The role of hindsight in the transient stem cell niche of the *Drosophila melanogaster* larval midgut

by

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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 electronically available to the public.
Abstract

The composition of the larval *Drosophila* midgut includes cells known as adult midgut precursors (AMPs) that represent the founder cells for the adult midgut. During the first and second larval instar stages, AMPs are solitary cells that proliferate and migrate along the length of the basal surface of the larval midgut (Jiang & Edgar, 2009). During the third instar larval stage, AMPs are observed as clusters that are encapsulated by a new differentiated cell type, the Peripheral Cell (PC). The PC is thought to function as the transient stem cell niche that prevents AMP differentiation and over-proliferation. During the larval-to-pupal transition, AMP differentiation results in the formation of a population of absorptive enterocytes (ECs) and the adult intestinal stem cells (ISCs). Subsequent to the establishment of the adult ISCs, ISCs divide asymmetrically to produce committed daughter cells known as enteroblasts (EBs). Further differentiation of EBs can result in an additional cell type of the adult midgut, the secretory enteroendocrine cell (EE), as well as further ECs. During AMP differentiation, AMPs are released from the PCs, some of which go on to form a transient pupal midgut. The transient midgut and other PCs are ultimately removed by programmed cell death.

In investigating the role of the transcription factor Hindsight (homolog of human Ras Responsive Element Binding Protein I) in this system, we have found that Hindsight is required in the larval midgut during the process of PC differentiation. Additional analysis addressed the cell lineage of the PC with respect to AMP and was inconclusive. Live imaging larval midgut explants revealed that the PC/AMP clusters are not autonomous, that these clusters can merge, and that PC cells are highly motile and dynamic.
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List of Abbreviations

AED: After Egg Deposition
AMPS: Adult Midgut Precursor (or Progenitors)
Br: Broad
CSL: Centromere-Binding Protein 1, Suppressor of Hairless, Longevity Assurance Gene 1
Dl: Delta
DIAP-1: *Drosophila* Inhibitor-of-Apoptosis Protein 1
EBs: Enteroblasts
ECs: Enterocytes
Ecd: Ecdysone
EEs: Enteroendocrine Cells
EGFR: Epidermal Growth Factor Receptor
FRT: Flippase Recognition Target
GAL4: Galactosidase enzyme 4
GFP$nls$: Green Fluorescent Protein with a Nuclear Localisation Signal
GOF: Gain-Of-Function
His2Av: Histone 2Av
Hnt: Hindsight
hsFLP: Heat Shock Flippase
ISCs: Intestinal Stem Cells
LOF: Loss of Function
Mam: Mastermind
MARCM: Mosaic Analysis with a Repressible Cell Marker
MET: Mesenchymal-to-Epithelial Transition
NICD: Notch Intracellular Domain
NRE: Notch Responsive Element
PCs: Peripheral Cells
RHG: Reaper – Hid – Grim protein complex
RFP: Red Fluorescent Protein
RREB-1: Ras-Responsive Binding Element 1
SOP: Sensory Organ Precursor
Su(H): Suppressor of Hairless
TubGAL80: Tubulin Galactose metabolism regulatory protein 80
UAS: Upstream Activation Sequence
Chapter 1: Introduction

1.1 Drosophila Midgut

1.1.1 Drosophila Midgut Development

The Drosophila gut is endodermally derived and is specified early in development. The endoderm is a population of cells that migrate to the interior of the embryo, during gastrulation. The migration of the cells creates two sites of invagination at the anterior midgut and posterior midgut. The Drosophila embryo undergoes extensive morphogenetic conformations including germ band extension, where the presumptive posterior region of the embryo comes to be situated directly behind the head region of the embryo (Campos-Ortega & Hartenstein, 1985, Tepass & Hartenstein, 1994). Late in development further morphogenetic movements bring the tail region back to the posterior of the embryo, a process known as germ band retraction. At the end of germ band retraction, the endoderm has distinct anterior and posterior regions. The anterior region is attached to the ectodermally derived foregut and the posterior region is attached to the hindgut, which are also ectodermal in origin. Similar to other metazoans, the endoderm of Drosophila gives rise to the epithelium of the digestive tract, the midgut (Takashima, Gold, & Hartenstein, 2013; Tepass & Hartenstein, 1994).

The endoderm forms three mesenchymal cell types early in development: the principle midgut epithelial cells (PMECs), the interstitial cell precursors (ICPs) and the adult midgut precursors (AMPs). The majority of the endodermal cells become PMECs that form the epithelium of the larval midgut. These cells undergo a mesenchymal-to-epithelial transition (MET). Afterwards, the ICPs and the AMPs migrate to the midgut epithelium, populating the apical surface of the epithelium, but at some point, they migrate to become situated on the basal surface (Fig. 1.1). A class of genes previously found to be involved in cell differentiation and the
regulation of specification of the neuroectoderm and the sensory organ precursors are required to establish the AMPs and ICPs (Tepass & Hartenstein, 1994, Tepass & Hartenstein, 1995).
Figure 1.1 Morphogenesis of the larval midgut. The endoderm is specified to three cell types: PMECs, AMPs, and ICPs. The majority of the endodermal cells become PMECs, the cells that form the epithelium of the developing midgut. A few cells become AMPs and ICPs and migrate to the apical surface of the rudimentary larval midgut (Tepass & Hartenstein, 1995).
1.1.2 Transient Stem Cell Niche

