Investigation of the role of Hindsight in the development of the *Drosophila* midgut

by

Brittany Baechler

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The *Drosophila* adult midgut is maintained through the division and differentiation of a population of intestinal stem cells (ISCs) (Issigonis and Matunis, 2010). The epithelial cells of the adult midgut are derived from adult midgut progenitors (AMPs), which are specified during embryogenesis, and remain in an undifferentiated state throughout larval stages by forming a transient niche (Mathur et al., 2010a). After puparium formation, the AMPs differentiate to form absorptive enterocyte (EC) cells, secretory enteroendocrine (EE) cells, or remain in an undifferentiated state as ISCs. The ISCs can then divide and differentiate throughout adulthood in order to regenerate and maintain the intestinal epithelium (Issigonis and Matunis, 2010).

Notch signaling and Epiderminal growth factor receptor (EGFR) signaling have been shown to play important roles in controlling cell proliferation and differentiation in the larval and adult midgut (Ohlstein and Spradling, 2007)(Xu et al., 2011). In the larval midgut, Notch signaling is required for peripheral cell differentiation and formation of the transient niche. In the adult midgut, Notch signaling is required for limiting ISC proliferation, and for promoting EC differentiation (Ohlstein and Spradling, 2007). EGFR signaling has been shown to be required for promoting AMP and ISC divisions in the larval and adult midgut, respectively, and it is also necessary for maintenance of the ISC population (Jiang and Edgar, 2009)(Xu et al., 2011).

The putative transcription factor Hindsight (Hnt) is a nuclear zinc-finger protein (Sun and Deng, 2007)(Wilk et al., 2004) that has been shown to be a direct transcriptional target of the Notch signaling pathway (Krejcí et al., 2009). Hnt is expressed in numerous tissues and has been implicated in a number of processes including the maintenance of epithelial integrity (Wilk et al., 2004) as well as promoting mitosis to endocycle transitions and cell differentiation (Sun and Deng, 2007). After discovering that Hnt is expressed throughout the larval and adult midgut, this

prompted further investigation of its role in the development and maintenance of these tissues. Using the GAL4/UAS system of inducible gene expression, we have shown that overexpression of Hnt was sufficient to promote premature differentiation of AMPs and ISCs into the Notch signaling-dependent ECs, likely downstream of Notch. In addition, RNAi-mediated knockdown of *hnt* revealed that expression of Hnt was necessary for the maintenance of stem cells in both the adult and larval midgut. We have also shown that EGFR signaling is required for maintaining Hnt expression in the ISCs, and that Hnt is required for promoting EC differentiation downstream of EGFR. These results suggest that Hnt is involved in mediating the EGFR and Notch signaling pathways in order to maintain the ISC population and to promote EC differentiation.

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List of Abbreviations

AMP	Adult midgut progenitor/precursor
cf	compare figure
Cyc	Cyclin
Dl	Delta
EB	Enteroblast
EC	Enterocyte
EE	Enteroendocrine cell
EGFR	Epidermal growth factor receptor
Esg	Escargot
Hnt	Hindsight
ISC	Intestinal stem cell
Mam	Mastermind
PC	Peripheral cell
PCNA	Proliferating cell nuclear antigen
Pros	Prospero
Su(H)	Suppressor of Hairless

Chapter 1. Introduction

1.1 Stem Cell Niches

Stem cells are undifferentiated cells that can undergo self-renewal as well as differentiate to form numerous cell types (Li and Xie, 2005). Stem cells are known to reside in a specialized microenvironment known as a niche. This microenvironment consists of the stem cells in addition to surrounding cells and/or structures which, through various signaling events, maintain the stem cell population in an undifferentiated state. The niche is also responsible for regulating stem cell proliferation and differentiation decisions (Resende and Jones, 2012). The concept of the stem cell niche was first proposed in 1978 based on studies involving the mammalian hematopoietic stem cells. However, due to the anatomical complexity of the mammalian system, *Drosophila* and *Caenorhabditis elegans* have served as attractive model systems for studying stem cells and stem cell niches (Li and Xie, 2005).

The mammalian and *Drosophila* guts contain intestinal stem cells (ISCs), and use similar signaling pathways to mediate ISC behaviour and differentiation (Apidianakis and Rahme, 2011). Therefore, the adult *Drosophila* midgut serves as an excellent model for studying stem cell maintenance and differentiation. The midgut undergoes regeneration and is maintained through the differentiation of approximately 1000 intestinal stem cells (Singh et al., 2012), which divide approximately once each day to maintain the stem cell population (Choi et al., 2011), and also differentiate to produce one of two possible cell types. Although the intestinal niche and its components are not entirely understood, numerous signaling pathways have been shown to regulate intestinal stem cell behaviour in the *Drosophila* midgut (Zeng et al., 2013).

1.2 AMPs and the larval midgut

Adult midgut progenitors (AMPs) are undifferentiated cells that are specified during embryonic stages, proliferate during larval stages, and are later used to generate the differentiated cells of the adult midgut as well as the undifferentiated intestinal stem cell population (Jiang and Edgar, 2009). The larval midgut is generated from the mitotically active cells of the embryonic endoderm, which have been shown to express the transcription factor encoded by *escargot* (*esg)* (Micchelli, 2012). During embryogenesis endoderm cells segregate to form an inner and outer layer of cells; the outer layer loses *esg* expression first as the cells differentiate to form the absorptive enterocytes (ECs) of the larval midgut, while the inner layer maintains *esg* expression in the AMPs, and *esg* expression is lost in cells that differentiate to form the larval secretory enteroendocrine cells (EEs). Escargot expression is maintained in the AMPs throughout larval stages, as well as in the intestinal stem cells (ISCs) of the adult midgut (Takashima and Hartenstein, 2012).

During the early larval stages, individual AMPs that were specified in the embryo disperse (Issigonis and Matunis, 2010) and undergo 3-4 symmetric divisions to expand the AMP population (Micchelli, 2012). AMP dispersal stops during late larval stages (Issigonis and Matunis, 2010), and AMPs undergo at least one asymmetric division to generate a differentiated peripheral cell (PC) (Fig. 1.1). The PCs surround the cluster of AMPs, and provide a niche environment that allows the AMPs to proliferate while also maintaining the AMPs in an undifferentiated state (Micchelli, 2012).

At the onset of metamorphosis, the AMP/PC clusters merge and the midgut appears as a three-layered structure. The AMPs are released from the PC niche and form the outside layer of cells, which will undergo differentiation following metamorphosis to generate the enterocytes (ECs), enteroendocrine cells (EEs) of the adult midgut, and a subpopulation of AMPs will

remain undifferentiated as the intestinal stem cells (ISCs). Meanwhile, the PCs flatten and join together to produce a "transient pupal midgut" layer between the AMPs that will generate the adult midgut, and the degenerating larval midgut cells. The PCs subsequently degenerate as well and are not found in the adult midgut (Takashima et al., 2011).

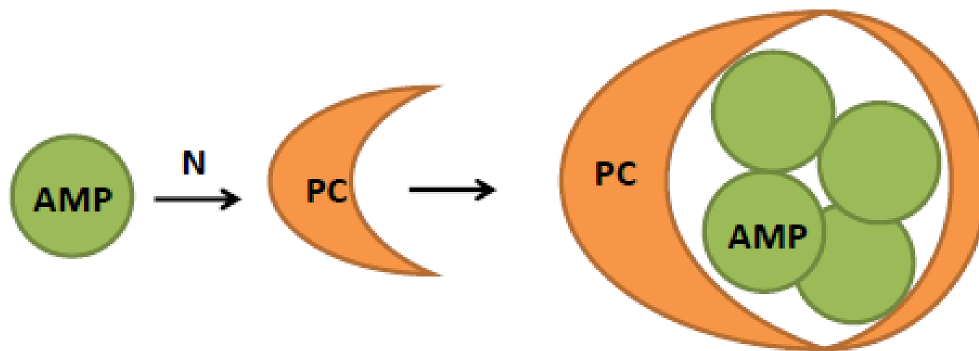

Figure 1.1 Notch signaling is required for proper formation of the AMP/PC niche. Notch signaling (N) is needed to promote peripheral cell (PC) differentiation through the asymmetric division of adult midgut precursor (AMP) cells. The AMPs express the Notch ligand, Delta, and activated Notch signaling occurs in the PCs. PC differentiation is needed to maintain the AMPs in an undifferentiated state throughout the larval stages, and prevents the AMP clusters from prematurely fusing. Figure adapted from Issigonis & Matunis, 2010; Mathur et al., 2010.

1.3 ISCs and the adult midgut

As mentioned in the previous section, most AMPs differentiate to form the absorptive, Pdm1-expressing enterocytes (ECs) as well as the Prospero-expressing secretory enteroendocrine cells (EEs) of the adult midgut (Zeng et al., 2013). A subpopulation of AMPs; however, do not undergo differentiation, maintain expression of Esg (Takashima and Hartenstein, 2012) and Delta, and become the intestinal stem cells (ISCs) of the adult midgut. The ISCs undergo asymmetric divisions daily, allowing them to produce an ISC daughter cell to maintain the ISC population, as well as an enteroblast (EB) cell, which will differentiate without dividing to form either an EC or EE cell. The ability to maintain an undifferentiated stem cell population of ISCs that are able to differentiate allows the midgut to undergo renewal and regenerate following intestinal injury or infection. The ISCs are maintained in an undifferentiated state through signaling from surrounding cells, and ISC differentiation and divisions are regulated by numerous signaling pathways including the Notch and EGFR signaling pathways, among others (Zeng et al., 2013).

1.4 Notch signaling is required for cell differentiation in the larval & adult midgut

The Notch signaling pathway is involved in many developmental processes and it regulates cell differentiation in numerous tissues. It accomplishes these tasks through the transcriptional regulation of many target genes. The Notch receptor is a transmembrane protein that is activated through interactions with its ligands, Delta and Serrate. Upon ligand binding, the Notch Intracellular Domain (NICD) is cleaved and translocates to the nucleus where it interacts with Suppressor of Hairless (Su(H)) and Mastermind. The formation of this complex results in the transcriptional activation of various target genes (Bray, 2006).

It is well established that Notch signaling plays a crucial role in mediating cell differentiation in both the larval and adult midgut. AMPs are known to express the Notch ligand, Delta, while the PCs express the Notch signaling reporter, *GBE-Su(H)lacZ*. The importance of Notch signaling in the establishment and maintenance of the AMP clusters was recognized through the generation of *Notch* mutant clones. *Notch* loss-of-function clones fail to generate PCs, resulting in the premature fusion of AMP clusters and subsequent premature differentiation of AMPs. A much different phenotype is observed with forced expression of activated Notch in the AMPs. Expression of activated Notch causes all AMPs to differentiate to form PC-like cells at the expense of maintaining a pool of undifferentiated AMPs. Therefore, Notch signaling is considered to be necessary and sufficient for PC differentiation. The levels of Notch signaling must be regulated in order to prevent all cells from differentiating to form PCs, while also ensuring the proper formation of PCs so that the individual clusters of AMPs are maintained in an undifferentiated state through PC-mediated signaling (Mathur et al., 2010).

Prior to discovering its role in maintaining the AMP clusters of the larval midgut, Notch signaling had been shown to be involved in controlling ISC divisions and EB formation in the adult midgut. Determining whether the EB differentiates to form an EC or EE cell is largely

dependent upon the level of Notch signaling activation, with strong induction of Notch signaling leading to EC differentiation, while low levels of Notch signaling result in EE differentiation (Fig. 1.2). The level of induction of Notch signaling in the EB is determined by the level of Delta expression in the ISC (Ohlstein and Spradling, 2007). Experiments have shown that Notch signaling is required to limit ISC proliferation, and that it is also sufficient to induce EC differentiation. Disruption of Notch signaling in ISCs using RNAi-mediated knockdown of Notch results in the formation of tumours consisting of ISCs and EEs, demonstrating that Notch signaling is not required for EE cell differentiation. In contrast, over-activation of Notch signaling is sufficient to cause all ISCs to differentiate to form ECs (Micchelli and Perrimon, 2005). The mechanism by which the level of Delta expression is regulated in ISCs and EBs is poorly understood. Following the mitotic division of ISCs, the ISC daughter cell retains expression of Delta, while Delta expression is repressed in the EB and the subsequent activation of Notch signaling from the ISC to the EB causes the EB to further differentiate to form either an EC or EE cell. Although the asymmetric distribution of Delta expression is not yet understood, it is a key factor in determining the differentiation fate of the EB (Jiang and Edgar, 2011).

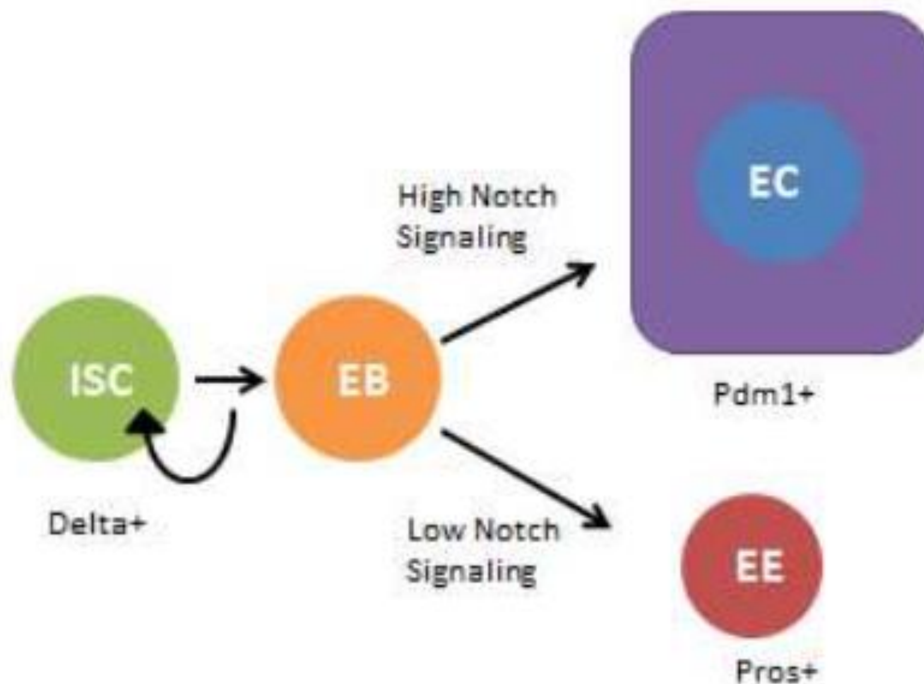

Figure 1. 2 Notch signaling is required for enteroblast formation and enterocyte differentiation in the adult midgut. The Notch ligand, Delta, is expressed in the intestinal stem cells (ISCs), and induces Notch signaling in the enteroblast (EB). Based on the level of Notch signaling activation, the EB can differentiate to form one of two possible cell types. Strong induction of Notch signaling leads to the differentiation of Pdm1-expressing enterocytes (ECs), while low levels of Notch signaling result in the formation of Prospero (Pros) positive enteroendocrine (EE) cells. Figure adapted from Lucchetta & Ohlstein, 2012; Ohlstein & Spradling, 2007.

1.5 EGFR signaling regulates cell proliferation in both the larval and adult midgut

The Epiderminal Growth Factor Receptor (EGFR) signaling pathway is also involved in regulating many developmental processes, and has been suggested to cooperate with and also antagonize Notch signaling depending on the context. EGFR signaling is initiated through the binding of an EGFR ligand to the receptor, which initiates a cellular response resulting in target gene expression. Activating ligands include Spitz, Keren, Vein, and Gurken. Ligand binding results in the phosphorylation and activation of EGFR, allowing EGFR to recruit the protein complex necessary for phosphorylating and activating Ras. Activation of Ras induces a phosphorylation cascade through the activation of numerous protein kinases, ultimately resulting in activation of the mitogen activated protein kinase (MAPK). Once MAPK has been activated, it moves from the cytoplasm to the nucleus where it phosphorylates the transcription factor Pointed, allowing Pointed to activate target gene expression (Doroquez and Rebay, 2008).

EGFR signaling plays an important role in promoting cell proliferation in both the larval and adult midgut. Not only does it promote cell divisions to maintain the AMP and ISC populations, but its over-activation is sufficient to cause extreme overproliferation phenotypes. AMPs undergo numerous divisions during larval stages in order to form clusters of cells that will differentiate following metamorphosis to generate the adult midgut. By knocking down expression of numerous EGFR signaling components such as *EGFR* and *Ras*, Jiang & Edgar (2009) observed a decrease in the number and size of the AMP clusters found throughout the larval midgut, suggesting that EGFR signaling is necessary for promoting AMP proliferation. Anti- diphospho-extracellular signal-regulated kinase (dpERK) staining was also used to demonstrate that EGFR signaling promotes AMP proliferation through activation of the MAPK pathway. AMP proliferation is likely mediated by the EGFR ligands Vein, Spitz, and Keren. It has been suggested that Vein expression in the visceral muscle appears to be most important

during early larval stages, while Spitz and Keren expression in the AMPs themselves encourages mitotic divisions during late larval stages, resulting in the formation of large AMP clusters prior to metamorphosis (Jiang and Edgar, 2009).

Similar to its role in promoting AMP proliferation in the larval midgut, EGFR signaling is also involved in mediating ISC divisions and maintaining the ISC population. Through activation of the MAPK pathway, EGFR signaling, along with additional regulatory pathways, allows for the regeneration of the midgut epithelium (Jiang et al., 2011). Various reporter lines have revealed that *Vein* and *Spitz* are expressed in the visceral muscle and ISCs/EBs, respectively (Strand et al., 2013), while *Keren* expression is primarily found in the ECs (Jiang et al., 2011) (Fig. 1.3). Anti-dpERK staining reveals that EGFR signaling is activated primarily in ISCs, but not in ECs or EE cells. As is the case in the larval midgut, over-activation of EGFR signaling in the adult midgut induces ISC divisions; however, it does not prevent cell differentiation from occurring. Although the ISCs over-proliferate, Notch signaling still occurs, and EBs can form and differentiate to produce EC-like cells (Xu et al., 2011).

The necessity of EGFR signaling for promoting ISC proliferation has been shown using *EGFR* mutants. Disruption of EGFR signaling can effectively suppress N-RNAi-induced tumour formation, resulting in smaller ISC/EE overgrowths, which demonstrates that EGFR signaling is necessary in allowing for ISC divisions to occur and that it does not interfere with cell differentiation. Additionally, EGFR signaling also assists with maintaining the ISC population. Clones expressing *UAS-EGFR-RNAi* (Biteau et al., 2011) or a *UAS-EGFR-dominant negative* do not divide sufficiently to maintain the ISCs and the ISC population is gradually lost through the shedding of the midgut epithelium during normal midgut regeneration. Although the mechanism by which EGFR signaling maintains the ISC population (while also allowing for cell

differentiation to occur) is not yet understood, studies suggest that EGFR signaling may promote expression of *delta* (Xu et al., 2011), which is expressed in the ISC and is important in promoting Notch signaling and cell differentiation (Ohlstein and Spradling, 2007). The generally accepted view is that over-activation of EGFR signaling leads to an increase in the number of Delta-positive ISCs, and this accounts for the induction of Notch signaling that promotes cell differentiation within the expanded ISC population (Xu et al., 2011).

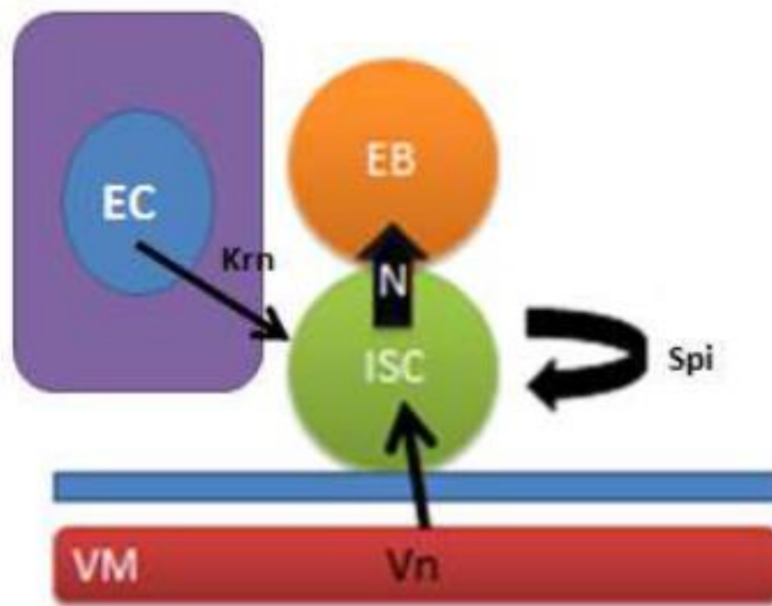

Figure 1.3 EGFR signaling promotes intestinal stem cell (ISC) proliferation in the adult midgut. EGFR signaling is activated in ISCs by interacting with its ligands which are expressed in the surrounding cell types, leading to ISC divisions. The enterocytes (ECs) express Keren (Krn), the visceral muscle (VM) expresses Vein (Vn), and the ISCs express Spitz (Spi). EGFR signaling is not involved in promoting enteroblast (EB) differentiation, but does maintain the ISC population and permits the necessary Notch (N) signaling to allow cell differentiation to occur. Figure adapted from Xu et al., 2011; Strand & Micchelli, 2013; Jiang et al., 2011.

