.dnRNA.P}^{14E}\). The \(\text{UAS-EGFR-RNAi}^{\text{line}}\) used was \(w^{1185}; P[\text{GD1654}]=\text{v}^{3267}\) and was obtained from the Vienna Drosophila RNAi Center. Most escargot \(\text{GAL4}^{\text{lines}}\) used (\(\text{esgGAL4}^{\text{only}}\), with \(\text{UAS-GFP}\), and with both \(\text{UAS-GFP}\) and \(\text{tubGAL80}^{\text{P}}\)) have been described previously (Micchelli and Perrimon, 2006). The \(\text{esgF}^{\text{X}}\) stock, described below, was provided by H. Jiang. The \(\text{hnt}^{\text{p99}}, \text{hnt}^{\text{F55}},\) and \(\text{UAS-GFP-hnt}^{\text{lines}}\) have been described (Ming et al., 2011; Reed et al., 2001). A recombinant\(\text{y}^{\text{w}^{1188}\text{hnt}^{\text{XESI}}}^{\text{FRT}^{19A}}\) line was recovered in our lab. \(\text{UAS-EgfrACT}^{\text{X}}\) was originally described as \(\text{UAS-Atop4.2}^{\text{Queenan et al., 1997}}\) and was obtained from T. Schüpbach. The NP6293 \(\text{GAL4}^{\text{line}}\) was obtained from the Kyoto Drosophila Resource Center. The \(\text{Yeti}^{1}\) enhancer trap line is from A. Michelson (Mohseni et al., 2009). The \(\text{UAS-esg}^{\text{line}}\) from S. Hayashi was provided by J. Korzelius (Korzelius et al., 2014). \(\text{UAS-mCherry-moesin}\) was provided by R. Jacobs (McMaster University, Canada). Stocks used for mosaic analysis included \(\text{tubGAL80}^{\text{hsFLP}}^{\text{FRT}^{19A}}\) (full description: \(P[\text{w}^{+}\text{mC}] = \text{tubP-GAL80.III.1}^{L} P[\text{ry}^{+7.2}] = \text{hsFLP}^{1.1} P[\text{ry}^{+7.2}] = \text{neoFRT}^{19A}\)) as well as \(Dp(1;2)4\text{FRD}^{\text{Dup}}\) originally from H. Salz (Case Western Reserve University, Ohio, USA).

The \(\text{esg}^{\text{X}}\) technique

A method for inducing gene expression within the ISCs/EBs of the adult midgut has been described (Michelucci and Perrimon, 2006) and is here abbreviated as the \(\text{esg}^{\text{X}}\) technique. Briefly, this method uses a chromosome that carries the \(\text{esgGAL4}^{\text{driver}}\) in addition to a \(\text{UAS-GFP}^{\text{reporter}}\) and a \(\text{tubGAL80}^{\text{P}}\) insertions. Rearing cultures at 18°C, which is permissive for \(\text{GAL80}^{\text{P}}\), prevents \(\text{GAL4}^{\text{activation}}\) and circumvents any effects associated with inducing gene expression during earlier stages of development. Shifting cultures to 29°C inactivates the \(\text{GAL80}^{\text{P}}\) repressor, resulting in \(\text{GAL4}^{\text{activation}}\) and consequently \(\text{UAS-reporter gene expression}\). To induce \(\text{UAS-reporter gene expression in the ISCs, cultures were kept at 18°C and shifted to 29°C when adults were 3-5 days old. In all experiments midguts of females were analyzed. For co-expression \(\text{esg}^{\text{X}}\) experiments, virgin females carrying the X-linked \(\text{UAS-N-RNAi}\) or \(\text{UAS-EgfrACT}^{\text{X}}\) insertions were crossed to males of the autosomal \(\text{UAS-GFP-hnt}^{\text{stock}}\). The male progeny of these crosses were subsequently crossed to \(\text{esg}^{\text{X}}\) virgin females.