1.6 Hindsight

The gene *hindsight (hnt)* encodes a nuclear zinc-finger protein (Sun and Deng, 2007)(Wilk et al., 2004), and it has been shown to be a direct transcriptional target of the Notch signaling pathway (Krejcí et al., 2009). Hnt is the *Drosophila* homolog of Ras Responsive Element Binding protein-1 (RREB-1) (Sun and Deng, 2007), which has been implicated in a number of human cancers (Costello et al., 2012). Hnt is expressed in a variety of tissues including the midgut, tracheae, and ovarian follicle cells, and has been shown to be involved in regulating various developmental processes (Ming et al., 2014). Follicle cell studies suggest that Hnt acts in a Notch-dependent manner to promote the mitosis-to-endocycle (M/E) transition (Sun and Deng, 2007), while studies focusing on eye development suggest that Hnt is involved in transcriptionally regulating *delta* expression, acting upstream of Notch (Pickup et al., 2009). Currently, Hnt is described as being a putative transcription factor. Although its nuclear localization and protein structure, along with genetic modifier screens, suggest that Hnt is a transcriptional regulator, direct transcriptional targets have not yet been rigorously established. Recent evidence; however, suggests that Hnt functions as a transcriptional attenuator of at least two target genes: itself and *nervy* (Ming et al., 2014).

1.7 Promoting M/E transitions in follicle cells and trachea

Hindsight expression is often used as a reporter for Notch signaling in follicle cells, and has been shown to act in a Notch-dependent manner to promote a mitosis-to-endocyle (M/E) transition in follicle cells (Sun and Deng, 2007). Hnt accomplishes this by negatively regulating the transcription factor Cut, String, a Cdc25 phosphatase (Jordan et al., 2006), and Hedgehog signaling, which is thought to promote exit from the mitotic cycle. The downregulation of Cut also alleviates the Cut-mediated suppression of Fizzy-related (Fzr), a component of the anaphase promoting complex required for the degradation of mitotic cyclins. Following the degradation of mitotic cyclins, follicle cells initiate endocycles, which allows for an increase in nuclear size in the absence of mitotic divisions (Klusza and Deng, 2011). In the follicle cells, overexpression of Hnt has also been shown to be sufficient to promote premature endocycles, and the necessity of Hnt for promoting the M/E transition has been shown using *hnt* mutants. Mutant clones of *hnt* fail to initiate endocycles at the appropriate stage of oogenesis and continue to stain positive for mitotic markers such as Cyclin A, Cyclin B, and phosphohistone H3 (PH3), suggesting that follicle cells remain mitotically active for an extended period of time in the absence of Hnt. However, although *hnt* mutant clones do not transition from mitotic divisions to endocycles at the correct stage of oogenesis, they eventually do endoreplicate. This suggests that although Hnt is needed to initiate the M/E transition at the appropriate stage of oogenesis, it is not the only factor involved in promoting this switch (Sun and Deng, 2007).

In addition to promoting the M/E transition through the downregulation of Cut, Hnt is also involved in regulating follicle cell differentiation (Sun and Deng, 2007). Follicle cells are maintained in an immature, actively proliferating state through Cut-mediated expression of FasIII and Eya (Sun, 2005). In the absence of Hnt, FasIII and Eya expression levels are maintained, which suggests that these cells fail to differentiate properly. In contrast,

overexpression of Hnt leads to premature downregulation of FasIII and Eya, indicating that Hnt expression can promote follicle cell differentiation prematurely (Sun and Deng, 2007).

As well as its role in promoting follicle cell differentiation, Hnt has also been implicated in the regulation of adult tracheal differentiation. The adult tracheal system is generated through the differentiation of tracheoblasts, a population of progenitor cells. Tracheoblasts are present during embryonic stages and remain in an undifferentiated state until they begin to proliferate and differentiate during late larval stages. During the larval stages there are four distinguishable zones of expression, with Hnt expression being highest in the cells found in the zone that also shows Fzr expression, where cells have endoreplicated and are differentiating. Cut is also expressed in the tracheoblast population, but is expressed at higher levels in the actively proliferative zone of cells, where Hnt expression is low (Pitsouli and Perrimon, 2010). The inverse expression pattern of Cut and Hnt expression found in this population of cells suggests that, similar to its role in promoting follicle cell endoreplication and differentiation, Hnt may be involved in promoting endocycles in tracheoblasts, potentially through the downregulation of Cut expression (Pitsouli and Perrimon, 2010)(Klusza and Deng, 2011).

Interestingly, Hnt and Cut are both expressed in the *Drosophila* midgut; Cut expression is found in the differentiated copper cells of the middle midgut region and absent in the anterior and posterior midgut regions (Strand et al., 2011), while Hnt expression is found throughout the entire midgut. Although Hnt could be involved in regulating Cut expression in the middle midgut, there is no evidence that this relationship exists in the anterior/posterior midgut regions, which were the areas of focus for the present study.

1.8 Promoting *delta* expression in the eye

Proper eye development in *Drosophila* is largely dependent upon the integration of the Notch and EGFR signaling pathways. The eye is composed of numerous subunits called ommatidia, with each subunit containing eight photoreceptors, four lens-secreting cone cells, pigment cells, as well as bristle cells (Doroquez and Rebay, 2008). Of particular interest is the fact that Hindsight has been shown to play an important role in cone cell development, acting in combination with both the EGFR and Notch signaling pathways (Pickup et al., 2009).

Cone cells are induced through EGFR and Notch-dependent signaling events initiated by photoreceptor cells. The photoreceptor cells express the EGFR ligand Spitz as well as the Notch ligand Delta, which are responsible for non-autonomously inducing *D-Pax2* expression in the cone cell precursors (Doroquez and Rebay, 2008). Proper cone cell induction is largely dependent on sufficient levels of Delta on the photoreceptor cells. The necessary level of *delta* expression is thought to be achieved through a combination of Hindsight-mediated *delta* expression as well as EGFR signaling-dependent *delta* expression (Pickup et al., 2009). In this context, Hindsight is acting along with the EGFR signaling pathway in order to induce the Notch signaling required for proper cone cell differentiation (Fig. 1.4).

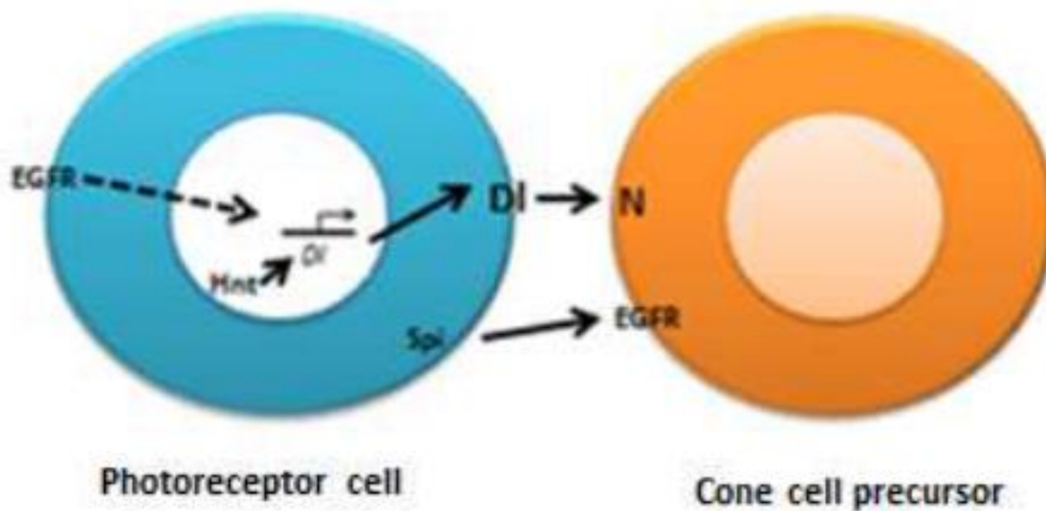

Figure 1.4 Cone cells are induced through EGFR and Notch-dependent signaling events initiated by photoreceptor cells. Photoreceptor cells express the EGFR ligand Spitz (Spi) and the Notch ligand Proper cone cell induction requires Delta (Dl) on the photoreceptor cells to induce Notch signaling in the cone cell precursor. Hindsight (Hnt) and EGFR signaling cooperate to transcriptionally up-regulate *delta* to allow for proper cone cell induction. Figure adapted from Pickup et al., 2009.

1.9 Potential role of Hnt in midgut development

Hnt is expressed in the embryonic midgut (Wilk et al., 2004); however, the potential role of Hnt in midgut development has not been determined. As described previously (Section 1.4), the PCs of the larval midgut are specified in a manner similar to that of cone cells (Pickup et al., 2009), requiring the Delta ligand from the AMPs in order to initiate the Notch signaling-dependent PC differentiation. The differentiation of ISCs to form the EEs as well as the ECs of the adult midgut is also dependent on Dl-Notch mediated signaling, with the level of Delta expression determining which cell fate is chosen (Issigonis and Matunis, 2010).

The overall aim of this study was to investigate the potential role of Hnt in the larval and adult midgut, focusing on Hnt's possible interaction with the Notch and EGFR signaling pathways.

Chapter 2. Materials and Methods

2.1 Fly culture and strains

All flies were raised on standard yeast and molasses-based food at 25°C on a 12 hour light/dark cycle, unless otherwise indicated. The following stocks were kindly provided by C. Micchelli (Washington University School of Medicine):*esgGAL4UASGFPtubGal80ts, esgGAL4, esgGAL4UASGFP,* and *UAS-*^{Nintra}. The *UAS-GFP-HNT*, *UAS-GFP-HNT; Gal80ts,* and *UAS-hnt-RNAi2A;2B* lines were gifts from H. Lipshitz (University of Toronto). The *UAS-Su(H)*^{VP16} stock was provided by S. Bray (University of Cambridge). The following stocks were obtained from Bloomington Drosophila Stock Center: *UAS-N-RNAi, UAS-mamN, UAS-Ras85D*^{V12}, *PCNA-GFP,* and *UAS-GFP*^{nls}. The *UAS-EGFR-RNAi* lines were obtained from the Vienna Drosophila RNAi Center, and the *NP3312-GAL4* driver was provided by the Kyoto Stock Center. The *EP55*;;*eyeless-GAL4/ TM6B, Tb*, *UAS-GFP-HNT*^{J18}, *tubGal80hsFLPFRT19A; esgGAL4UASGFPnls/(CyO), hnt*^{308}*FRT19A,* and *hnt*^{XE81}*FRT19A* stocks were made by B.H. Reed.

2.2 The GAL4/UAS system

The GAL4/UAS system of targeted gene expression uses the GAL4 yeast transcription factor to induce expression of genes placed under the control of an upstream activating (UAS) sequence. The GAL4 gene, along with regulatory elements to determine its temporal- and tissue-specific expression pattern, is carried by the driver line. The driver line can be crossed with a responder line carrying the *UAS-gene of interest* construct, and the resulting progeny that carry both the GAL4 and UAS constructs will express the gene of interest (Elliott and Brand, 2008).

GAL4-induced gene expression can be regulated by using the yeast GAL80 protein. GAL80 binds to the transactivation domain of the GAL4, preventing it from binding to the UAS

sequence. The gene encoding the GAL80 protein is placed under the control of a *tubulin 1α* promoter, allowing for universal repression of the GAL4 protein. A temperature-sensitive GAL80 (GAL80ts) allows for temperature-dependent control of the GAL4 protein. When flies are grown at 18 ºC, GAL80ts binds to GAL4 to inactivate it and prevent UAS-controlled gene expression. Shifting flies to 29 ºC prevents Gal80ts from binding to and inhibiting the GAL4 protein, allowing GAL4-induced gene expression to occur (Elliott and Brand, 2008).

2.3 Temperature shift experiments

Crosses were established and cultured at 18°C until adulthood. After reaching adulthood, flies were aged for 2-3 days at 18°C and then shifted to and maintained at 29°C until being used for imaging. Flies were shifted for the following periods of time: *UAS-GFP-HNT* (1-14 days); *UAS-hntRNAi* (1-16 days); *UAS-EGFR-RNAi* (5-18 days); *UAS-Ras85DV12* (1-4 days); *EP55* + *UAS-Ras85D*^{V12} (1-4 days); *UAS-N*^{intra} (2 days); *UAS-N-RNAi* (3-12 days); *UAS-N-RNAi* + *UAS-GFP-HNT* (4-12 days); *UAS-N-RNAi* + *UAS-hnt-RNAi* (3-10 days); *UAS-λtop4.2* (1-7 days); *UAS-λtop4.2* + *UAS-GFP-HNT* (1-3 days); *UAS-λtop4.2* + *UAS-hnt-RNAi* (1-3 days), *UAS-Su(H)*^{VP16} (11 days). Note: for *UAS-Ras* experiments, shifts were performed during larval stages.

2.4 Immunostaining

Larvae and adult flies were dissected in 1 X PBS. The entire gastrointestinal tract was removed and fixed in a solution of 3.7% formaldehyde in PBS for 20-30 minutes. The guts were washed in PBT (1 X PBS + 0.1% Triton X 100) and 1% Bovine serum albumin (BSA) for 3x 10 minutes, followed by a 1 h wash in a blocking solution consisting of PBT, 1% BSA, and normal goat serum. Following the blocking step, the samples were incubated overnight at 4°C in a fresh solution of PBT, 1% BSA, and normal goat serum, along with primary antibodies. The guts were then washed in fresh PBT + 1% BSA for 3x 10 minutes before being placed in fresh blocking solution for 1-2 h. After blocking, a fresh solution of PBT, 1% BSA, and normal goat

serum was added to the samples, along with the secondary antibody. The guts were incubated for 2-3 h at room temperature. Finally, the samples were washed in PBT for 3x 10 minutes, and in PBS for 3x 3 minutes. After the PBS washes, the guts were put through a glycerol-PBS series consisting of increasing glycerol concentrations, ending with 95% glycerol and DABCO. The midguts were then dissected further and imaged. Note: for anti-Pdm1 staining, 0.5% BSA was used in place of 1% BSA.

2.5 Antisera

Primary antibodies: mouse monoclonal anti-Hnt (H. Lipshitz, University of Toronto), 1:25; mouse anti-Prospero (Developmental Studies Hybridoma Bank (DSHB), University of Iowa)1:100; mouse anti-Cyclin A A19 (P. O'Farrell, University of California) 1:50; mouse monoclonal anti-Cyclin B F2F4 (DSHB), 1:5; rabbit anti-Pdm1 (X. Yang, Zhejiang University) 1:1000.

Secondary antibodies: TRITC-conjugated goat anti-mouse and TRITC-conjugated donkey anti-rabbit (Cedarlane Labs) were used at a 1:500 dilution.

2.6 Mosaic analysis

The mosaic analysis with a repressible cell marker (MARCM) system was used in order to generate GFP-labelled AMP clones in the larval midgut. This method is dependent upon site-specific recombination at *FRT* sites during mitosis, which is catalyzed by the enzyme FLP recombinase (Wu et al., 2007). This method uses transgenes for homologous FRT sites, a *UAS-GFP* reporter,,a *GAL4* driver*, FLP-recombinase* and *tubGal80*. The *tubGal80* transgene is placed distal to the FRT site in *trans* to a mutant gene of interest. In heterozygous cells, the Gal80 prevents the GAL4 from inducing expression of UAS-GFP. In homozygous mutant cells, the transgene encoding the Gal80 is lost, preventing it from suppressing GAL4-driven expression of UAS-GFP. This allows for GFP-labelled visualization of the mutant cells and distinguishes

them from the heterozygous cells (Singh et al., 2012). A nuclear stain can then be used in order to label all cell nuclei, and allow for comparisons of mutant and wild type cells.

hnt clones were generated by crossing male flies of genotype *tubGal80hsFLP19A; esgGAL4UASGFP*^{nls}*/(CyO)* with either *hnt*^{308}*FRT19A* or *hnt*^{XE81}*FRT19A* virgin females. The resulting progeny were maintained at 25°C until larval stages. Second/early third instar larvae were then heat shocked at 37°C in a circulating water bath for 2x 30 minutes. After heat shock, larvae were maintained at 25°C. The midguts were then dissected out from wandering third instar larvae 1-2 days after heat shock, and were stained using the immunostaining protocol described previously. Clones were then imaged using confocal microscopy.

2.7 Laser confocal microscopy

The midguts from third instar larvae or adults were used for imaging. For each experiment, 5-10 midguts were dissected out in halocarbon oil or mountant on a microscope slide. Images were obtained using a Nikon Eclipse 90i fitted with a Nikon D-eclipse C1 scan head using Nikon EZ-C1 software at 20x and 40x objectives. Image processing was performed using Nikon EZ-C1 software and images were edited and assembled using Microsoft PowerPoint 2010.

Chapter 3. Experimental Results

3.1 Hnt is expressed throughout the larval and adult midgut

The embryonic expression pattern of Hnt has been reasonably well described and includes expression throughout the developing embryonic midgut (Yip et al., 1997). In other contexts, most notably the adult follicular epithelium and the adult muscle progenitors, *hnt* has been identified as a downstream target of the Notch signaling pathway (Sun and Deng, 2007)(Krejci et al., 2009). Interestingly, differentiation of the larval and adult midgut is largely mediated by Notch signaling (Issigonis and Matunis, 2010). Any connection between Notch signaling and *hnt* expression in this tissue, however, remains unexplored. Initial experiments were therefore carried out to examine the expression of *hnt* in the larval and adult midgut.

The *hnt-GAL4* driver (hnt^{NP3312}) driving nuclear-GFP resulted in expression throughout the larval midgut in several cell types (Fig. 3.1A). The faithfulness of the hnt^{NP3312} *GAL4* expression pattern to the endogenous pattern of Hnt was confirmed by immunostaining. Wholemount anti-Hnt immunostaining of fixed larval midguts confirmed that Hnt is expressed in several cell types throughout the anterior and posterior regions of the larval midgut (Fig. 3.1B). Further experiments were carried out to identify which cells types of the larval midgut are associated with Hnt expression. Subsequently, additional cell markers (including other enhancer trap *GAL4* lines and antibodies) were used to identify which midgut cell types express Hnt.

Differentiated larval enterocytes (ECs) are easily identified because they are the only large polyploid cells within the larval midgut. ECs clearly express Hnt at high levels, and this was observed in both the hnt^{NP3312} *GAL4* expression pattern as well as anti-Hnt immunostainings (Figs. 3.1A-C). The peripheral cells (PCs) and the adult midgut precursors (AMPs) were also Hnt-positive, although there was a clear elevation of Hnt expression in the PCs relative to the

AMPs (Fig. 3.1C). Anti-Prospero staining in the background of $hnt^{NP3312} > GFP^{nls}$, indicated that the enteroendocrine (EEs) cells also have low levels of Hnt expression (Figs. 3.1D-D’’).

Having established that Hnt is expressed in all cell types of the anterior and posterior regions of the larval midgut, albeit at lower levels in the AMPs and the EEs, it was subsequently of interest to determine if Hnt is also expressed in the adult midgut. Similar to the observations of $hnt^{NP3312} > GFP^{nls}$ and anti-Hnt immunostaining in larvae, Hnt was found to be expressed throughout the anterior and posterior adult midgut (Fig. 3.2A, B). Further analysis using anti-Hnt immunostaining of adult midguts expressing GFP under the control of *esgGAL4*, a marker for intestinal stem cells (ISCs) and enteroblasts (EBs) (Beebe et al., 2010a), demonstrated that Hnt expression was found in both of these cell types. In addition to the *esgGAL4* expressing ISCs and EBs (GFP positive), Hnt expression was also observed in the non-*esgGAL4* expressing cells (GFP negative) of the adult midgut. These GFP negative cells correspond to the large differentiated polyploid ECs and the smaller diploid differentiated EEs. The identification of the EEs relied on their small size and complete absence of GFP; in contrast, newly differentiated ECs were noticeably polyploid (i.e. large) but still maintained GFP (Fig 3.2 C, C’).

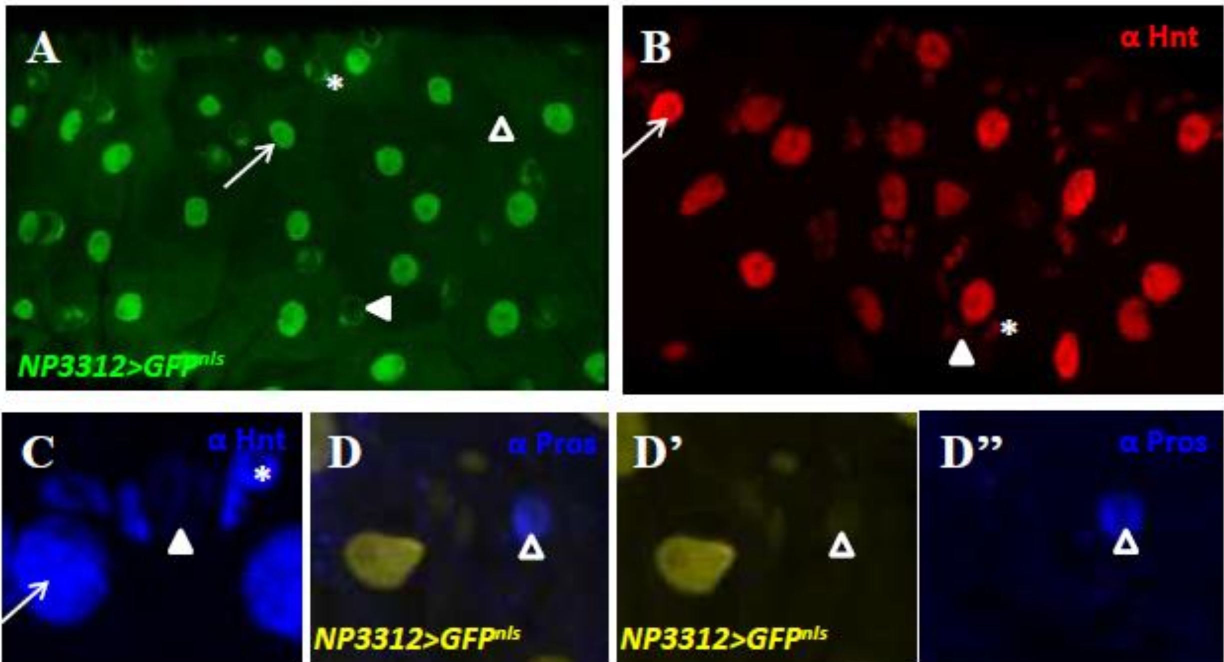

Figure 3.1 Hnt is expressed throughout the larval midgut. Expression of the *hntGAL4* enhancer trap line hnt^{NP3312} driving GFP^{nls} (A) and anti-Hnt immunostaining (B, C) demonstrate that *hnt* is expressed in all cell types of the larval midgut. *hnt* expression is strongest in the differentiated absorptive enterocytes (ECs, arrows) and the peripheral cells (PCs, asterisks). *hnt* is expressed albeit weakly, in the adult midgut precursors (AMPs, solid arrowheads) and the differentiated secretory enteroendocrine cells (EEs, open arrowhead). Immunostaining using the enteroendocrine cell marker Prospero (anti-Pros) confirms that *hnt* is expressed at low levels in this cell type (D, D', D").

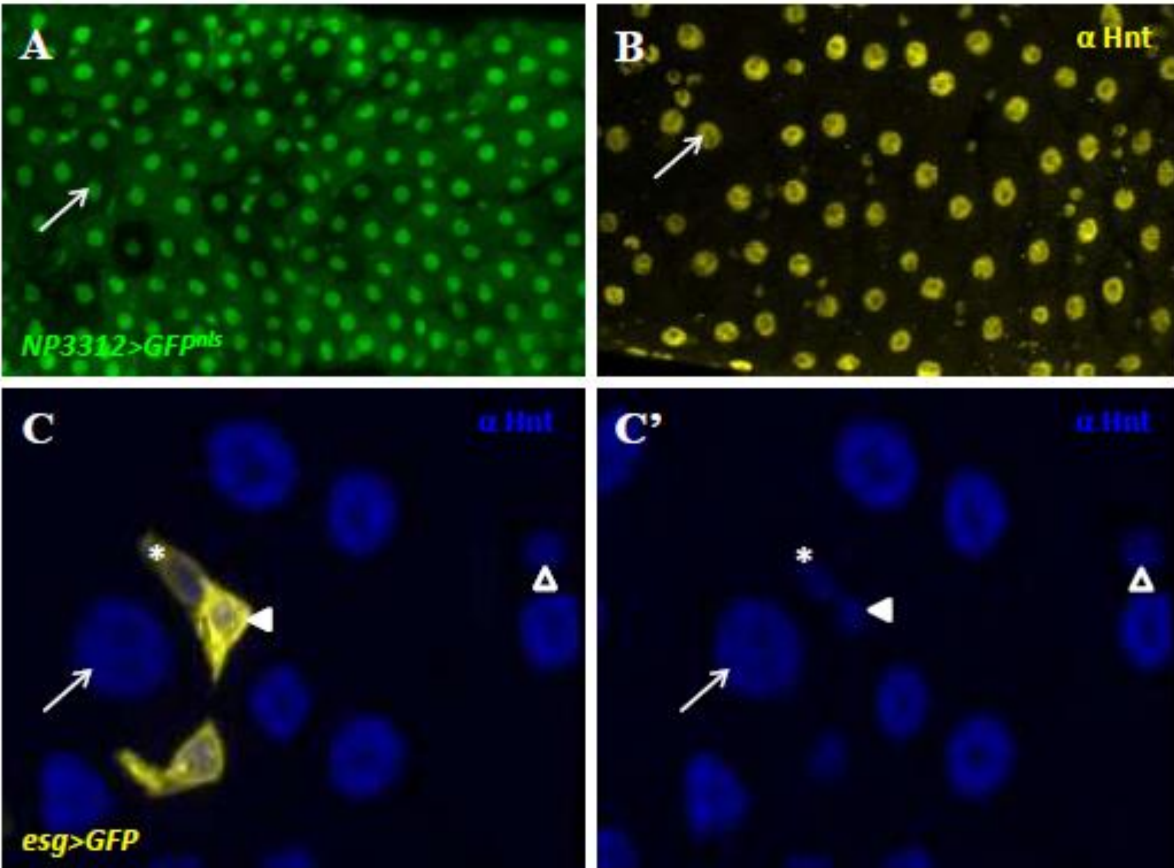

Figure 3.2 Hnt is expressed throughout the adult midgut. Expression of the *hntGAL4* enhancer trap line hnt^{NP3312} driving GFP^{nls} (A) and anti-Hnt immunostaining (B, C) demonstrates that *hnt* is expressed in all cell types of the adult midgut, including the large polyploid enterocytes (ECs, arrows) and smaller cell types. Anti-Hnt immunostaining of the adult midgut of the *escargotGAL4* line driving *GFP* (C, C'), which marks the adult intestinal stem cell population (ISCs, arrowheads) and the enteroblasts (EBs, asterisks), demonstrates that *hnt* is expressed in these cells as well as the smaller GFP-negative enteroendocrine cells (EEs, open arrowheads).

3.2 Hnt is required for proper PC differentiation and formation of the AMP clusters of the larval midgut

The organization of AMP clusters in the larval midgut can be visualized using the *esgGAL4* driver, as well as the *hnt-GAL4 driver* (hnt^{NP3312}). As shown in Figures 3.3A-B, *esgGAL4* driven expression of GFP^{nls} suggests that Esg expression is elevated in the PCs relative to the AMPs. A similar expression pattern of elevated PC expression was also observed using the hnt^{NP3312} driver (Fig. 3.3C), and this was confirmed by immunostaining (see Fig. 3.1C).

The MARCM system (see materials and methods) was used in order to examine the consequences of reduced *hnt* expression in the larval midgut. This system allows us to identify *hnt* mutant AMP clones based on *esgGAL4* driven GFP expression, and to compare the mutant clonal patches (GFP positive) with neighbouring wild type AMP clusters (GFP negative). Initially, hnt^{308} (a hypomorphic allele of *hnt*) clones were generated and anti-Hnt immunostaining was performed to ascertain if Hnt expression is reduced within the mutant clonal patches using the neighbouring wild type AMPs as an internal positive control. In general, the expression of Hnt within the hnt^{308} mutant clonal patches was uniformly low, whereas the neighbouring wild type immunostaining revealed a less uniform expression pattern (Fig 3.3D-D''). In addition, the hnt^{308} mutant AMP clusters were atypical in that they did not show any obvious increased expression of either the *esgGAL4* driver (Fig 3.3A *cf.* 3.3D') or Hnt (see circled region Fig 3.3D''). These observations suggest that the hnt^{308} mutant AMP clusters are incapable of promoting PC differentiation, which is normally associated with increased *esgGAL4* and Hnt expression. A stronger phenotype was observed when complete loss-of-function *hnt* clones were generated using hnt^{XE81}. Anti-Hnt staining confirmed that Hnt was not expressed in the mutant clonal patches, and allowed for the identification of the wild type AMP clusters (Figs. 3.3E-E''). Complete loss of Hnt (hnt^{XE81} clones) disrupted AMP cluster formation,

and the mutant clusters appeared to be more dispersed and disorganized than what was observed in the hypomorphic condition. Intriguingly, the *hnt*^{XE81} mutant clonal patches were often associated with strong *esgGAL4* expression but, based on morphology, appeared not to contain PCs. This suggests that *hnt* loss-of-function of mutant clones fail in PC differentiation, which in turn leads to the observed dispersal of undifferentiated AMPs.

Unfortunately an attempt to recover *hnt*^{XE81} mutant clones in the adult midgut was unsuccessful, as mutant clonal (GFP positive) cells were never observed (data not shown). Clonal patches in the adult midgut using the hypomorphic *hnt*^{308} allele were not attempted, but such an analysis might be warranted in the future.

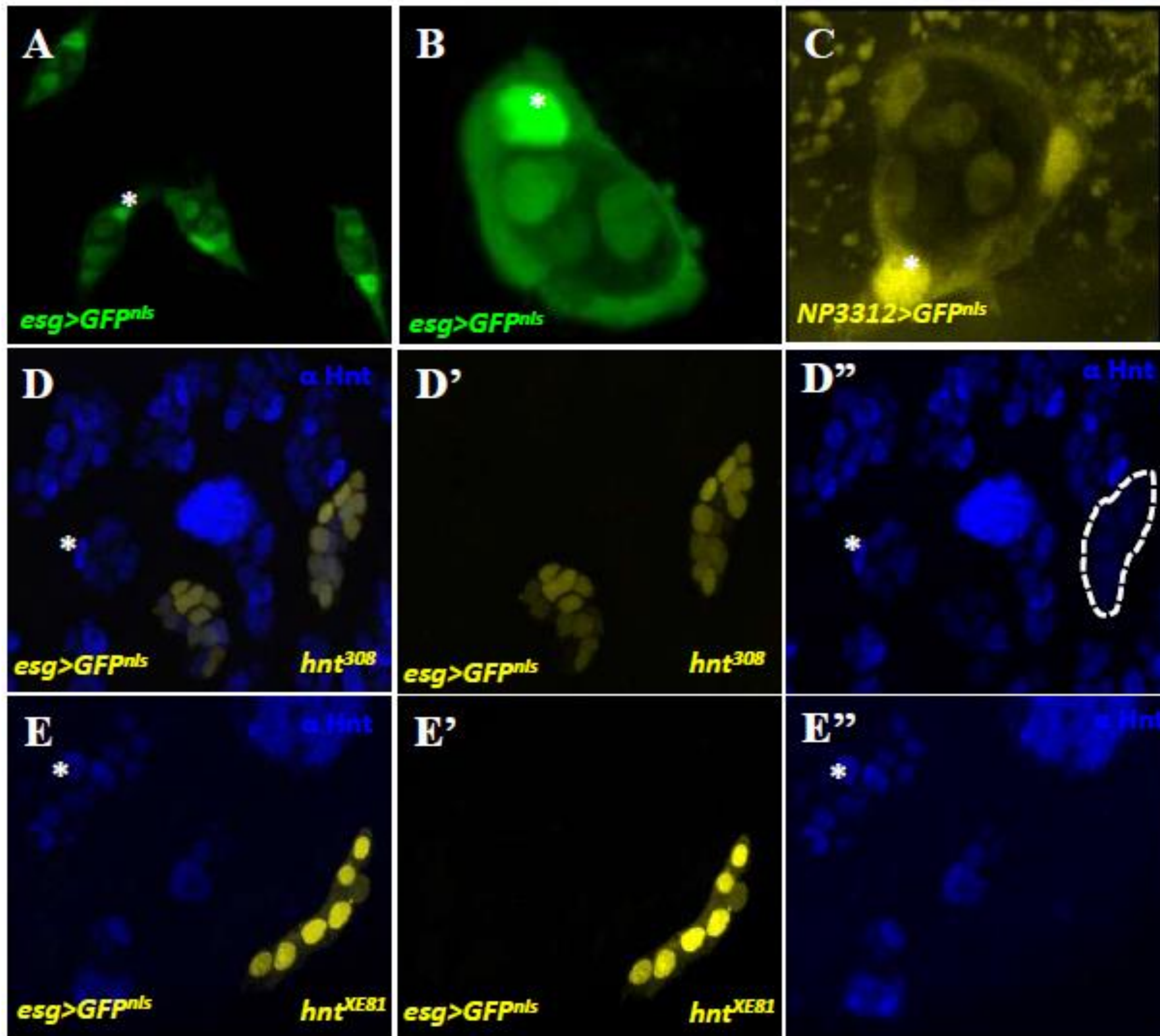

Figure 3.3 Hnt is required for proper organization of the AMP cluster. Hnt is required for proper organization of the adult midgut precursors (AMP) and associated peripheral cells (PCs) in larvae. The wild-type organization of the AMP/PC cluster is readily visualized in the larval midgut of *esgGAL4>UASGFP*^{nls} (A and B). The *esgGAL4* expression is typically elevated in the PCs (asterisks in A and B). A similar pattern where *hnt* expression is elevated in PCs, is also seen in *hnt*^{NP3312}*>UASGFP*^{nls} (C). Larval midguts containing somatic clones (marked by GFP^{nls} expression; see Materials & Methods) homozygous for either the hypomorphic loss-of-function *hnt*^{308} allele (D-D"), or the antibody-null allele, *hnt*^{XE81} (E-E"), do not form PCs and are typically dispersed. *hnt* mutant clones display uniform expression of *esgGAL4>GFP*^{nls} (D and E) as well as uniform expression of *hnt* in the *hnt*^{308} clones (D-D", white dotted line).

3.3 Peripheral cells induced by activated Notch signaling express Hnt

It has been established that Notch signaling is required for PC differentiation, and that expression of activated Notch in AMPs is sufficient to cause all AMPs to differentiate into PCs (Mathur et al., 2010). Hnt expression was elevated in PCs relative to AMPs (see Fig. 3.1C and Fig. 3.4A). Anti-Hnt immunostaining of larval midguts in the background of *esgGAL4* driving expression of GFP and activated Notch (N^{intra}, Notch intracellular domain) (Micchelli and Perrimon, 2005), showed strong Hnt expression in all PC-like cells (Fig. 3.4B, B'). Although this does not establish any relationship between Notch signalling and Hnt expression in the larval midgut, it does confirm that activated Notch signaling causes PC differentiation, and further demonstrates that Hnt is strongly expressed in the differentiated PCs, some of which in this context are likely to be ectopic.

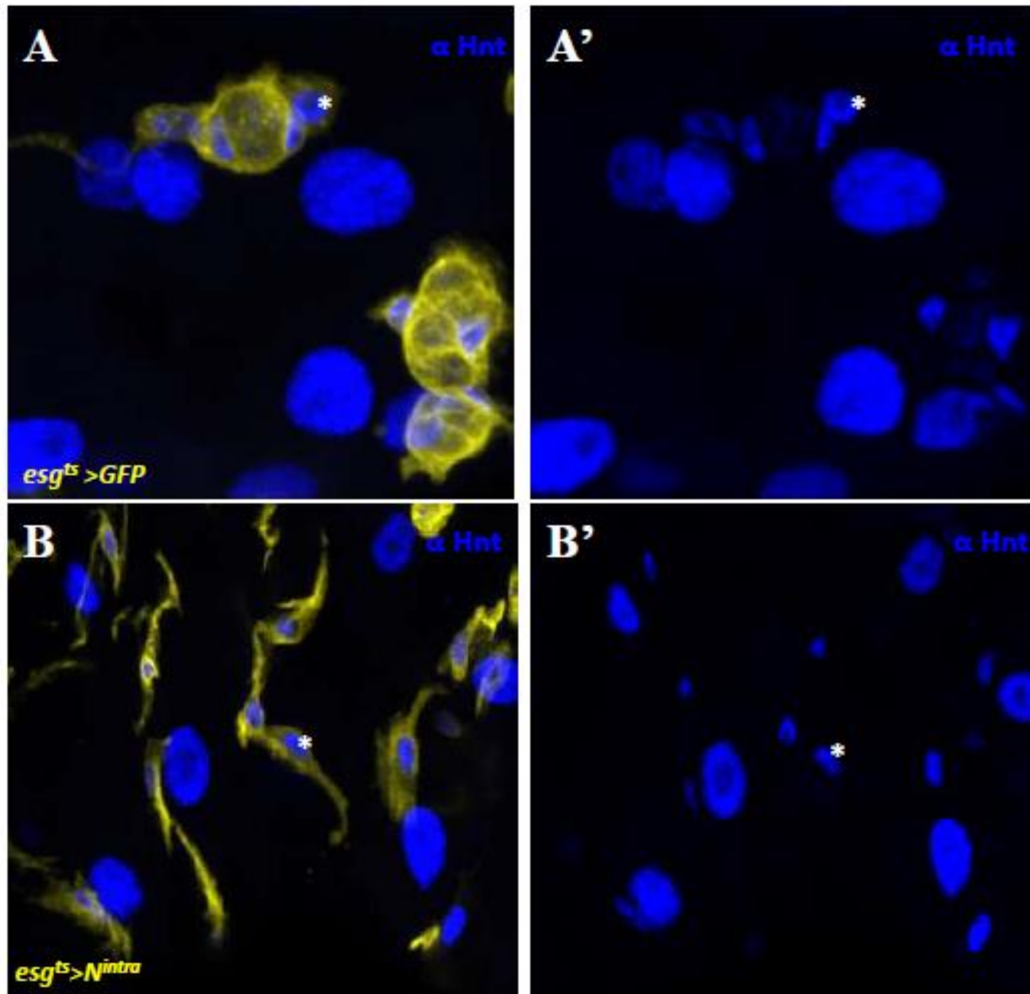

Figure 3.4 Activation of the Notch signaling pathway promotes peripheral cell differentiation. Anti-Hnt immunostained larval midgut of *esgGAL4>GFP + tubGAL80^{ts} (esg^{ts})* following a 2 day shift to 29 °C without (A, A') or with expression of N^{intra} (B, B'). The control (A, A') shows typical AMP/PC clusters (marked by *esg>GFP)* with elevated Hnt in the PCs (asterisks). Larval midguts expressing N^{intra} (B, B'') lack typical AMP/PC clusters and all AMPs differentiate to form PC-like cells with strong Hnt expression (asterisks).

3.4 Overexpression of Hnt is sufficient to promote premature enterocyte differentiation in the larval midgut

Previous results (this study) show that Hnt is upregulated in the differentiated PCs, raising the possibility that Hnt expression is sufficient to promote PC differentiation and thereby phenocopy activated Notch, where all AMPs differentiate to form PCs. Initial experiments were performed in the larval midgut using the *esgGAL4* driver to increase the levels of Hnt in AMPs using HNT reporters, hnt^{EP55} and $UAS\text{-}GFP\text{-}HNT^{J18}$, which are associated with low levels of expression. In contrast to the activated Notch phenotype, these larvae showed a very different disruption of PC-AMP niche formation (Figs. 3.5A *cf.* Fig3.5B,C). In the context of low level Hnt overexpression, some AMPs appeared to have large polyploid nuclei, suggesting premature EC differentiation, as opposed to the PC differentiation associated with Notch activation.

Additional experiments were performed in the larval midgut using the *esgGAL4* driver to further increase the expression of Hnt in AMPs using the *UAS-GFP-HNT* reporter, which is associated with a much higher level of expression (data not shown, see Fig. 3.11A-A''). Given that *esgGAL4* driving expression of the strong *UAS-GFP-HNT* reporter is embryonic lethal, these experiments required the addition of the $tubGAL80^{ts}$ insertion and a temperature shift from 18º to 29º during larval stages (see materials and methods). With strong Hnt overexpression, the above phenotype was dramatically enhanced in that all GFP positive cells were visibly large and polyploid, resembling differentiated ECs (Fig. 3.5D). In this context of strong HNT overexpression, it was interesting to note that no AMP-like cells remained within the clusters. In addition, the overall number of GFP positive AMP clusters was dramatically reduced, which was more obvious with prolonged exposure to 29º. One possible explanation for this apparent disappearance of the GFP-positive Hnt over-expressing AMPs is their possible premature differentiation into ECs, which may be associated with a downregulation of the *esgGAL4* driver.

In order to address the possible premature EC differentiation phenotype, anti-Pdm1 immunostaining, a well-known method of marking differentiated adult ECs (Singh et al., 2012), was performed. This experiment confirmed that overexpression of Hnt results in expression of the adult EC marker Pdm1 (Figs. 3.5E-E'). This striking result supports the interpretation that Hnt overexpression results in the premature differentiation of larval AMPs into adult ECs. Overall, the level of Hnt overexpression correlated with the severity of the premature differentiation phenotype, suggesting that a threshold effect operates in this differentiation pathway.