The \(\text{esg}^{\text{X}}\) technique

ISC lineages were marked using the \(\text{esg}^{\text{X}}\) (flip-out) technique (Jiang et al., 2009). Briefly, the \(\text{esgF}^{\text{X}}\) stock carries the same elements as the \(\text{esg}^{\text{X}}\) stock (\(\text{esgGAL4}, \text{UAS-GFP}, \text{and tubGAL80}^{\text{P}}\)) in addition to \(\text{UAS-Flp}^{\text{(Flp recombinase)}}\) and a ubiquitous actin or tubulin based promoter designed to drive \(\text{GAL4}^{\text{expression}}\) but prevented from doing so by a \(\text{CD2 cassette}^{\text{flanked by FRT sites}}\) (i.e. \(\text{Act}^{\text{P}}\text{-CD2}^{\text{2}}\text{-GAL4}^{\text{A}}\)). Shifting cultures from 18°C to 29°C is permissive to \(\text{esgGAL4}^{\text{expression}}\) of \(\text{UAS-Flp}\), resulting in the removal or ‘flip-out’ of the \(\text{CD2 cassette}\). This permanently activates \(\text{ActGAL4}^{\text{or tubGAL4}}^{\text{within ISCs/EBs}}\) and their daughter cells. For \(\text{hnt}^{\text{X}}\) and \(\text{esgX}\)-co-expression experiments, virgin females carrying the X-linked \(\text{hntF55}\) insertion were crossed to the autosomal \(\text{UAS-esg}^{\text{line}}\) and resulting male progeny were crossed to \(\text{esg}^{\text{X}}\) virgin females.

Mosaic analysis: \(\text{His}2A\text{-GF}^{\text{P/His}2A\text{-RF}^{\text{P}}}\) twin spots

Experiments involving \(\text{His}2A\text{-GF}^{\text{P/His}2A\text{-RF}^{\text{P}}}\) twin spots used Bloomington stock 30563, fully described as \(y w P[w^{+}\text{mC}] = \text{His}2A\text{-TF: Aovic}^{\text{GFP}-\text{S65T}}^{1.1} P[\text{ry}^{+7.2}] = \text{hsFLP}^{1.1} P[\text{ry}^{+7.2}] = \text{neoFRT}^{19A} P[w^{+}\text{mC}] = \text{His}2A\text{-mRF}^{\text{P1}}\text{III.1} \text{TM6B, Tb}^{.}\). For the generation of control and \(\text{hnt}^{\text{XESI}}^{\text{mutant clones, males of the above stock were crossed to virgin females of}}\) \(y w \text{hntXESI}^{\text{FRT}^{19A}} = \text{Dp}(1;2)4\text{FRD}^{\text{Dup}}\)\(, Tb^{.}\) females. For co-expression experiments, both the \(\text{white (w)}\) and \(\text{hnt genes, were used}}\) as controls. Sibling progeny lacking the duplication were used to generate \(\text{hntXESI}^{\text{mutant clones}}\).

For adult ISC clone induction, 3-5 day old adult female progeny were heat shocked twice for 40 min in a 37°C water bath, separated by a 1 h interval at room temperature. For larval AMP clone induction, progeny were heat shocked as described above when cultures contained wandering third instar larvae. For analysis of clones induced during larval stages (AMP clones), progeny eclosing either 5 or 6 days following heat shock treatment were dissected 1-2 days post-eclosion (cultures were maintained at 25°C following heat shock).

Mosaic analysis: \(\text{esg-MARC}^{\text{M}}\) and \(\text{NP6293-MARC}^{\text{M}}\) techniques

Somatic clones were recovered using modified MARCM techniques (Lee and Luo, 2001). Rather than using a ubiquitous \(\text{GAL4}^{\text{driver}}\), \(\text{esgGAL4}\) and \(\text{NP6293 GAL4}^{\text{drivers}}\) were used to positively mark clones as either ISCs/EBs or ECs, respectively. For \(\text{esg-MARC}^{\text{M}}\) experiments \(\text{tubGAL80}^{\text{P}}\) was provided by R. Jacobs (McMaster University, Canada).
hs-FLP FRT19A; esgGAL4 UAS-GFP<sup>flp</sup>/CyO males were crossed to either virgin females of the y w <sup>hsX<sub>FLP</sub></sup> FRT19A; Dp(1;2)FRTDup/+ or y w <sup>hsX<sub>FLP</sub></sup> FRT19A/FM7 and y w FRT19A stocks. For NP6293-MARC quantitative experiments tubGAL80<sup>ts</sup> hs-FLP FRT19A; NP6293 UAS-GFP<sup>flp</sup> His2Av-RFP/CyO males were crossed to the same y w <sup>hsX<sub>FLP</sub></sup> FRT19A; Dp(1;2)FRTDup/+ (ISC exg-MARC) and AMP NP6293-MARC Stock and y w <sup>hsX<sub>FLP</sub></sup> FRT19A; Dp(1;2)FRTDup/+ males. All exg-MARC and NP6293-MARC experiments used the heat shock induction regimes as described above for His2av-GFP/ His2Av-RFP twin spots, with the exception that adult heat shock treatment was repeated for 3-5 consecutive days and midguts were dissected 7 days following the final heat shock treatment.