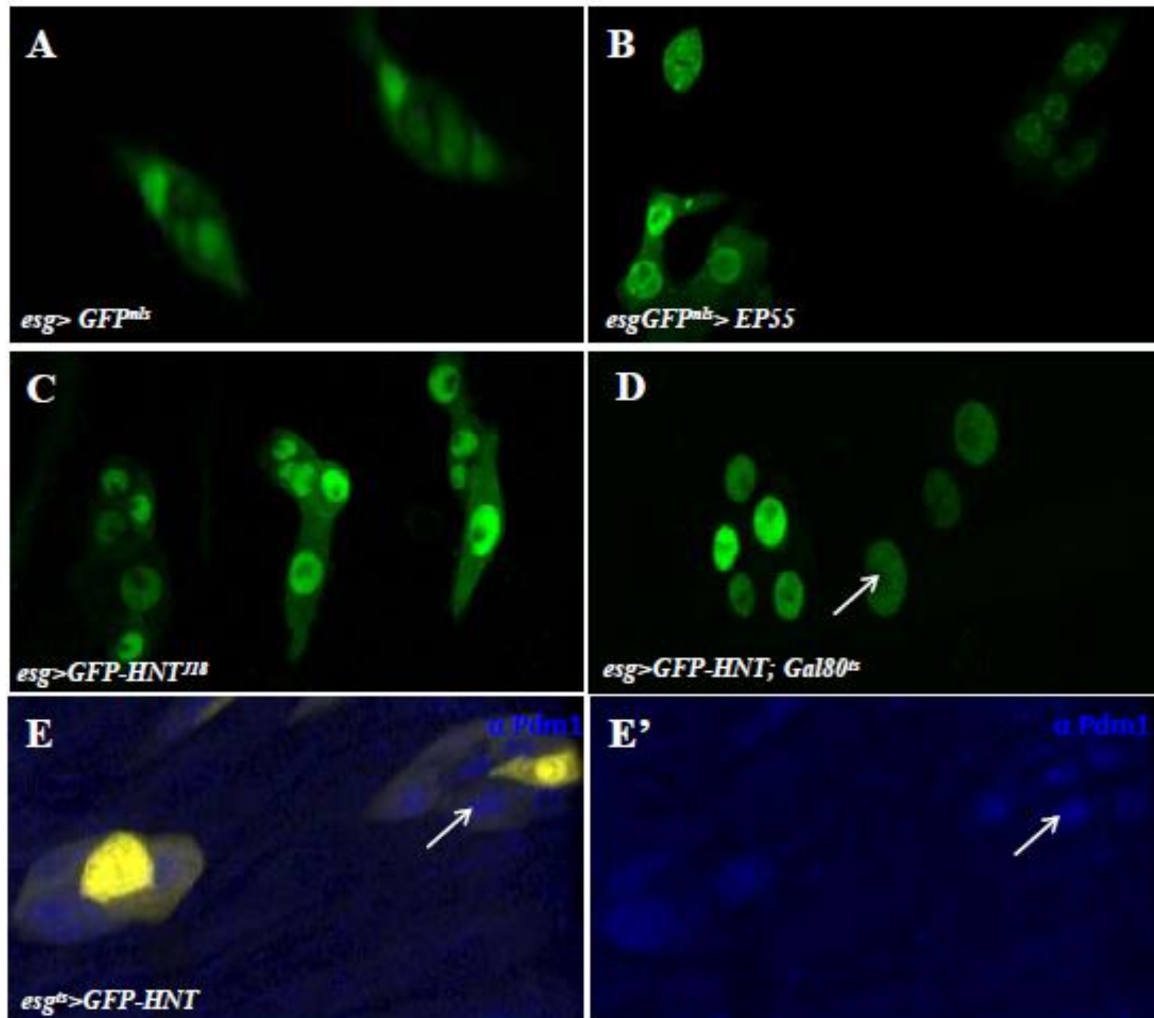

Figure 3.5 Overexpression of Hnt in the larval AMP/PC cluster promotes enterocyte differentiation. The wild-type AMP/PC cluster morphology visualized by *esgGAL4>GFP*^{nls} (A) is perturbed by co-expression of Hnt (B-D). Backgrounds associated with weak (*EP55*; B), medium (*UAS-GFP-HNT*^{J18}; C), or strong (*UAS-GFP-HNT*; C) *hnt* over-expression were associated with progressively stronger perturbations in AMP/PC organization. In the strongest Hnt over-expression background (*UAS-GFP-HNT;* D) all AMP/PC cells normally marked by *esgGAL4>GFP* are large and appear to be polyploid (arrows). Immunostaining the latter background using the enterocyte marker anti-Pdm1 (E, E') confirms that strong over-expression of Hnt is associated with inappropriate differentiation of AMP/PCs into enterocytes (arrows).

3.5 Reduced Hnt in AMP clusters resembles the *Notch* loss of function phenotype

Previous studies have found that AMPs undergo an asymmetric division to generate the differentiated PC in a Notch-dependent manner. Disruption of Notch signaling using *Notch* loss-of-function mutants prevents PC formation (Mathur et al., 2010). Similar results were obtained using the *esgGAL4* driver to disrupt Notch signaling through the expression of either *N-RNAi* or *mamN,* the latter of which produces a dominant negative truncated version of the Notch signaling co-activator Mastermind (Mam) (Vied et al., 2009).

These results were repeated in order to better compare them with the phenotype resulting from knockdown by *hnt-RNAi*. As published, AMP clusters with impaired Notch signaling showed abnormal PC-AMP cluster organization and a clear absence of PCs (Fig 3.6A *cf.* Figs. 3.6B,C). Knockdown of Hnt using the *esgGAL4* driver and a *hnt-RNAi* reporter (see materials and methods for full stock descriptions) revealed that RNAi-mediated knockdown of Hnt phenocopies the *Notch* loss-of-function phenotype. More specifically, this background also lacked any clear PC differentiation (Fig. 3.6D). The Hnt knockdown phenotype was not entirely similar to the Notch loss-of-function phenotype in that the Hnt knockdown was associated with a dramatic reduction in the overall number of GFP expressing clusters, whereas the Notch loss-of-function clusters were prevalent. The decreased number of clusters associated with Hnt knockdown could result from cell death, lack of AMP proliferation associated with fusion of solitary AMPs, or premature differentiation due to the absence of PC cells and their associated stem cell niche properties.

The phenotype of the individual AMP clusters in the Hnt knockdown larvae did share properties with the Notch loss of function phenotype, and this could be associated with a failure to differentiate PCs, which could relate to Notch signaling. The AMP cluster reduction, however, is likely not associated with Notch signaling but could be associated with other

signaling pathways such as the EGFR signaling pathway which is required for AMP proliferation (Jiang and Edgar, 2009).

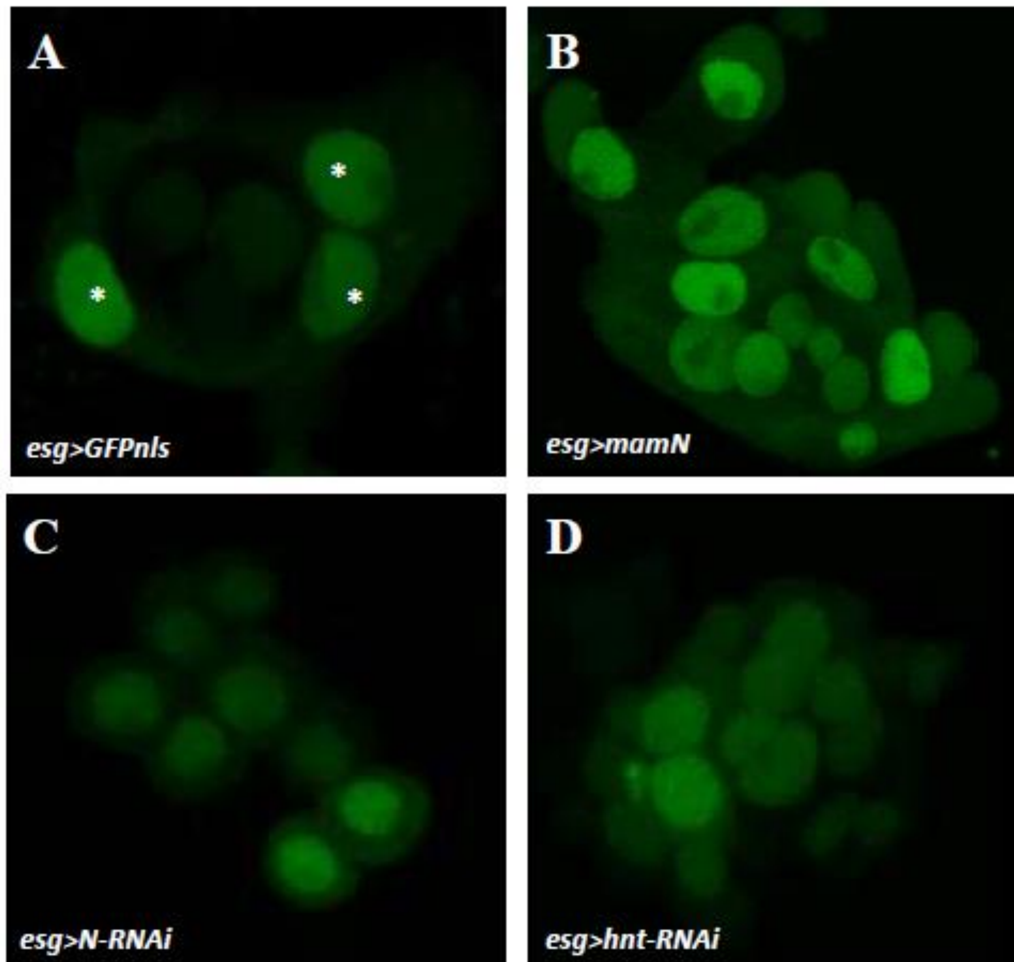

Figure 3.6 Notch signaling is required for peripheral formation in the larval midgut. The wild-type AMP/PC arrangement visualized by live imaging using *esgGAL4>GFP^{nls}* (A) shows three PCs (asterisks) enveloping the AMPs. The same background co-expressing *mamN* (B) or *N-RNAi* (C), both of which negatively disrupt Notch signaling, displays disorganized AMP clusters that lack PCs. The RNAi-mediated knockdown of Hnt by *esgGAL4* (D) results in a decrease in the overall number of AMP clusters; the remaining clusters display a phenotype similar to the disruption of Notch signaling (compare D to B/C).

3.6 Hnt is expressed at lower levels in mitotically active cells but does not inhibit mitotic divisions

Based on prior results showing that reduced Hnt results in a reduction in the number of AMPs, and given that Hnt overexpression leads to premature differentiation of polyploid ECs (this study), it was possible that Hnt could play a role in the regulation of AMP proliferation. Also relevant to this line of investigation is the reported finding that Hnt, in combination with the Notch signaling pathway, promotes mitotic-to-endocycle (M/E) transitions in the follicle cells of the adult ovary (Sun and Deng, 2007). Furthermore, this previous work used anti-phosphohistone H3 immunostaining (an M phase marker) to show that Hnt is not expressed in mitotically active follicle cells. In addition, overexpression of Hnt in the follicle cells halts mitotic divisions and induces premature endocycles(Sun and Deng, 2007). Therefore, in order to see if Hnt is involved in regulating mitotic divisions of the AMPs in the larval midgut, anti-Cyclin A and anti-Cyclin B immunostaining was performed. The co-expression of the S-phase marker PCNA-GFP and Hnt was also examined. In these experiments anti-Cyclin B and anti-Cyclin A were used as markers of the mitotic cell cycle (Lee and Orr-Weaver, 2003). Immunostaining the larval midgut for Cyclin A and B in the background of *hnt-GAL4* (*hnt^{NP3312}*) driving expression of *UAS-GFP^{nls}* revealed that cells expressing either Cyclin A or B generally show low levels of Hnt expression (Figs. 3.7A & B). Similarly, larval midguts expressing *PCNA-GFP* generally showed lower levels of Hnt expression in the GFP-positive AMPs (S phase) relative to neighbouring GFP-negative AMPs (Fig. 3.7C). However, there were occasionally instances of GFP-positive cells with high levels of Hnt expression (data not shown). These rare cases presumably represented post-mitotic PCs which were possibly undergoing S phase during an endocycle, although the ploidy of PCs remains unknown (Takashima et al.,

2011a). Interestingly, if PCs do indeed undergo endocycles, it is possible that Hnt is involved in promoting the M/E transition in this cell type.

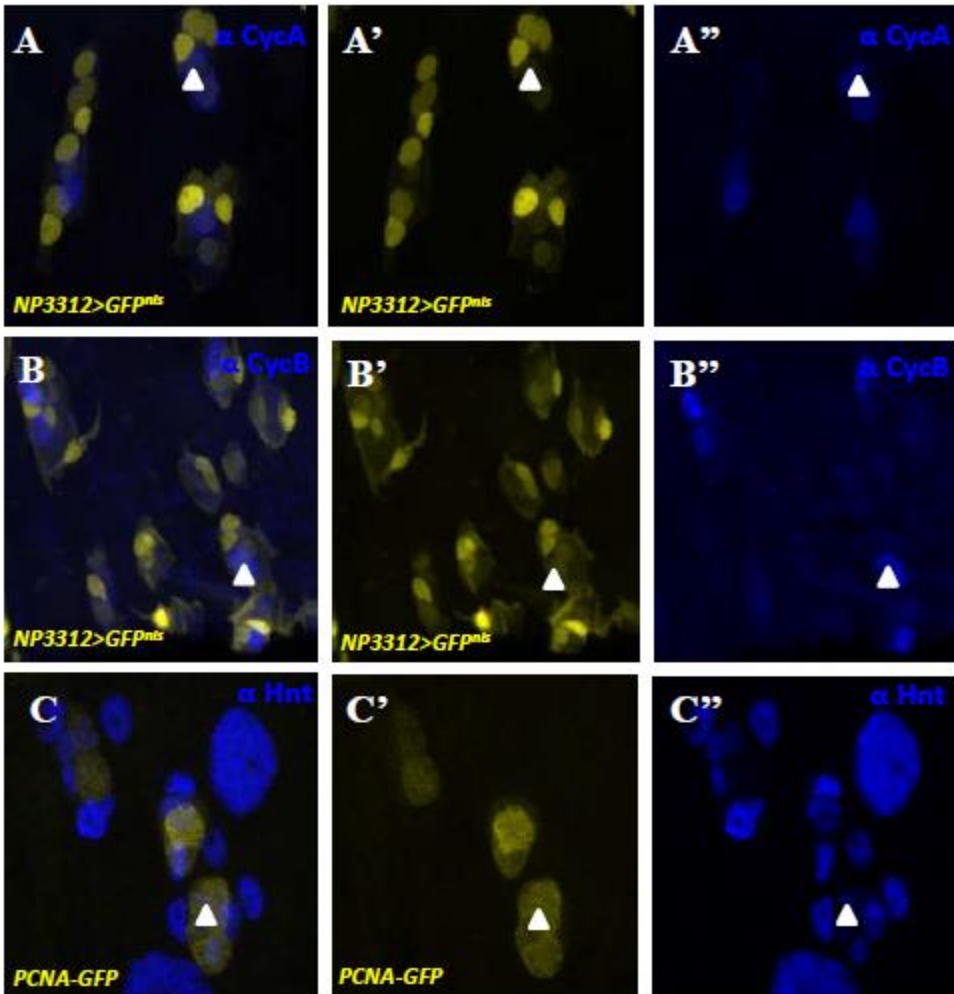

Figure 3.7 Mitotically active AMPs in the larval midgut are associated with lower levels of *hnt* expression. Larval midguts of hnt^{NP3312}>GFP^{nls}, which reports *hnt* transcription, immunostained with the cell cycle markers anti-Cyclin A (A-A'') or anti-Cyclin B (B-B'') display reduced *hnt* expression in the mitotically active Cyclin A or Cyclin B positive cells (arrowheads). Larval midguts expressing the S phase marker *PCNA-GFP* (yellow) and immunostained with anti-Hnt (blue) also show an inverse correlation between PCNA-GFP and Hnt expression (C-C'', arrowheads).

Although the results shown in Fig. 3.7 suggest that there is an inverse relationship between Hnt expression and mitotic activity, these observations do not establish that Hnt is involved in the control of AMP proliferation in the larval midgut. In order to address whether Hnt expression is sufficient to halt AMP mitotic divisions, an activated *Ras* (*UAS-Ras85D^{V12}*) was expressed in AMPs using the *esgGAL4* driver. These experiments also required the use of the *tubGAL80^{ts}* insert and temperature shifting (see materials and methods). Expression of activated *Ras* for several days resulted in extreme AMP overproliferation to the extent that it was impossible to identify individual AMP clusters (Figs. 3.8A, C, E). This confirmed the previously published phenotype which includes occlusion of the larval midgut by the massive AMP overproliferation (Jiang and Edgar, 2009). In order to determine if overexpression of Hnt can prevent AMP divisions, *hnt^{EP55}* was co-expressed with *UAS-Ras85D^{V12}* using the *esgGAL4* driver (see materials and methods). AMP overproliferation through the expression of activated Ras was suppressed by co-expression of Hnt both at 3 and 4 days following GAL4 activation by temperature shift (Fig. 3.8B, D). After 4 days of co-expression, GFP-positive cells with larger nuclei where readily observed, suggesting that these cells were differentiated ECs and had undergone the M/E transition (arrow in Fig. 3.8D). This striking suppression of AMP overproliferation supports the interpretation that Hnt is sufficient to suppress the effect of activated Ras. Interestingly, Hnt immunostaining of the activated Ras over-proliferating midgut showed that dividing AMPs also express Hnt (Fig. 3.8E, E'). This observation suggests that while Hnt overexpression can suppress overproliferation induced by activated Ras, Hnt expressed at normal levels does not prevent overproliferation. Overall, these observations suggest that the levels of Hnt expression are relevant to the AMP cell fate.

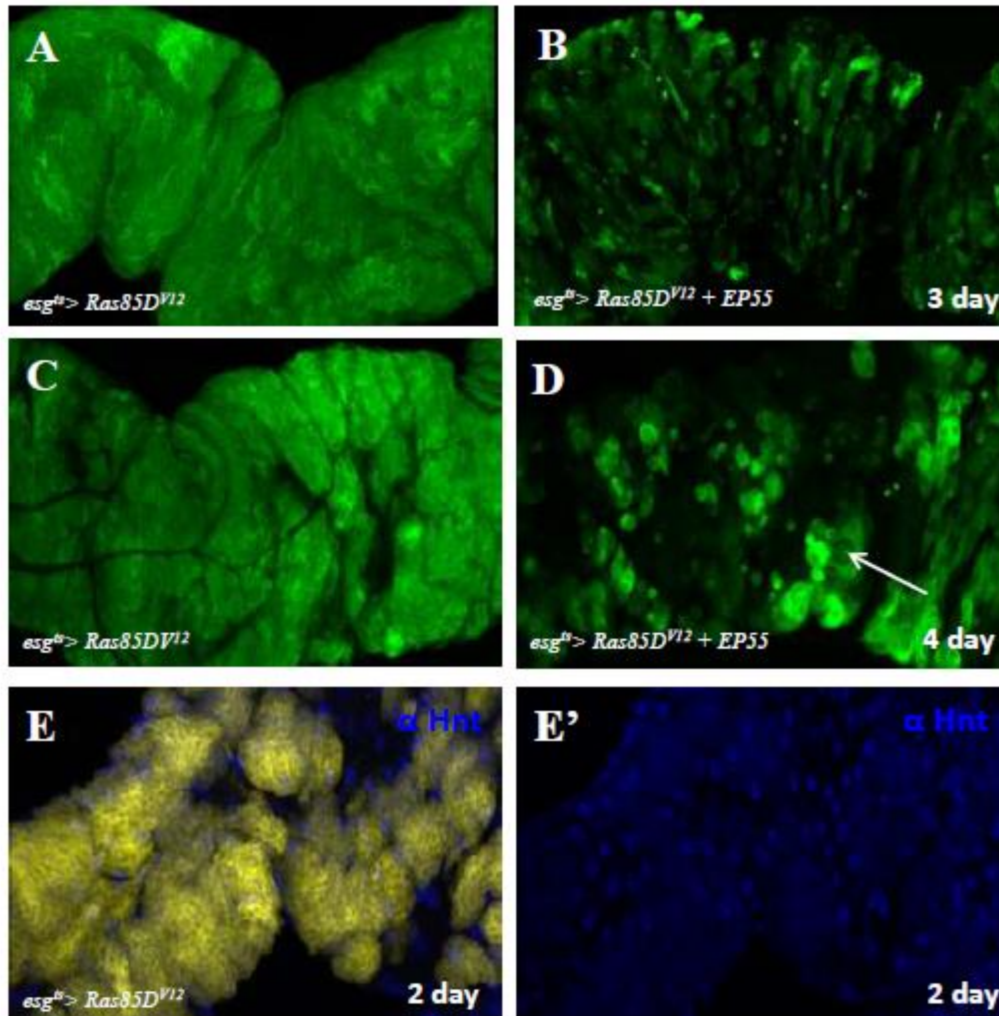

Figure 3.8 Overexpression of Hnt in the larval midgut suppresses Ras-induced AMP overproliferation. Live images of larval midguts expressing GFP and activated Ras (Ras85D^{V12}) under the control of *esgGAL4* in combination with *tubGal80*^{ts} three days (A) and four days (C) following the shift to 29 °C to activate GAL4. The expression of activated Ras results in massive AMP proliferation (compare to Figure 3.1A) that may occlude the larval midgut. Co-expression of Hnt using the *EP55* insert under the same conditions (B and D) suppresses the massive AMP over-proliferation. At four days, the midguts of larvae co-expressing *Ras85D*^{V12} and *EP55* display large GFP-expressing cells not observed in the control midguts (arrows; D), suggesting that Hnt expression can promote enterocyte differentiation in the context of Ras-induced AMP over-proliferation. Two day control larval midguts immunostained using anti-Hnt show that Hnt is expressed in the over-proliferating and mitotically active AMPs. The level of Hnt expression in the over-proliferating AMPs; however, is lower than what is observed in the differentiated larval enterocytes.

3.7 Hnt expression is required for ISC maintenance in the *Drosophila* adult midgut and is EGFR dependent

Analysis of the larval midgut, although interesting, is limited by the onset of pupation, during which the larval midgut degenerates and is replaced by the adult midgut. The adult midgut is a more attractive system for stem cell research in that ISC maintenance and proliferation can be monitored throughout the lifespan of the fly. In order to determine if Hnt is required in the adult ISCs, similar to its role in the maintenance of the larval AMPs, RNAi-mediated knockdown of Hnt in the ISC/EB was performed using the *esgGAL4* driver in combination with $tubGAL80^{ts}$. Midguts were immunostained using anti-Hnt and imaged to evaluate the GFP-positive adult ISC/EB population at various time-points following temperature shift from 18° C to 29° C. This revealed a progressive decline in the number of ISCs throughout the midgut following RNAi-mediated knockdown of Hnt expression (data not shown). The comparison of control midguts (Fig. 3.9A) with Hnt knockdown midguts (Fig. 3.9B) seven to fourteen days post-shift showed a dramatic decrease in the number of ISCs throughout the midgut. Remaining GFP-positive cells were often round, solitary cells, rather than the normal ISC/EB doublets, suggesting a cessation in ISC division accompanying the loss of the ISC population. The loss of the ISC population by RNAi-mediated knockdown of Hnt is consistent with the previous failure to observe hnt^{XE81} mutant clonal patches, as such mutant ISCs were likely also lost.

It was previously shown that EGFR signaling is required to promote AMP and ISC divisions, and that a loss of EGFR signaling in the adult midgut leads to a progressive loss of the ISC population through attrition (Xu et al., 2011). In contrast, RNAi-mediated reduction of Notch in the adult ISCs results in an increase in the number of ISCs (Micchelli and Perrimon, 2005). Both of these results were repeated in this study and the corresponding phenotypes were confirmed and compared to control and Hnt knockdown midguts (Figs. 3.9A-D). Overall, the

loss of Hnt and loss of EGFR were found to display remarkably similar ISC loss phenotypes, which were dramatically different from the overproliferation observed in the Notch knockdown. The ISCs in each of these backgrounds (control, Hnt, EGFR, and Notch knockdown) were imaged at higher magnification to assess Hnt expression. Interestingly, disrupting Notch signaling in the adult ISCs (Figs. 3.9E-E'') did not prevent Hnt expression, which suggests that Hnt expression is independent of Notch signaling in this context. Moreover, ISC Hnt expression in the EGFR knockdown was dramatically reduced (Fig. 3.9F-F''). EGFR knockdown midguts were examined at several time points following shift to 29°, which demonstrated that the reduction in Hnt expression in the ISCs was gradual and by fourteen days virtually no Hnt expression was detectable (data not shown). EGFR is normally expressed in the ISCs and activation by secreted ligands leads to ISC divisions (Xu et al., 2011). This gradual loss of EGFR expression via RNAi likely reflects a gradual reduction in the overall amount of receptor present on the surface of these cells. Subsequently the reduction in EGFR signaling leads to reduced ISC division as well as reduced Hnt expression. Thus, in this context Hnt expression has been established as being downstream of EGFR signaling and independent of Notch signaling.

This result was surprising, given that previously published work on the follicle cells and adult muscle progenitors had shown that Hnt expression was responsive to activation of Notch signaling (Sun and Deng, 2007)(Krejcí et al., 2009). To further confirm the Notch independent nature of Hnt expression in the midgut, anti-Hnt immunostaining of larval AMPs with disrupted Notch signaling was also performed. Consistent with the above results for the adult ISCs, Hnt expression in this context was also maintained and was not visibly reduced (See Appendix B Fig. B1.1). Overall, these results support the interpretation that Hnt expression in AMPs/ISCs is not

mediated through Notch signaling, but relies on a different pathway, mostly likely the EGFR pathway.

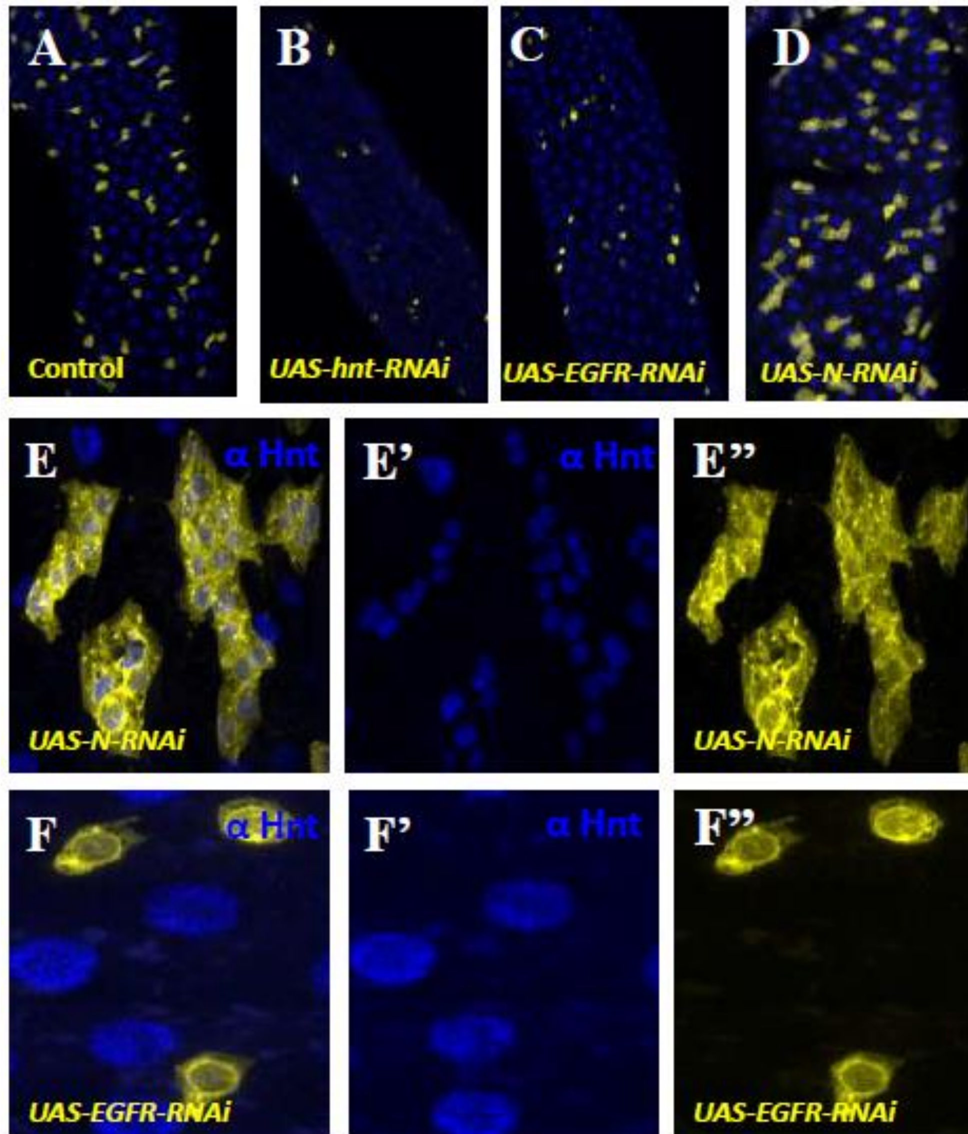

Figure 3.9 Hnt expression in the adult ISCs is EGFR-dependent and Notch-independent. Anti-Hnt (blue) immunostained adult midgut expressing GFP (yellow) under the control of *esgGAL4* and *tubGAL80*^{ts} (A), which marks the ISC population scattered throughout the midgut. 1-2 weeks following GAL4 activation by shifting to 29 °C, co-expression of *UAS-hnt-RNAi* (B) or *UAS-EGFR-RNAi* (C) results in a loss of the ISC population, while co-expression of *UAS-N-RNAi* leads to ISC over-proliferation (D). The over-proliferating ISC population associated with *N-RNAi* expression continues to express Hnt (E-E'') whereas Hnt expression is absent in the *EGFR-RNAi*-expressing ISC population (F-F'').

3.8 Notch-signaling dependent enteroblasts (EBs) show increased Hnt expression

Although Notch signaling is not required for Hnt expression in the ISC population, our previous work using the larval midgut suggested that Hnt is involved in mediating Notch-dependent PC differentiation. Therefore, we sought to determine if Hnt might also be involved in promoting Notch-dependent EB formation and subsequent EC differentiation in the adult midgut. To clarify, the hypothesis would be that Hnt expression in the ISC is Notch-independent, but that following ISC division the daughter cell destined to become the EB is associated with Notch-dependent Hnt expression.

To test the above hypothesis, anti-Hnt immunostaining was carried out on wild type midguts in which the ISC/EB population was marked using the usual *esgGAL4 >GFP + tubGAL80*^{ts} method. Following ISC division, it is known that the Notch signaling pathway is more active in the EB daughter cell, and that higher Notch signaling in the EB promotes EC differentiation whereas lower levels of Notch signaling are associated with EE differentiation (Ohlstein and Spradling, 2007). It was therefore of interest to determine if Hnt expression shows any increase in the EB daughter cell relative to the ISC. In this experiment it was also possible to distinguish the ISC cell from the EB cell by virtue of GFP expression (driven by esgGAL4) which is always stronger in the ISC. Consistent with the above hypothesis, it appeared that Hnt expression was increased in the EB relative to the ISC (Fig. 3.10A-A'').

Previous experiments have shown that expression of activated Notch (*UAS-N*^{intra}) in ISCs causes the ISCs to differentiate into mature ECs in the adult midgut (Beebe et al., 2010). In our experiments we used expression of Su(H)^{VP16} to mimic Notch activation. Normally Su(H) functions as a repressor of Notch responsive genes, but addition of the activation domain VP16 converts this protein into a transcriptional activator of Notch responsive genes (Krejci et al., 2009). Expression of the *Su(H)*^{VP16} activator using *esgGAL4 >GFP + tubGal80*^{ts} was associated

with EC differentiation, as expected, and the newly differentiated ECs were found to have strong Hnt expression (Figs. 3.10B, B'). Also, some ISCs were lost following $Su(H)^{VP16}$ expression as newly differentiated GFP positive EC doublets were observed (arrow, Fig. 3.10B-B''). Overall, these observations suggest that Hnt expression is increased during the process of EC differentiation; given that EC differentiation is Notch-dependent, it remains possible that Hnt expression in this context is Notch-dependent. These experiments; however, do not conclusively establish any such relationship as Hnt expression could occur in parallel to Notch activation.

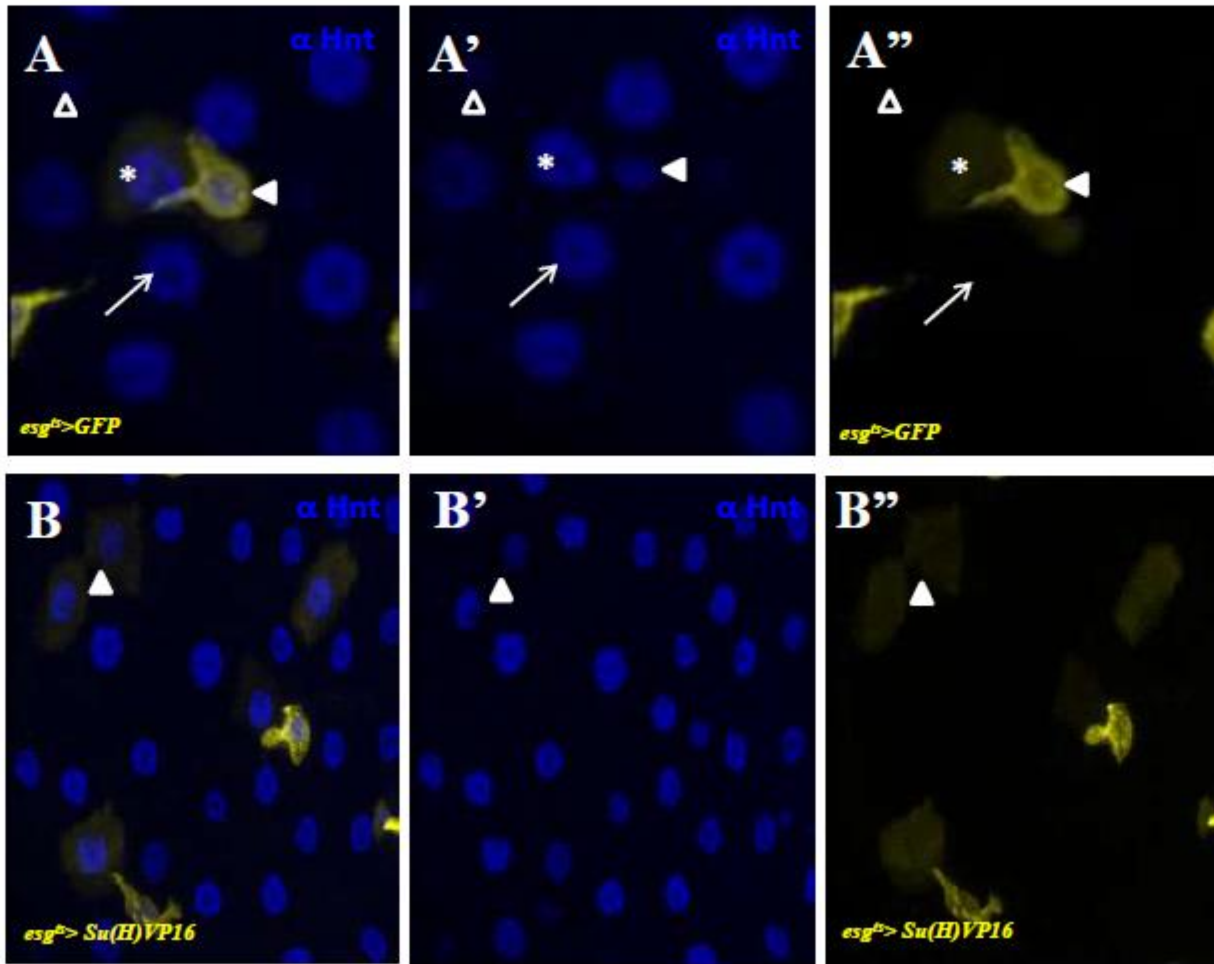

Figure 3.10 The activation of Notch target genes in the adult midgut results in a failure to maintain ISC stem cell divisions and promotes enterocyte differentiation. Anti-Hnt (blue) immunostained adult midgut expressing GFP (yellow) under the control of *esgGAL4* and *tubGAL80^{ts}* showing the expression of Hnt in all four cell types of the adult midgut (A-A'') 11 days following GAL4 activation by shifting to 29 °C. The ISC is associated with strong GFP expression and moderate Hnt expression (arrowhead), while the newly formed enteroblast is associated with a decrease in *esgGAL4>GFP* expression and increased Hnt expression (asterisks). The differentiated enterocytes (arrow) and enteroendocrine cells (open arrowhead) also lack GFP expression and are associated with strong and weak Hnt expression, respectively. In adult midguts co-expressing the Notch target gene activator *Su(H)-VP16*, twin cells displaying weak GFP expression and strong Hnt expression are observed (arrowheads in B-B''). This is in contrast to the one strong/one weak GFP expression pattern associated with ISC maintenance during normal ISC asymmetric cell divisions.

3.9 Overexpression of Hnt is sufficient to promote enterocyte (EC) differentiation in the adult midgut

The above results (section 3.8) suggested that Hnt expression is up-regulated in cells undergoing EC differentiation in the adult midgut. In addition, overexpression of Hnt in the larval midgut was found to promote premature EC differentiation (section 3.4), leading us to predict that overexpression of Hnt would yield a similar result in the adult midgut. Micchelli & Perrimon (2006) have shown that activated Notch causes ISCs to differentiate into EC-like cells. In order to determine if overexpression of Hnt has a similar effect, *esgGAL4 >GFP +GFP-HNT + tubGal80^{ts}* was used to overexpress Hnt in the ISCs, and midguts were analyzed at various time points following GAL4 activation by shift to 29°. At 14 hours post-shift a normal distribution of ISCs throughout the midgut was observed, but anti-Hnt immunostaining confirmed that the ISCs were already beginning to show higher levels of Hnt expression (Fig. 3.11A-A''). The Hnt expression in the GFP-positive cells in this experiment was distinctly increased relative to the neighbouring ECs, a situation that is never observed in wild type midguts (arrowheads in Fig. 3.11A-A''). At 4 days post-shift ISCs with nuclear sizes similar to those of neighbouring ECs, and also showing a significant decrease in GFP expression, were observed (Fig. 3.11B-B''). At this time point the expected small strongly GFP-positive ISCs were not observed, and all GFP-positive cells resembled newly differentiated ECs, suggesting that ISCs overexpressing Hnt were differentiating to form ECs. At 14 days post-shift midguts contained no visible GFP-positive ISCs, and the adult midgut epithelium appeared to be composed entirely of large polyploid differentiated ECs (Fig. 3.11C-C''). Anti-Pdm1 immunostaining (a marker for mature ECs) of the midguts at 4 days post-shift confirmed that ISCs over-expressing Hnt are Pdm1 positive, leading us to conclude that Hnt expression promotes EC differentiation (Fig. 3.11D-D''). It is interesting to note that the effect of Hnt overexpression on promoting EC differentiation was

much stronger than what was reported by Micchelli & Perrimon (2006) using expression of activated Notch. Micchelli & Perrimon expressed activated Notch for a longer period of time (7 to 10 days), and although they reported that ISCs appeared to undergo EC differentiation, this differentiation was not complete as *esgGAL4* > *GFP* expression persisted in the ISC population in their experiments. The differences between the extent of EC differentiation associated with Hnt overexpression verses activated Notch could be attributed to the different levels of UAS-reporter expression. It is also possible, however, that robust expression of Hnt in the ISC leads to complete EC differentiation, and that that lower levels of Hnt are permissive to ISC maintenance. Overall, these observations suggest that the levels of Hnt must be tightly regulated in order to prevent the undifferentiated ISC population from being completely lost through EC differentiation.