Experiments comparing the behavior of <sup>hsX<sub>FLP</sub></sup> and <sup>hsX<sub>GFP</sub></sup> clones (Fig. S2) combined both esgGAL4 and NP6293-MARC techniques. Here GFP-positive clones indicate either ISCs/EBs due to esgGAL4 expression (small GFP-positive cells) or ECs due to NP6293 expression (large GFP-positive cells). Cells were scored as large if the nuclear diameter was greater than approximately 7 μm. The recovery of adult females carrying both esg- and NP6293-MARC was achieved by crossing y w <sup>hsX<sub>FLP</sub></sup> (or <sup>hsX<sub>GFP</sub></sup>) FRT19A; Dp(1;2)FRTDup/+ females to NP6293 UAS-GFP<sup>flp</sup> His2Av-RFP/ CyO males to recover y w <sup>hsX<sub>FLP</sub></sup> (or <sup>hsX<sub>GFP</sub></sup>) FRT19A; Dp(1;2)FRTDup/ NP6293 UAS-GFP<sup>flp</sup> His2Av-RFP males. These males were subsequently crossed to tubGAL80 hsFLP FRT19A; esgGAL4 UAS-GFP<sup>flp</sup> females. Clones were induced as described for the GFP/RFP twin spot analysis. Following heat shock, flies were maintained at 25°C and transferred onto fresh food every 2-3 days. Adult females of the desired genotype y w <sup>hsX<sub>FLP</sub></sup> FRT19A/tubGAL80 hsFLP FRT19A; His2Av-RFP NP6293 UAS-GFP<sup>flp</sup> esgGAL4 UAS-GFP<sup>flp</sup> were identified by the absence of the Confuens phenotype associated with Dp(1;2)FRTDup in addition to using His2Av-RFP as a marker post-dissection. Females were dissected and midguts examined by live imaging confocal microscopy on days 3, 7, and 14 after clone induction. Control clones were similarly recovered by crossing y w FRT19A/Y, His2Av-RFP NP6293 UAS-GFP<sup>flp</sup>/+ males to tubGAL80 hsFLP FRT19A; esgGAL4 UAS-GFP<sup>flp</sup> females.

**Immunostaining**

Immunostaining of adult midguts was carried out as described (Singh et al., 2012). The following primary antibodies were used at the indicated dilutions: mouse monoclonal anti-Hindsight (Hnt) 27B8 1G9 (1:25; from H. Lipshitz, University of Toronto, Canada); mouse monoclonal anti-Prospero (Pros) MR1A (1:100; DSHB); rabbit anti-Pdm1 (1:1000; from X. Yang, Zhejiang University, China); mouse monoclonal anti-Armadillo (Arm) N2 7A1 (1:100; DSHB). TRITC-conjugated goat anti-mouse and TRITC-conjugated donkey anti-rabbit secondary antibodies (1:500; Jackson Immunoresearch).

**Microscopy**

For experiments that did not require immunolabeling, midguts were dissected in a drop of halocarbon oil (1:1 mixture of series 56 and series 700, Halocarbon Products Corp.), covered with a coverslip and imaged live. Immunostained midguts were mounted in 70% glycerol in PBS containing 2.5% DABCO (Sigma-Aldrich). Confocal microscopy and image processing were performed as previously described (Cormier et al., 2012).

**Quantitative analysis**

To quantify the esg-MARC and NP6293-MARC results shown in Fig. 6 GFP-positive cells were counted for the entire length of each midgut. Box plot diagrams were configured using Microsoft Excel, with whiskers indicating the minimum and maximum values of the data set. To quantify the percentage of large GFP-positive cells shown in Fig. S2, the large GFP-positive cells and total GFP-positive cells were counted along the top surface for the entire length of each midgut. To quantify EE frequency in midguts of control and midguts containing <sup>hsX<sub>GFP</sub></sup> or <sup>hsX<sub>FLP</sub></sup> mutant clones (shown in Fig. S2F) Pros-positive midgut cells within one field of view (20× objective) of the midgut/hindgut junction were counted. A minimum of five midguts was scored for each genotype. Standard deviation and P values were calculated using Microsoft Excel.