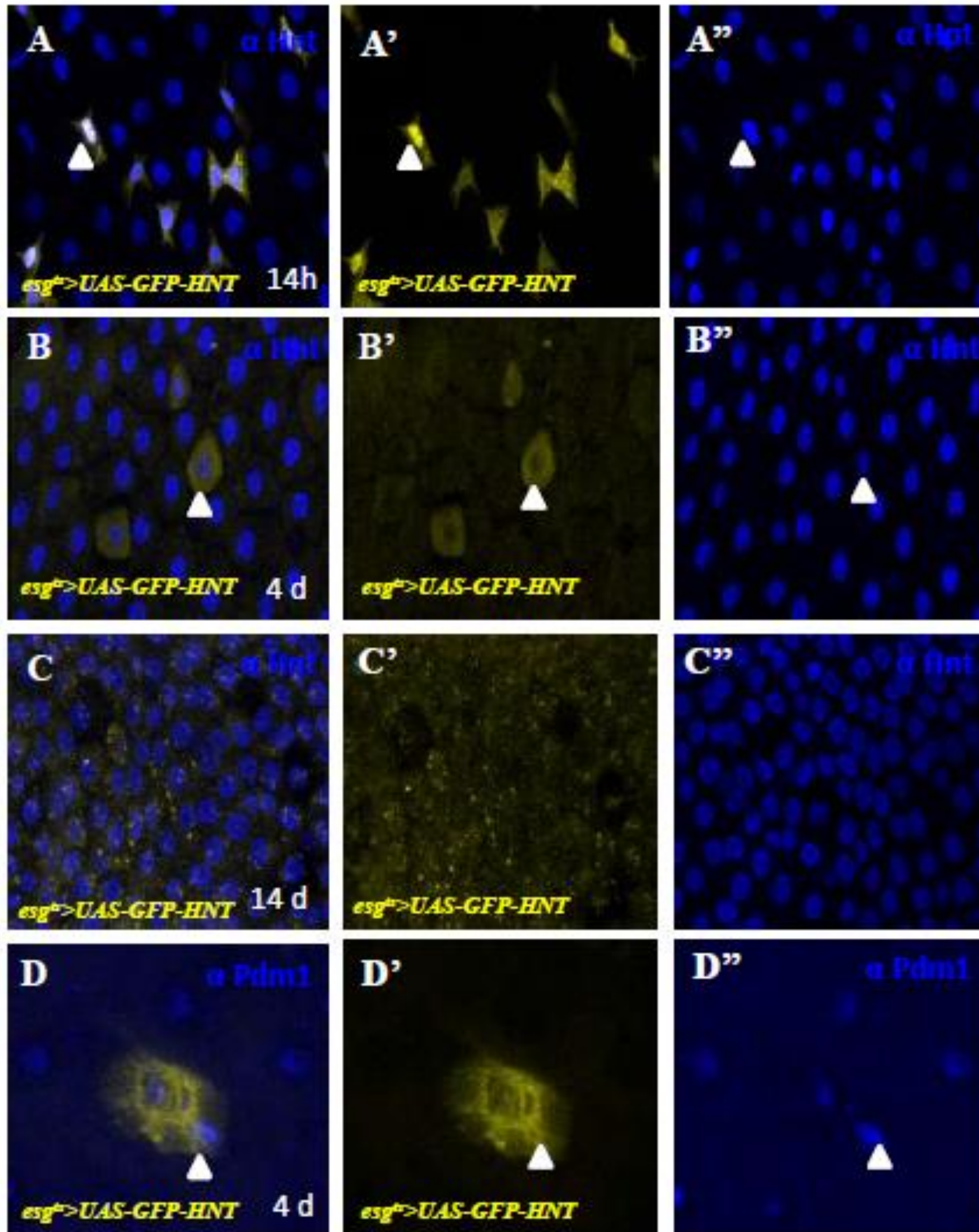

Figure 3.11 Overexpression of Hnt in the adult midgut promotes enterocyte differentiation. Anti-Hnt (blue) immunostained adult midgut expressing GFP-HNT and GFP under the control of esgGAL4 and tubGal80ts 14 hours (A-A”), 4 days (B-B”), and 14 days (C-C”) following the activation of GAL4 by shifting to 29 °C. At 14 hours, ISCs show strong Hnt expression (arrowheads in A-A”). The loss of the *esgGAL4>GFP* expressing ISC population is associated with enterocyte differentiation (arrowheads in B-B”). At 14 days the ISC population is completely absent (C-C”). Immunostaining using the enterocyte marker anti-Pdm1 (blue) 4 days following shift to 29 °C confirms the differentiation of ISCs into ECs associated with increased Hnt expression (D-D”).

The above results suggest that both Hnt and Notch signaling can promote EC differentiation in the adult midgut. In addition to increasing the ISC population, a reduction in Notch signaling has also been reported to be associated with an increase in EE differentiation at the expense of EC differentiation (Takashima et al., 2011a). It seemed appropriate to consider if decreasing the levels of Hnt would have a similar effect. Therefore, in order to determine if the loss of ISCs in the adult midgut in response to decreased Hnt (discussed in section 3.7) is associated with increase EE differentiation, anti-Prospero immunostaining (an EE marker) was performed on Hnt knockdown midguts. Reducing the level of Hnt expression did not cause an increase in the number of Prospero-positive EEs throughout the midgut (Fig. 3.12). In addition, ISCs (strongly GFP-positive) and newly differentiated EBs (weakly GFP-positive) were never observed to be Prospero-positive. This suggests that loss of the ISC population following Hnt knockdown is not attributable to increased EE differentiation.

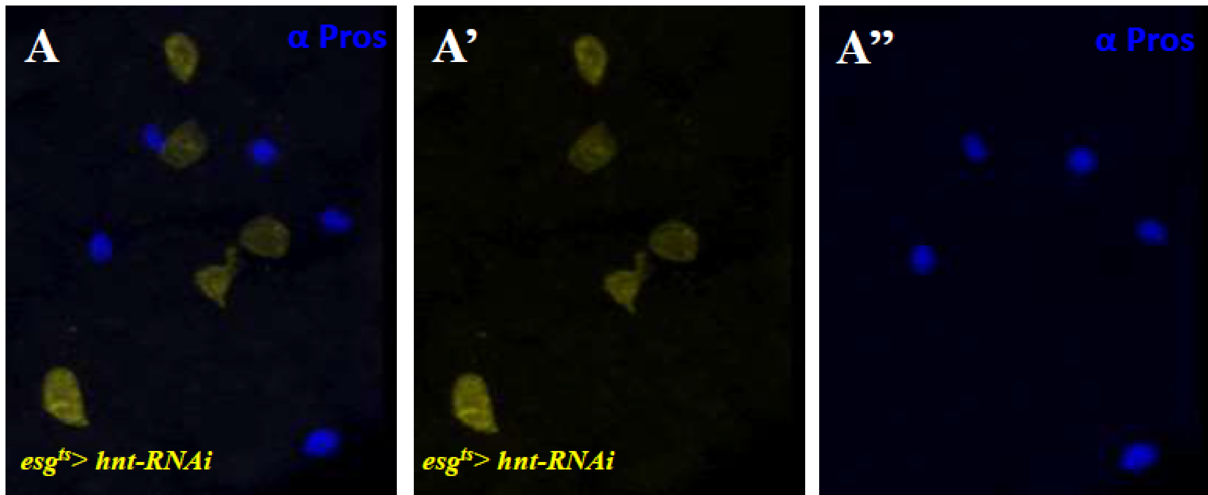

Figure 3.12 The RNAi-mediated reduction in Hnt expression is associated with a progressive loss of the ISC population without a concomitant increase in the number of enteroendocrine cells. Anti-Prospero (Pros) immunostained (blue) adult midgut expressing GFP (yellow) and *hnt-RNAi* under the control of *esgGAL4 and tubGal80^{ts}* 7 days following GAL4 activation by shift to 29 °C (A-A'') shows that ISCs in this context do not express Prospero and are not differentiating as enteroendocrine cells.

3.10 Overexpression of Hnt is sufficient to promote EC differentiation in the absence of Notch signaling

It has previously been shown, and confirmed in this study, that Notch signaling is required to limit ISC proliferation in the adult midgut. RNAi-mediated knockdown of Notch leads to the formation of tumours composed of ISCs and EEs at the expense of differentiated ECs(Beebe et al., 2010). Given that Hnt overexpression proved to be sufficient to cause all ISCs to differentiate into ECs, the next step was to determine if Hnt could promote EC differentiation in the absence of Notch signaling. Expression of *UAS-N-RNAi* using *esgGAL4 > GFP + tubGal80^{ts}* yielded profound ISC tumours, as expected (Fig. 3.13A, C, E). Interestingly, this overproliferation phenotype was dramatically suppressed by Hnt co-expression as all ISCs were observed to differentiate into ECs (Fig. 3.13B, D, F). This striking result suggests that Hnt overexpression is epistatic to the loss of Notch signaling. If operating in the same regulatory pathway, Hnt would, therefore, function downstream of Notch signaling. Regardless of the pathway relationship, this result confirms that Hnt can promote EC differentiation in the absence of Notch signaling.

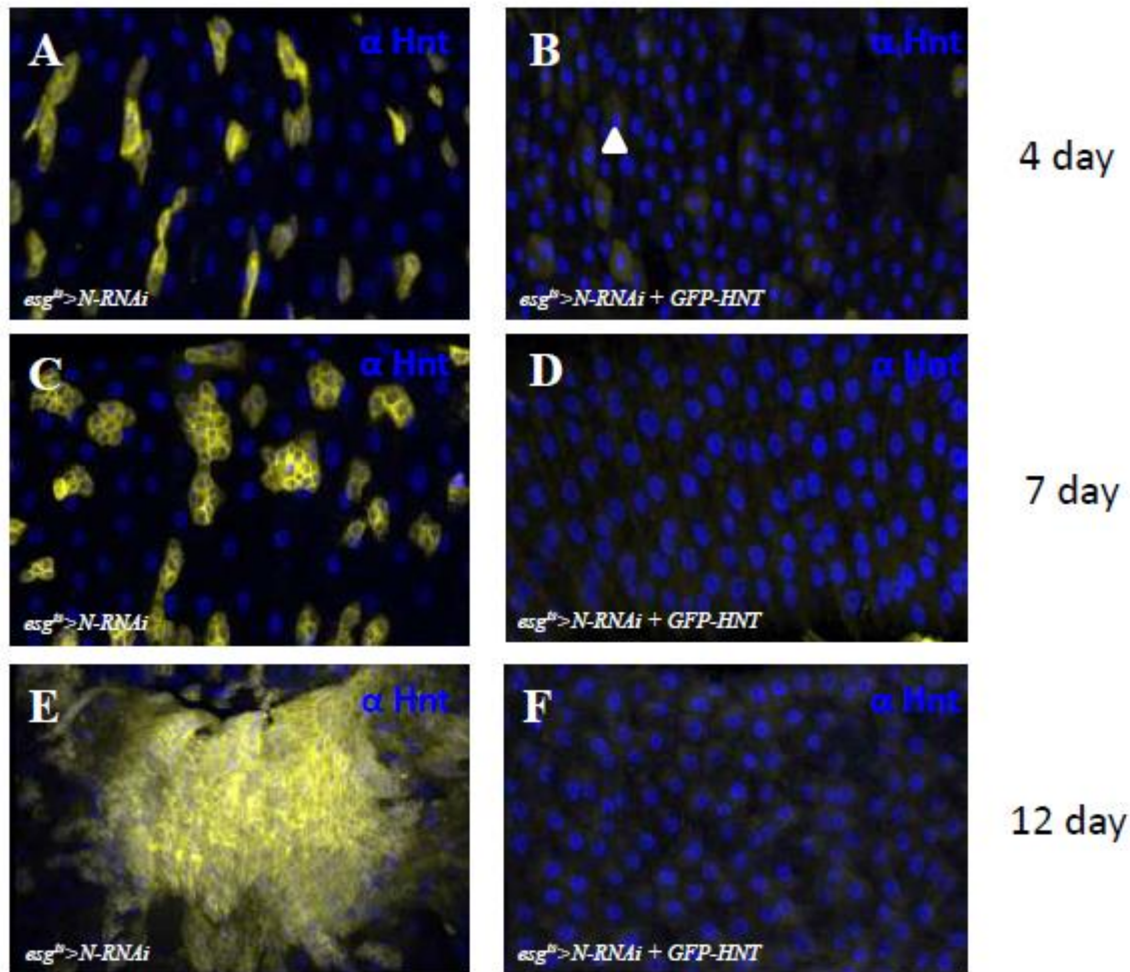

Figure 3.13 Overexpression of Hnt is sufficient to promote enterocyte differentiation in the absence of Notch. Anti-Hnt (blue) immunostained adult midgut expressing *GFP* (yellow) and *N-RNAi* under the control of *esgGAL4* and *tubGal80ts* at 4 days (A), 7 days (C), and 12 days following GAL4 activation by shift to 29 °C. Note the progressive inappropriate ISC proliferation. The same conditions with co-expression of *GFP-HNT* abrogate ISC proliferation (B, D, F). Note the weak GFP expression at day 4 and the apparent differentiation of ISCs into enterocytes (arrowheads in B).

Earlier experiments showed that Hnt is necessary for maintaining the ISC population (discussed in section 3.4), which prompted further investigation as to whether ISC tumours resulting from inactivation of Notch signaling are formed in the absence of Hnt. RNAi-mediated knockdown of Notch resulted in ISC tumours, as expected (Figs. 3.14A, C). Co-knockdown of Notch and Hnt resulted in a decrease in the size of ISC tumours (Figs. 3.14B, D). While the overall suppression of ISC tumours was dramatic, some remaining GFP-positive ISC tumours showed residual Hnt expression (data not shown), presumably due to incomplete knockdown of Hnt. These results indicate that Hnt is not only involved in the differentiation of ISCs, but that it is also required for the maintenance and division of ISCs in the tumour-like condition associated with the absence of Notch signaling.

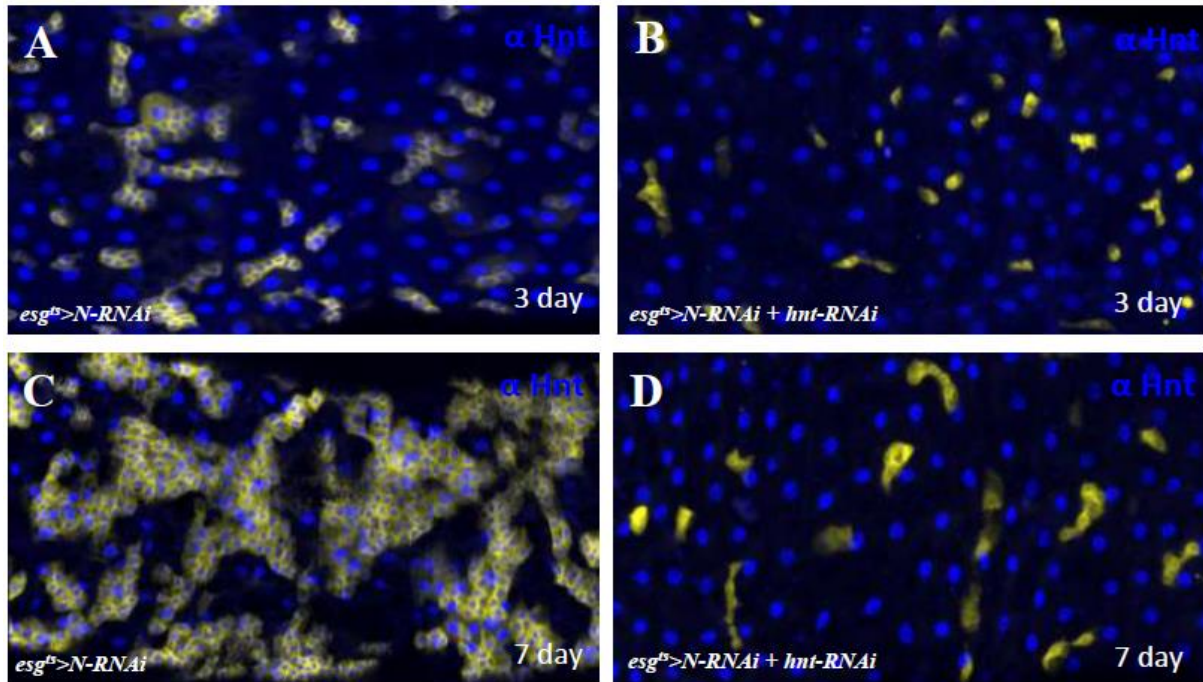

Figure 3.14 RNAi-mediated reduction in Hnt expression suppresses *N-RNAi* induced ISC over-proliferation. Anti-Hnt (blue) immunostained adult midgut expressing *GFP* (yellow) and *N-RNAi* under the control of *esgGAL4* and *tubGal80*^{ts} at 3 days (A) and 7 days (C) following GAL4 activation by shift to 29 °C. The same conditions with co-expression of *hnt-RNAi* (B and D) results in a suppression of the ISC over-proliferation phenotype associated with the reduction of Notch signaling.

3.11 Overexpression of Hnt suppresses ISC overproliferation induced by activated EGFR signaling

EGFR signaling is required to promote ISC proliferation in the adult midgut, but it does not play a prominent role in the regulation of differentiation following ISC division (Jiang and Edgar, 2011). Expressing an activated form of EGFR (*λtop4.2)* results in overproliferation of ISCs, but does not prohibit EB formation and subsequent EC/EE differentiation (Xu et al., 2011). Based on prior observations that Hnt overexpression can suppress ISC overproliferation, *esgGAL4 > GFP + tubGal80*^{ts} was used to co-express *GFP-HNT* and activated EGFR (*λtop4.2*). At 1 day post-shift *λtop4.2* caused mild ISC overproliferation, and this became extreme by 3 days post-shift (Fig. 3.15A, C). The 3 day post-shift midguts showed numerous GFP-positive cells having variable nuclear sizes, indicative of a heterogeneous GFP-positive population, likely including all adult midgut cell types (ISCs, EBs, ECs, EEs). The GFP-positive cells with large nuclei showed strong Hnt immunostaining similar to what is observed in EBs and differentiated ECs (Fig. 3.15A', C'). Co-expression of *GFP-HNT* and *λtop4.2* prevented the accumulation of GFP-positive cells and resulted in all ISCs differentiating to produce EC-like cells (Figs. 3.15B, B', D, D'). Similar to the observations of reduced Notch signaling, this result suggests that Hnt overexpression possibly acts downstream of EGFR signaling and that increasing Hnt can effectively promote EC differentiation.

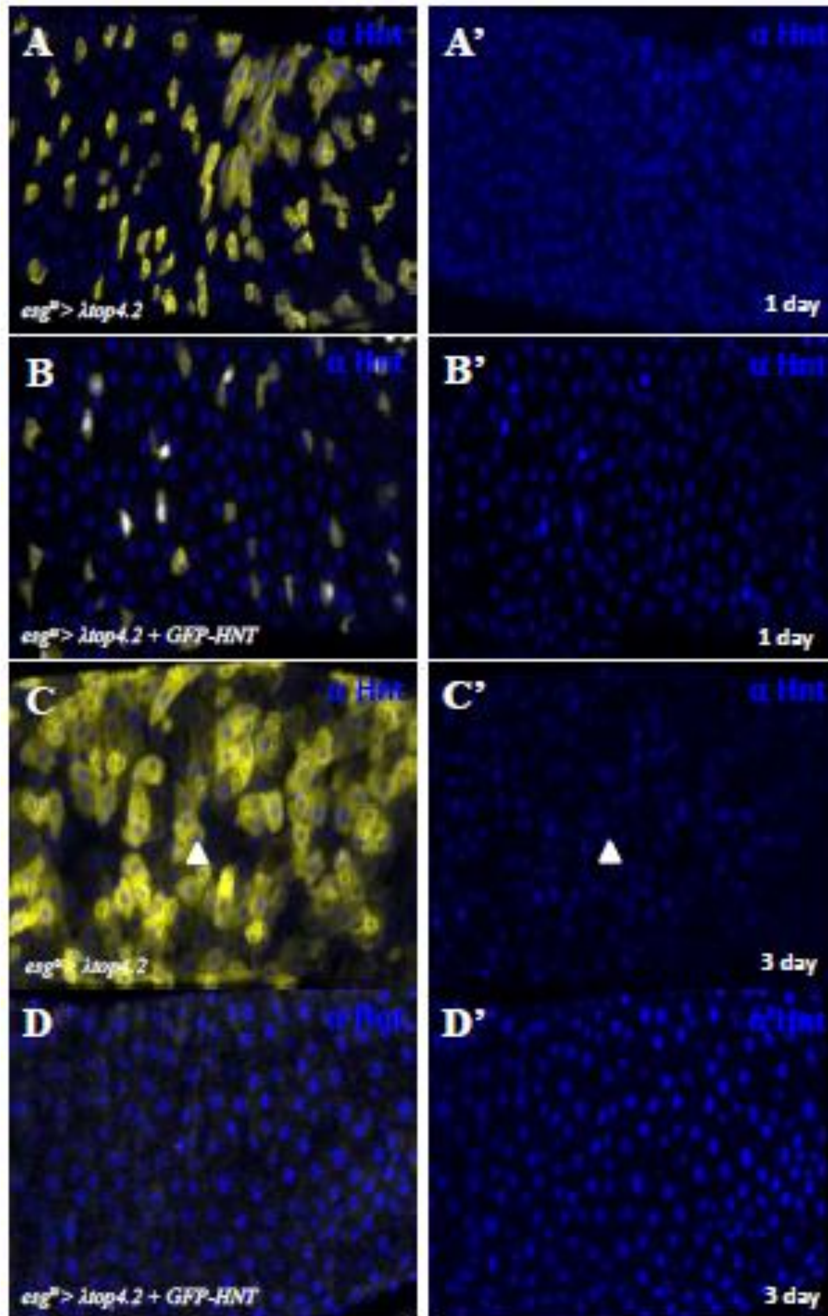

Figure 3.15 Hnt over-expression suppresses ISC proliferation associated with the activation of EGFR signaling. Anti-Hnt (blue) immunostained adult midgut expressing *GFP* and activated EGFR (*λtop4.2)* under the control of *esgGAL4* and *tubGal80^{ts}* at 1 day (A, A') and 3 days (C, C') following GAL4 activation by shift to 29 °C. As is readily apparent at day 3 (C), the activation of EGFR signaling results in over-proliferation of ISCs, but ISC progeny are still capable of differentiating, as evidenced by the frequent observation of large nuclei among the tumourous ISC population (arrowheads in C). The same conditions with co-expression of *GFP-HNT* (B and D) results in a complete suppression of the ISC over-proliferation phenotype associated with activation of EGFR signaling. The absence of GFP expression and preponderance of large, Hnt positive nuclei at day 3 (D and D') suggests that Hnt expression promotes enterocyte differentiation in the context of activated EGFR signaling.

To address the requirement for Hnt in promoting EC differentiation in the context of activated EGFR signaling, *hnt-RNAi* was co-expressed with *λtop4.2* using the *esgGAL4 > GFP + tubGal80*^{ts} system. As was previously shown in Fig. 3.15, expression of *λtop4.2* results in numerous GFP-positive cells having variable nuclear sizes (Fig. 3.16A). In contrast to the Hnt knockdown phenotype shown in Fig. 3.9B, Hnt knockdown with co-expression of *λtop4.2* did not eliminate the ISC population, but resulted in numerous GFP-positive clusters composed of uniformly smaller cells (Fig 3.16C). Anti-Hnt immunostaining confirmed that the large GFP-positive cells in the *λtop4.2*-only background show strong Hnt expression, whereas the smaller GFP-positive cells in the *hnt-RNAi* expression background lacked Hnt expression (Fig. 3.16B, B', D, D'). Thus, in the absence of Hnt, activation of EGFR signaling promotes ISC proliferation, but based on the size of the GFP positive cells, EC differentiation is inhibited. The maintenance of the ISC population in the co-expression background is intriguing, but could be associated with maintenance of a low level of Hnt expression in the ISCs. The finding that EGFR signaling is required for the Hnt expression in the ISCs (discussed in section 3.7) raises the possibility that Hnt expression in the ISC is responsive to EGFR signaling. In combination with the *hnt-RNAi*, this might have the effect of maintaining a low level of Hnt that is sufficient to maintain the ISC population but insufficient to promote EC differentiation. Future experiments will address this question as well as the question of possible EE differentiation in this background.

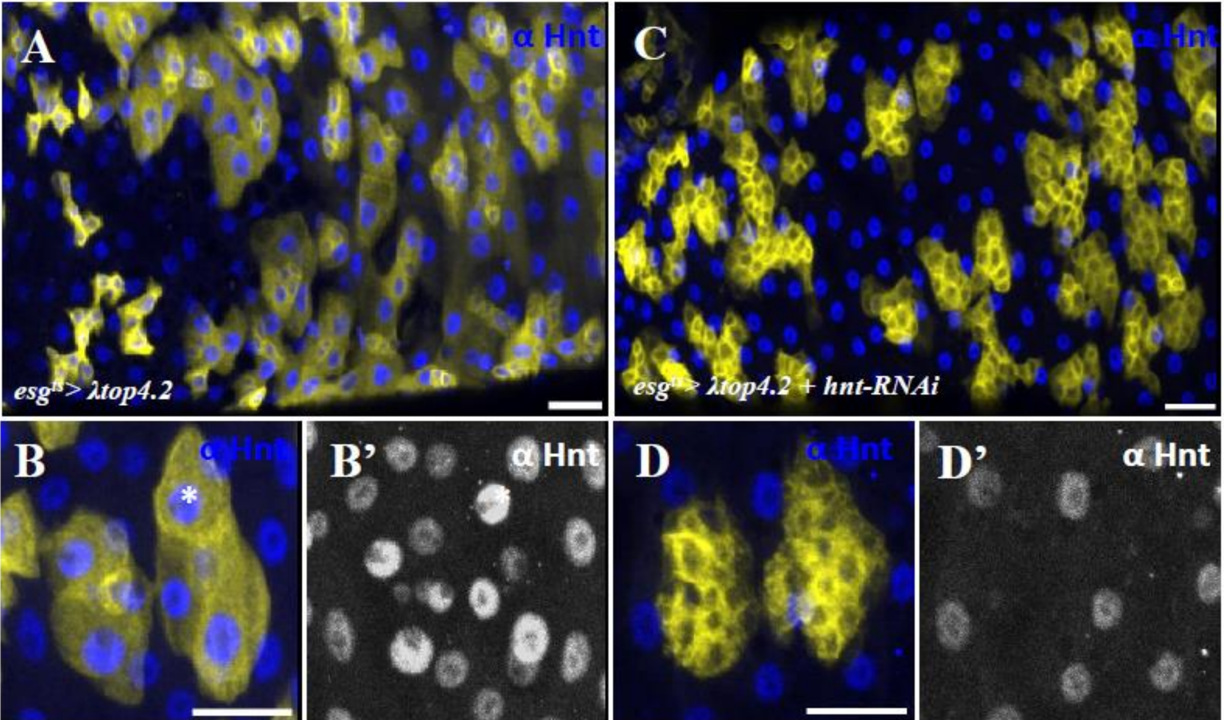

Figure 3.16 RNAi-mediated reduction in Hnt expression precludes enterocyte differentiation. Anti-Hnt (blue) immunostained adult midgut expressing *GFP* (yellow) and activated EGFR (*λtop4.2*) under the control of *esgGAL4* and *tubGal80^{ts}* at 3 days following GAL4 activation by shift to 29 °C (A and B). The GFP-positive zones of over-proliferation include large, Hnt-positive nuclei (asterisks) in (B and B'). The same conditions with co-expression of *hnt-RNAi* (C and D) result in GFP-positive zones of over-proliferation that are devoid of large nuclei, consistent with a failure in EC differentiation within the zones of over-proliferation associated with EGFR activation. Scale bars: 20 μm.

Chapter 4. Discussion

4.1 Hnt is highly expressed in PCs of the larval midgut and is required for AMP cluster organization

Anti-Hnt immunostaining and *hnt-GAL4* expression patterns revealed that Hnt was strongly expressed in the differentiated ECs and PCs of the larval midgut, and expressed at lower levels in the undifferentiated AMPs (Fig. 3.1A-C). This result was intriguing because it had previously been shown that Notch signaling is required for PC differentiation, and that AMPs express the Notch ligand Delta. In addition, *Notch* mutant clones in the larval midgut lack PC differentiation and AMP clusters merge prematurely, resulting in larger clusters of AMPs (Mathur et al., 2010). In this study a similar phenotype was observed using *N-RNAi* or *mamN* to disrupt Notch signaling (Fig. 3.6B & C). RNAi-mediated knockdown of Hnt produced a similar phenotype to the loss of Notch signaling with respect to the lack of PC differentiation, but differed in that a reduction in the overall number of clusters was evident (Fig. 3.6D). Similarly, hnt^{308} and hnt^{XE81} clonal mutant patches appeared as dispersed AMPs that also did not have distinguishable PCs (Fig. 3.3D & E). These results suggest that in the absence of Hnt, PC differentiation is disrupted, resulting in the premature fusion of AMP clusters, similar to what is observed with the loss of *Notch* (Mathur et al., 2010).

The phenotype of the *hnt* mutant or knockdown AMP clusters could be associated with the failure of PC differentiation. One of the proposed functions of PCs is the prevention of AMP cluster dispersal and fusion (Mathur et al., 2010). While the absence of PCs can explain the AMP dispersal that was observed in the absence of Hnt, this does not account for the overall reduction in AMP clusters (seen in the Hnt knockdown). In the absence or reduction of Notch signaling PC differentiation does not occur, but AMP proliferation is unaffected. Although PC formation is necessary for maintaining the AMPs in an undifferentiated state throughout the

larval stages, AMPs are able to proliferate in the absence of PCs (Mathur et al., 2010). AMP proliferation has been shown to be dependent on EGFR signaling (Jiang and Edgar, 2009).

Thus, a model that could explain the Hnt mutant phenotype is that EGFR signaling maintains a low level of Hnt expression in the undifferentiated AMPs, whereas Notch signaling results in the up-regulation of Hnt expression in the PCs. Consequently, it could be proposed that the low level of Hnt expression in the AMPs is required for their maintenance, whereas a high level of Notch-dependent Hnt expression is required for PC differentiation. Although, the relationship between EGFR signaling and Hnt expression in the AMP clusters has not yet been examined, we speculate that this relationship might exist based on the requirement for EGFR signaling to maintain Hnt expression in the adult ISCs. Consistent with the above model, Hnt expression in the AMPs is unaffected in the context of reduced Notch signaling (see Appendix Fig. B1.1). Therefore, although activated Notch signaling may be required to promote Hnt expression for PC formation, it is possible that in addition to its role in PCs, Hnt may play a role in the AMPs in a Notch-independent manner.

4.2 Hnt expression and cell cycle regulation

Previous studies suggest that Hnt promotes mitosis-to-endocycle (M/E) transitions in follicle cells (Sun and Deng, 2007) and tracheoblasts (Pitsouli and Perrimon, 2010), and that Hnt is not expressed in mitotically active cells (Sun and Deng, 2007). AMPs actively proliferate during larval stages and undergo a M/E cell cycle transition during EC differentiation in the pupal stage (Jiang and Edgar, 2009). This prompted us to look at whether Hnt is expressed in mitotically active AMPs and if Hnt expression promotes the M/E transition during EC differentiation. Immunostaining for Cyclin A and Cyclin B revealed that Hnt expression, as monitored using the *hntGAL4* enhancer trap line (*hnt*^{NP3312}), was found be lower in mitotically active (Cyclin-

positive) AMPs (Fig. 3.7A & B). This result, however, reflects only a correlation and does not establish any causal relationship between Hnt expression and AMP proliferation.

In an attempt to determine if expression of Hnt was sufficient to prevent the mitotic division of AMPs, Hnt was overexpressed using hnt^{EP55} in combination with $UAS\text{-}Ras85DV^{12}$, the latter of which causes extreme AMP overproliferation. Hnt co-expression suppressed AMP overproliferation induced by activated Ras (Fig. 3.8), and some cells had larger nuclei (Fig. 3.8D), suggesting that Hnt expression could, in this context, promote the M/E transition. Alternatively, Hnt expression could promote cell differentiation, in which case the induction of an endocycle would be a secondary indirect effect.

Anti-Hnt immunostaining of midguts expressing activated Ras (Fig. 3.8E) showed that Hnt is expressed in the population of overproliferating AMPs; in addition, Hnt is expressed, albeit at a low level, in wild type AMPs. Overall, in the context of the larval midgut, a low level of Hnt expression is, therefore, not sufficient for exit from the mitotic cell cycle. This does not, however, preclude the possibility that a high level of Hnt expression could promote exit from the mitotic cell cycle and entry into the endocycle.

4.3 Hnt is required for ISC maintenance in the adult midgut

The results of this study show that Hnt is required for maintenance of undifferentiated AMP clusters in the larval midgut, and that it is also necessary for maintenance of the undifferentiated ISCs of the adult midgut. Knockdown of Hnt in the adult midgut led to a progressive decline in the number of ISCs throughout the adult midgut (Fig. 3.9B), and those that remained were often single cells, suggesting that the ISCs were not dividing to produce EBs that would undergo subsequent cell differentiation. A similar phenotype has been reported for disruption of EGFR signaling in ISCs. Mutant ISC clones of *EGFR* or EGFR pathway signaling components are

gradually lost over time, not due to cell death, but likely because of a failure to divide to maintain the ISC population. EGFR mutant clones are either shed from the midgut or undergo differentiation during normal epithelial homeostasis (Xu et al., 2011). Based on the similar phenotypes of midguts expressing *hnt-RNAi* and those with defective EGFR signaling, we sought to determine if Hnt is involved in regulating EGFR signaling associated with ISC proliferation and maintenance. Using *EGFR-RNAi* we were able to generate a phenotype similar to that which was previously shown (Biteau et al., 2011). Anti-Hnt immunostaining of midguts with reduced EGFR in the ISCs revealed that Hnt expression was significantly reduced or absent. This striking result suggests that EGFR signaling is required for the maintenance of Hnt expression in ISCs (Fig. 3.9F). Therefore, Hnt expression in ISCs is dependent on EGFR signaling. Interestingly, the human homolog RREB-1 has been shown to be activated through the Ras/MAPK pathway (Kent et al., 2012). In addition, given the persistence of Hnt expression in the Notch knockdown midgut, Hnt expression in the ISCs is independent of Notch signaling (Fig. 3.9E).

Disruption of Notch signaling in ISCs leads to overproliferation of ISCs and ISC tumour formation. However, disruption of EGFR signaling can effectively suppress the formation of N-RNAi-induced ISC tumours, suggesting that EGFR signaling is necessary for ISC divisions (Biteau et al., 2011). Interestingly, *Notch-RNAi* induced ISC tumour formation was also suppressed by co-expression of *hnt-RNAi* (Fig. 3.14). Therefore, we speculate that EGFR-dependent expression of Hnt may be required for ISC proliferation, and that this requirement would likely relate to the competence of the ISC with respect to its ability to proliferate.

An important consideration with respect to the possible connection between Hnt expression and EGFR signaling and the question of ISC competence (i.e. the ability of a cell to

respond to a particular signal) is expression of the Notch ligand Delta. ISCs normally express Delta, which is required for activation of Notch signaling in EBs which, in turn, promotes their subsequent differentiation to form either ECs or EEs. Based on the observation that over-activation of EGFR signaling leads to an increase in the number of Delta positive ISCs, it has been suggested that EGFR signaling may directly and/or indirectly promote expression of *delta*, (Xu et al., 2011). Interestingly, studies on development of the pupal eye identified Hnt as a potential candidate for promoting *delta* expression (Pickup et al., 2009). Therefore, it seems reasonable to speculate that Hnt may be acting downstream of EGFR to promote *delta* expression in the ISC. Delta expression in the ISC is associated with maintenance of ISC identity and the activation of Notch signaling in the ISC daughter cells (EBs). Subsequently, it is possible to propose that in the ISC EGFR signaling promotes *hnt* expression, which in turn could promote expression or activation of Delta. According to this model, ISCs would be lost when either EGFR signaling or Hnt expression are reduced due to a failure to achieve sufficient levels of Delta expression or activation. The phenotype of reduced Delta in the ISC, however, resembles the loss of Notch and is associated with small cell tumour-like formation (Ohlstein and Spradling, 2007). The absence of Notch signaling in this context leads to symmetric, rather than asymmetric ISC division, and this phenotype is not observed in the Hnt knockdown. Thus, while Hnt could be involved in the up-regulation of Delta expression in the ISC, Hnt's role in responding to EGFR signaling, and possibly enhancing the response to EGFR activation, could lead to the failure in ISC proliferation. In this way, because EGFR signaling predominates in the regulation of ISC division, which is upstream of Notch-Delta signaling, the Hnt loss of function phenotype could primarily reflect the impairment of EGFR signaling in the ISC.

It is also worth noting that although hyperactivation of EGFR signaling using *λtop4.2* leads to ISC overproliferation, the ISCs are still able to differentiate and often appear as EC-like cells with larger nuclei (Fig. 3.16A & C). This makes sense if hyperactivation of EGFR signaling leads to an up-regulation of Delta expression, since high levels of Delta are known to promote EC differentiation (Ohlstein and Spradling, 2007). Co-expression of *GFP-HNT* and *λtop4.2* results in a phenotype similar to expression of *GFP-HNT* alone, where all cells differentiate to form ECs. This suggests that Hnt might act downstream of EGFR to promote EC differentiation (Fig. 3.15D). However, co-expressing *hnt-RNAi* with *λtop4.2* appeared to suppress cell differentiation without disrupting ISC proliferation, resulting in ISC overgrowths consisting entirely of small ISCs and no visibly larger EC-like cells (Fig. 3.16B & D). This could be explained through maintenance of a low level of Hnt expression, which in this context could be activated by *λtop4.2* and override the RNAi-mediated knockdown. Thus, a low level of Hnt expression may be sufficient to allow EGFR dependent ISC division, but insufficient to allow EC differentiation.

While the observed phenotype associated with Hnt knockdown resembled the loss of EGFR signaling, the loss of integrin mediated cell adhesion also presents a similar phenotype. Integrins are essential for ISC maintenance and studies using RNAi-mediated knockdown of Myospheroid have shown that there is a reduction in the number of ISCs throughout the midgut with reduced integrin expression (Lin et al., 2013). Hnt has also been implicated in the regulation of integrin mediated cell adhesion (Pickup et al., 2002). Additionally, N-RNAi-induced tumour formation is effectively suppressed in the absence of integrin signaling, suggesting that integrins are an absolute necessity in allowing for ISCs to proliferate and be maintained. Additionally, the reduction in the number of ISCs cannot be ameliorated by

overexpressing signaling components known to induce ISC proliferation, such as the EGFR signaling pathway component *Ras* (Lin et al., 2013). Given the similarity of the phenotype associated with the disruption of integrin signaling and the loss of Hnt, future experiments addressing a possible role for Hnt in regulating cell adhesion in the *Drosophila* midgut would be warranted.

4.4 Hnt may be involved in mediating Notch-signaling dependent cell differentiation

In the larval midgut PC differentiation has been shown to be dependent on Notch signaling, and in the absence of *Notch*, these cells are not generated. Notch signaling is not only required for PC differentiation, but expression of an activated form of Notch in AMPs is sufficient to cause all AMPs to differentiate into PC-like cells, resulting in the loss of the undifferentiated progenitor cell population (Mathur et al., 2010). Hnt is highly expressed in PCs (Fig. 3.1C), where Notch signaling is active, suggesting that Hnt expression in PCs could be Notch-dependent. In this scenario Hnt could be acting as a downstream target of Notch signaling to promote PC differentiation. Consistent with this possibility, anti-Hnt immunostaining of larvae expressing activated Notch revealed strong Hnt staining in all PC-like cells, comparable to the high levels of Hnt normally found in the differentiated ECs (Fig. 3.4B). Although this does not confirm that up-regulation of Hnt is Notch-dependent, it does show that Hnt is strongly expressed in these differentiated cells. In contrast to the all-PC phenotype associated with overexpression of activated Notch, overexpression of Hnt caused AMPs to differentiate into large EC-like cells having reduced esgGAL4 expression (Fig. 3.5D). The observed loss of AMP and subsequent EC differentiation does not rule out the possibility that Hnt functions in a Notch-dependent manner, given that EC differentiation is also largely dependent on Notch signaling (Ohlstein and Spradling, 2007). We speculate that the levels of Hnt expression are tightly

regulated, and that a high level of Hnt expression is required for EC differentiation rather than PC differentiation. Similar threshold types of effects relating to the levels of Hnt overexpression have also been observed in the developing embryo (Reed lab, unpublished results).

The TGFβ/BMP signaling pathway has been identified as a key pathway used by PCs in order to maintain the AMPs in an undifferentiated state. Dpp (a Drosophila homolog of TGFβ) is secreted by the PCs, and RNAi-mediated knockdown of this Dpp expression results in the premature breakdown of the PC niche and subsequent premature differentiation of AMPs into EC-like cells (Mathur et al., 2010), a phenotype strikingly similar to the Hnt overexpression phenotype.

Dpp signaling from the PCs (which have been described as a transient stem cell niche) has been proposed to maintain the AMPs in an undifferentiated state (Mathur et al., 2010). Interestingly, Hnt has been shown to down-regulate *dpp* expression in the embryonic amnioserosa and the developing pupal eye imaginal disc (Wilk et al., 2004). Therefore, it is possible that Hnt could function to down-regulate *dpp* expression in the PC as well, and could be required for AMP niche breakdown and AMP differentiation at the appropriate stage of development. At this point, the signal required to initiate breakdown of the PC niche has not been identified (Issigonis and Matunis, 2010). The premature differentiation of the AMPs into EC-like cells (Fig. 3.5B-D) could, therefore, be attributed to the possible down-regulation of Dpp expression in the PCs.

The specification of EBs and subsequent differentiation of ECs has been shown to be largely dependent upon the Notch signaling pathway. Studies employing the activated Notch signaling reporter, *GBE-Su(H)lacZ*, along with anti-Delta staining, have shown that high levels of Notch signaling are associated with EC differentiation, while low levels result in the

formation of EE cells. ISCs that show high levels of Delta also show strong expression of *GBE-Su(H)lacZ* in the EB daughter cell, indicative of strong induction of the Notch signaling pathway (Ohlstein and Spradling, 2007). Anti-Hnt immunostaining revealed that Hnt is expressed in both ISCs and EBs (Fig. 3.2C), and it is also strongly expressed in the differentiated ECs. This suggests that Hnt expression is up-regulated in EBs differentiating to form EC-like cells. Overall, these observations allow us to speculate that Hnt expression is up-regutated in cells in which Notch signaling is strongly activated.

4.5 Overexpression of Hnt is sufficient to promote EC differentiation

Earlier work done by Micchelli & Perrimon (2005) had shown that Notch signaling is not only required to limit ISC proliferation, but that it is also sufficient to cause all ISCs to differentiate to form EC-like cells at the expense of maintaining the undifferentiated ISC population. These authors demonstrated that reduced Notch signaling allows for overproliferation of ISCs, resulting in the formation of ISC tumours that also, on the basis of Prospero expression, contain differentiated EEs. This suggests that Notch signaling is needed to promote EC differentiation and limit ISC divisions, but that it is not required for EE differentiation. In contrast, expressin of activated Notch was reported to cause the ISCs to form EC-like cells, suggesting that Notch signaling is sufficient to cause ISCs to differentiate to form ECs (Micchelli and Perrimon, 2005). This was later confirmed by immunostaining for the EC marker, Pdm1 (Beebe et al., 2010). Based on these results, it seemed appropriate to perform a similar experiment using *GFP-HNT* in place of N^{intra} in order to determine if overexpression of Hnt is sufficient to cause ISCs to differentiate to form ECs. The results showed Hnt overexpression is particularly potent in causing all ISCs to differentiate to form EC-like cells; EC-like cells were confirmed as being Pdm1 positive, having reduced Esg expression, and containing large nuclei comparable in size to

neighbouring ECs. Even more striking was the fact that this occurred within four days of expressing *GFP-HNT* induction whereas the published results for Notch activation required at least seven days to observe a similar effect (Micchelli and Perrimon, 2005). One possible explanation for this is that Hnt expression in the EB is up-regulated in a Notch-dependent manner, and that a high level of Hnt expression is required to mediate the necessary downstream responses that promote EC differentiation. In support of this model was the observation that co-expression of *N-RNAi* and *GFP-HNT* (Fig. 3.13) led to EC differentiation in the absence of Notch. This suggests that Hnt acts downstream of or in parallel to the Notch signaling pathway to promote EC differentiation.

4.6 Proposed model for the role of Hnt in the maintenance and differentiation of stem cells within the adult midgut.

Overall, this study supports the interpretation that Hnt is required in regulating the stem cell population in the adult midgut, as well as the larval AMPs. In the adult midgut EGFR signaling is required for ISC maintenance and proliferation. The results presented in this study show that Hnt is also required for ISC maintenance and proliferation, and that Hnt expression in the ISC is dependent on EGFR signalling and independent of Notch signaling. ISC maintenance and proliferation likely require a low level of Hnt expression, which could be facilitated through EGFR signaling. This study also shows that Hnt overexpression is a potent effector of EC differentiation, and we suggest that the high levels of Hnt expression in the EB could be dependent on increased Notch signaling in this cell (Fig. 4.1).

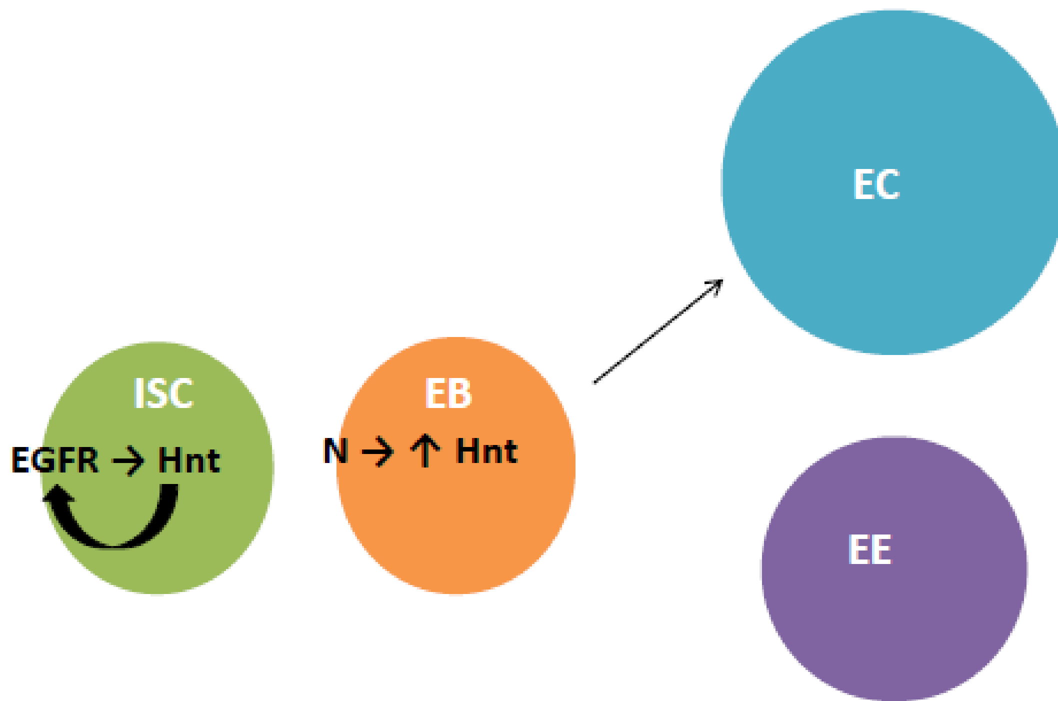

Figure 4.1 Proposed model that Hnt acts both upstream and downstream of Notch to regulate intestinal stem cell (ISC) maintenance and enterocyte (EC) differentiation. EGFR signaling in the ISC promotes ISC proliferation and maintenance, and is necessary for maintaining Hnt expression. Hnt is necessary for ISC maintenance, and could be required for promoting EGFR-dependent proliferation of ISCs. Delta expression on the ISC activates Notch signaling in the enteroblast (EB) cell, and the level of Notch signaling determines if the EB differentiates to form an EC or enteroendocrine (EE) cell. This study revealed that strong Hnt expression leads to EC differentiation, similar to what occurs with strong Notch signaling, suggesting that Hnt could mediate the necessary Notch signaling to promote EC differentiation.

Chapter 5. Future Directions

5.1 Role of Hnt in mediating EGFR-dependent AMP proliferation

Adult midgut precursors (AMPs) proliferate during larval stages and AMP divisions are regulated by EGFR signaling. EGFR signaling is induced in the larval midgut through the binding of the ligand Vein from the visceral muscle, as well as Spitz and Keren from the AMPs themselves. By reducing the level of EGFR signaling in AMPs, studies have shown that EGFR signaling is necessary to regulate both the size and number of AMP clusters throughout the larval midgut (Jiang and Edgar, 2009).

RNAi-mediated knockdown of Hnt in the larval midgut resulted in a reduced number of AMP clusters, which could be attributed to reduced proliferation of AMPs. Additionally, anti-Hnt staining of adult midguts expressing *UAS-EGFR-RNAi* revealed that EGFR signaling is required for maintaining Hnt expression in the intestinal stem cells (ISCs) of the adult midgut (Fig. 3.9F). Therefore, it is reasonable to predict that EGFR signaling is required to regulate or maintain Hnt expression in the AMPs as well, and that Hnt may be required to mediate EGFR signaling to promote AMP divisions. In order to address this relationship, the first step would be to perform anti-Hnt staining on larval midguts with varying levels of EGFR signaling and monitor changes in Hnt expression. Based on preliminary findings, subsequent rescue experiments could be performed such as co-expression of *UAS-EGFR-RNAi* with *UAS-GFP-HNT* to see if the reduced EGFR signaling/reduced AMP proliferation can be ameliorated with expression of Hnt, as well as co-expression of *UAS-λtop4.2* (activated EGFR) and *UAS-hnt-RNAi* to see if the AMP overproliferation phenotype induced by hyperactivation of EGFR signaling is suppressed by reducing the level of Hnt in the AMPs. Performing these experiments would allow us to determine if Hnt is acting along with the EGFR signaling pathway to regulate AMP

divisions and maintenance in the larval midgut, which would be consistent with its proposed role in maintaining the ISCs of the adult midgut.

5.2 EGFR- and Hnt-dependent *delta* expression in AMPs and ISCs

Previous studies suggest that EGFR signaling maintains the intestinal stem cell (ISC) population by promoting *delta* expression (Xu et al., 2011), similar to its role in promoting *delta* expression to encourage cone cell induction in the developing eye (Pickup et al., 2009). This suggestion was made because there is an accumulation of many Delta+ ISCs with over-activation of EGFR signaling (Xu et al., 2011), and the ISCs are able to differentiate to form EC-like cells, a cell fate that is largely dependent upon high levels of Delta to induce strong Notch signaling in the enteroblast (Ohlstein and Spradling, 2007). However, although this suggestion has been made, this relationship has not yet been proven. Therefore, it would be beneficial to measure *delta* expression levels using a *delta-lacZ* reporter to see if manipulating the levels of EGFR signaling afffects the expression of *delta* in the midgut. Additionally, it would be useful to combine *UAS-λtop4.2* (activated EGFR) with a *UAS-delta-dominant negative* or *UAS-delta-RNAi* to see if the loss of Delta prevents the ISC overproliferation phenotype that is observed with expression of *UAS-λtop4.2* alone (Fig. 3.15C) using the *esgGAL4UASGFPtubGal80*^{ts} driver. If the overproliferation and accumulation of ISCs and EC-like cells does not occur in the absence of Delta, then we could speculate that EGFR signaling promotes ISC divisions by upregulating *delta* expression.

In addition to examining whether *delta* expression in ISCs is EGFR signaling-dependent, it will also be important to see if Hnt is involved in promoting *delta* expression. In the developing eye, Hnt and EGFR-signaling are both needed to achieve the necessary level of *delta* expression in photoreceptor cells to induce Notch signaling in the neighbouring cone cell

precursor to promote cell differentiation (Pickup et al., 2009). Based on the experimental results suggesting that Hnt expression in ISCs is EGFR signaling-dependent (Fig. 3.9F), and that reduced Hnt expression in ISCs leads to a progressive loss of the ISC population, similar to what is observed with reduced EGFR signaling (Fig. 3.9B and C), we speculate that Hnt may be acting in an EGFR-dependent manner to promote *delta* expression in ISCs and maintain the ISC population. Future studies will examine *delta* expression levels using a *delta-lacZ* reporter or Anti-Delta staining with varying levels of Hnt expression. Subsequent studies would also attempt to rescue the loss of ISCs observed following Hnt knockdown in ISCs (Fig. 3.9B) by co-expressing *UAS-delta*. Additionally, it would be interesting to see if the EC differentiation that is observed with overexpression of Hnt (Fig. 3.11) is suppressed with co-expression of *UAS-delta-dominant negative* or RNAi-mediated knockdown of Delta. These experiments would allow us to suggest that Hnt's role in ISC maintenance and differentiation in the midgut is to promote sufficient levels of *delta* expression.

5.3 Role of Hnt in mediating JAK/STAT signaling for promoting EC differentiation

It has long been accepted that Notch signaling plays an important role in promoting EC differentiation. More recent studies have shown that the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway acts downstream of Notch and is necessary for promoting EC differentiation. However, unlike Notch signaling, JAK/STAT signaling is also required for EE cell differentiation (Beebe et al., 2010b).

Microarray data obtained by our lab had identified *unpaired 3 (upd3)*, an activating ligand of the JAK/STAT signaling pathway (Beebe et al., 2010), as a potential direct/indirect transcriptional target of Hnt (unpublished data). Therefore, it is possible that Hnt may promote JAK/STAT signaling in the midgut. Overexpression of Hnt causes all ISCs to differentiate to

form ECs, so it is possible that this effect may be achieved through activation of the JAK/STAT signaling pathway. Therefore, it would be beneficial to combine overexpression of Hnt with knockdown of STAT92E to see if disruption of JAK/STAT signaling prevents the elevated level of Hnt from promoting EC differentiation. If this occurs, then we could speculate that Hnt acts downstream of Notch and upstream of JAK/STAT signaling to promote EC differentiation.

JAK/STAT signaling is required for EC differentiation, and also for EE cell differentiation (Beebe et al., 2010). EE cell differentiation occurs with low levels of Notch signaling (Bahl et al., 2012), and can occur in the absence of Notch signaling. RNAi-mediated knockdown of Notch prevents ECs from forming, and instead produces ISC tumours consisting of both Esg-positive ISCs as well as Pros-positive EE cells. Although EE differentiation occurs in the absence of Notch, it does not occur if JAK/STAT signaling is disrupted (Beebe et al., 2010). To test if Hnt is involved in regulating the JAK/STAT signaling necessary for EE cell differentiation in the *Drosophila* midgut, the first step would be to see if RNAi-mediated knockdown of Hnt prevents EE differentiation from occurring in a loss-of-Notch background. Although we have shown that reduced Hnt suppresses the Notch RNAi-induced tumour formation, anti-Prospero staining would be useful to confirm that these cells are not forming. Additionally, staining of the small cell tumours that resulted with co-expression of *UAS-λtop4.2* and *UAS-hnt-RNAi* might also show that the tumours consist entirely of Esg+ ISCs, which would suggest that EE cell differentiation does not occur in the absence of Hnt. If our results show that Hnt is needed for EE cell differentiation as well, then we could predict that Hnt mediates the JAK/STAT signaling required for both EC and EE cell differentiation.

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Appendix A Stocks and Crosses

A1.1 Crossing Schemes

Figure 3.1 *w[*] P{w[+mW.hs]=GawB}NP3312 / FM7c x w[1118]; P{w[+mC]=UAS-GFP.nls}14*

Figure 3.2A *w[*] P{w[+mW.hs]=GawB}NP3312 / FM7c x w[1118]; P{w[+mC]=UAS-GFP.nls}14*

Figure 3.2C *esgGAL4 + UASGFP*

Figure 3.3A, B *esgGAL4 + UASGFPnls/CyO*

Figure 3.3C *w[*] P{w[+mW.hs]=GawB}NP3312 / FM7c x w[1118]; P{w[+mC]=UAS-GFP.nls}14*

Figure 3.3D *tubGal80hsFLP19A; esgGAL4UASGFP*^{nls}*/(CyO)* x *hnt*^{308}*FRT19A*

Figure 3.3E *tubGal80hsFLP19A; esgGAL4UASGFP*^{nls}*/(CyO) x hnt*^{XE81}*FRT19A*

Figure 3.4 *esgGAL4UASGFPtubGal80*^{ts} *x UAS-N*^{intra}*/CyO*

Figure 3.5A *esgGAL4 + UASGFPnls/CyO*

Figure 3.5B *esgGAL4+UASGFPnls/CyO x EP55;;eyeless-GAL4/Tn6, Tb*

Figure 3.5C *esgGAL4 x UAS-GFP-HNT*^{J18}

Figure 3.5D *esgGAL4 x UAS-GFP-HNT; tubGal80ts*

Figure 3.5E *esgGAL4UASGFPtubGal80*^{ts} *x UAS-GFP-HNT*

Figure 3.6A *esgGAL4 + UASGFPnls*

Figure 3.6B *esgGAL4+UASGFPnls x w[*]; P{w[+mC]=UAS-mamN}3*

Figure 3.6C *esgGAL4+UASGFPnls x P{w[+mC]=UAS-N.dsRNA.P}14E, w[*]*

Figure 3.6D *esgGAL4+UASGFPnls x UAS-hnt-RNAi2A; UAS-hnt-RNAi2B*

Figure 3.7A, B *hnt*^{NP3312} *x UAS-GFPnls*

Figure 3.7C *w[1]; P{w[+mC]=PCNA-EmGFP}T137*

Figure 3.8 A,C, E *esgGAL4UASGFPtubGal80*^{ts} *x UAS-Ras85DV12*

Figure 3.8 B, D

- *EP55;; eyelessGAL4/Tn6, Tb x UAS-Ras85DV12 → EP55;; UAS-Ras85DV12/+*
- *esgGAL4UASGFPtubGal80*^{ts} *x EP55;; UAS-Ras85DV12/+ x*

Figure 3.9A *esgGAL4UASGFPtubGal80*^{ts}

Figure 3.9B *esgGAL4UASGFPtubGal80*^{ts} *x UAS-hnt-RNAi2A; UAS-hnt-RNAi2B*

Figure 3.9C *esgGAL4UASGFPtubGal80*^{ts} *x UAS-EGFR-RNAi*

Figure 3.9D *esgGAL4UASGFPtubGal80*^{ts} *x P{w[+mC]=UAS-N.dsRNA.P}14E, w[*]*

Figure 3.9E *esgGAL4UASGFPtubGal80*^{ts} *x UAS-EGFR-RNAi*

Figure 3.10A *esgGAL4UASGFPtubGal80*^{ts}

Figure 3.10B *esgGAL4UASGFPtubGal80*^{ts} *x UAS-Su(H)VP16*

Figure 3.11 *esgGAL4UASGFPtubGal80*^{ts} *x UAS-GFP-HNT*

Figure 3.12 *esgGAL4UASGFPtubGal80*^{ts} *x UAS-hnt-RNAi2A; UAS-hnt-RNAi2B*

Figure 3.13A, C, E *esgGAL4UASGFPtubGal80*^{ts} *x P{w[+mC]=UAS-N.dsRNA.P}14E, w[*]*

Figure 3.13B, D, F

- *UAS-N-RNAi x UAS-GFP-HNT → UAS-N-RNAi; UAS-GFP-HNT/+* (males)
- *UAS-N-RNAi; UAS-GFP-HNT/+ (males) x esgGAL4UASGFPtubGal80*^{ts}

Figure 3.14A, C *esgGAL4UASGFPtubGal80*^{ts} *x UAS-N-RNAi*

Figure 3.14B, D

- *UAS-N-RNAi x UAS-hnt-RNAi2A; UAS-hnt-RNAi2B → UAS-N-RNAi; UAS-hnt-RNAi2A/+; UAS-hnt-RNAi2B/+* (males)
- *UAS-N-RNAi; UAS-hnt-RNAi2A/+; UAS-hnt-RNAi2B/+ (males) x esgGAL4UASGFPtubGal80*^{ts}

Figure 3.15A, C *esgGAL4UASGFPtubGal80*^{ts} *x UAS-λtop4.2*

Figure 3.15B, D

- *UAS-λtop4.2 x UAS-GFP-HNT → UAS-λtop4.2; UAS-GFP-HNT/+* (males)
- *UAS-λtop4.2; UAS-GFP-HNT/+* (males) x *esgGAL4UASGFPtubGal80*^{ts}

Figure 3.16A, B *esgGAL4UASGFPtubGal80*^{ts} *x UAS-λtop4.2*

Figure 3.16C, D

- *UAS-λtop4.2 x UAS-hnt-RNAi2A; UAS-hnt-RNAi2B → UAS-λtop4.2; UAS-hnt-RNAi2A/+; UAS-hnt-RNAi2B/+* (males)
- *UAS-λtop4.2; UAS-hnt-RNAi2A/+; UAS-hnt-RNAi2B/+* (males) x *esgGAL4UASGFPtubGal80*^{ts}

Appendix B. Additional Experimental Data

B1.1 Hnt expression in larval AMPs does not require Notch signaling

As described previously in Section 3.7 of the Experimental Results section, Hnt expression in the ISCs of the adult midgut is dependent on EGFR signaling and Notch-independent. Given that the larval AMPs are also regulated by EGFR (Jiang and Edgar, 2009) and Notch signaling (Mathur et al., 2010), anti-Hnt immunostaining was performed to determine if Hnt expression in AMPs is Notch-dependent. Using *esgGAL4* driven nuclear *GFP* in combination with *UAS-mamN* to disrupt Notch signaling (Vied et al., 2009) in AMP clusters, revealed that Notch signaling is not required for Hnt expression in AMPs (Fig. B1.1).

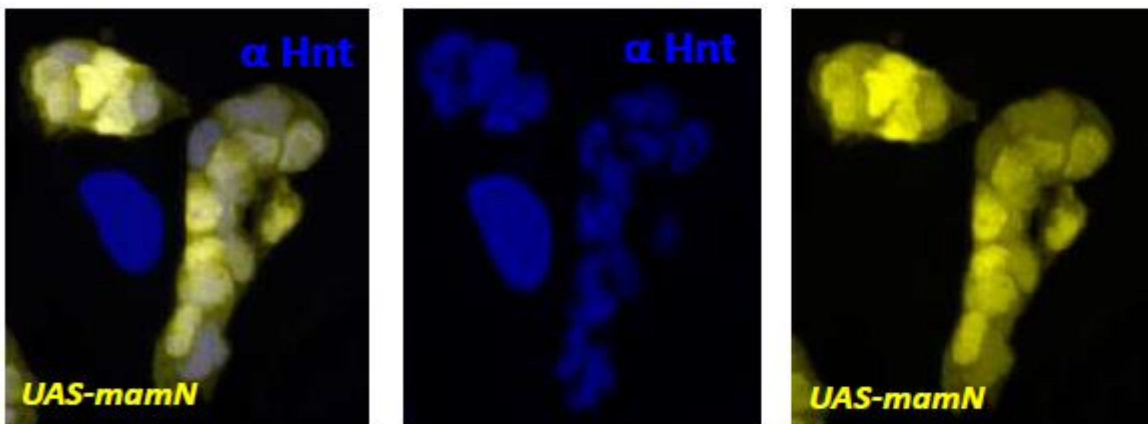

Figure B1.1 Hnt expression in the larval AMPs is Notch-independent. Anti-Hnt (blue) immunostained larval midgut expressing GFP (yellow) and *UAS-mamN,* under the control of *esgGAL4* + GFP^{nls}, which marks the AMPs, revealed that Notch signaling is not required for Hnt expression in AMPs.