Ultimately, it is the AMPs of the larval midgut that differentiate into the three adult midgut cell types. These are the absorptive enterocytes (ECs), the secretory enteroendocrine cells (EEs), and the intestinal stem cells (ISCs) (Hartenstein et al., 1992). AMPs start off as solitary cells along the length of the larval midgut epithelium during the first instar stage of development. Each AMP then goes one round of symmetric division resulting in each solitary cell paired with its daughter. A second and third round of symmetric division occurs in each pair of AMPs before one AMP from each island undergoes asymmetric division to produce a niche cell known as a Peripheral Cell (PC) (H. Jiang & Edgar, 2009; Micchelli, Sudmeier, Perrimon, Tang, & Beehler-Evans, 2011). Sometimes, clusters have two to three PCs, an observation that remains largely unexplored (Takashima et al., 2011). The mechanism of PC differentiation is not completely understood. It is known that Notch signaling is required and sufficient for PC differentiation (Mathur et al., 2010). However, there is not much else known definitely beyond this point analysing the mechanism of differentiation of the PC is a topic of this investigation.

The PC forms a transient stem cell niche, preventing the AMPs from undergoing premature differentiation and from over-proliferation – it has been suggested to serve as a ‘holding pen’ (Mathur et al., 2010). AMP islands devoid of a PC prematurely differentiate into adult ECs during the late third instar stage of development. This results in a complete lack of ISCs since all AMPs are specified to become ECs (Mathur et al., 2010). The PC maintains the undifferentiated state of the AMPs via signaling of the Drosophila homolog of bone morphogenetic protein, Decapentaplegic (Dpp), that is secreted from the PCs (Mathur et al., 2010).
Each AMP cluster, by the time the larva pupates, contains about 30 cells (H. Jiang & Edgar, 2009). AMPs are released from their clusters during metamorphosis, at which point they differentiate into ECs in a Notch-dependent manner. At this stage, the PCs either form a transient pupal midgut or they die by programmed cell death (Takashima et al., 2011). Overall, the transient stem cell niche created by the PCs is important to establish the appropriate adult intestinal stem cell population. Ultimately, all PC cells, regardless of whether or not they form part of the transient pupal midgut, are removed from development by programmed cell death. At present, the pathways regulating this death have not been investigated in any detail.

Lateral inhibition through Notch signaling drives the asymmetric division between an AMP and a PC (see Notch Signaling). The presumptive PC expresses Notch receptors in its membrane and the AMP daughter expresses Delta ligands. PCs have a high level of Notch signaling, as demonstrated by the high level of expression of downstream target genes (Mathur et al., 2010). One of these downstream target genes having expression exclusive to PCs is Su(H) (Bray, 2006; Mathur et al., 2010). Also, expression of an activated form of Notch in the AMP population causes all AMPs to take on a PC-like fate (Mathur et al., 2010). Notch signaling is reported as being required and sufficient for PC differentiation.

EGFR signaling is the main driver for AMP proliferation both before and after PC differentiation. EGFR signaling drives AMP proliferation along the length of the midgut epithelium as solitary cells. It also drives the symmetric division of AMPs at the second instar stage. The primary ligand that initiates EGFR signaling in these two stages is vein, which is secreted from the visceral muscle surrounding the midgut epithelium (H. Jiang & Edgar, 2009). Once a niche is established, EGFR signaling drives proliferation of AMPs. However, the primary ligand is no longer Vein but involves both Spitz and Keren. The source of secreted Spitz and/or
Keren, whether the AMPs or the PCs remains unknown. The dynamics of AMP proliferation, in other words whether there is a single stem-cell like AMP, or whether all AMPs are capable of division, also remains a largely unexplained area of investigation (H. Jiang & Edgar, 2009).

As mentioned above, the ultimate fate of the PCs is removal by programmed cell death (PCD). In support of this, during the larval-to-pupal transition PCs been found to be immuno-positive for active caspases (Takashima et al., 2011). The death of the PCs coincides with adult EC differentiation, as indicated by the adult EC-specific marker, PDM-1 (Mathur et al., 2010).
1.1.3 Adult Drosophila Midgut

In general, the consensus is that the development of the adult midgut in *Drosophila* is largely regulated by proneural genes (genes necessary for the formation of neural cells that include transcription factors encoding genes *achaete* and *scute*) and Notch activity (Tepass & Hartenstein, 1995). In the midgut, proneural gene expression promotes the differentiation of secretory EE cells whereas Notch signaling promotes EC differentiation. The two pathways establish a proper ratio of cells through the process of lateral inhibition (see Notch Signaling) (Takashima et al., 2013). The majority of AMPs take on the EC fate while a few are specified to become adult ISCs (Zeng & Hou, 2012). EEs are only derived from an existing ISC. Thus, while AMPs can directly differentiate to ECs, the same is not true for EEs. The formation of EEs occurs between 44 and 96 hours after pupal formation. At this time, the ISCs undergo an asymmetric division, which generates one ISC and one EE cell. Each newly formed daughter then undergoes a symmetric division. Different levels of Notch signaling are required to promote different cell fates in the adult midgut. The formation of an EE from an ISC is driven by low Notch signaling. The low signal is also required to maintain the intestinal stem cell identity (Fig. 1.2A). This low level of Notch signaling precludes EC differentiation, which requires high levels of Notch signaling (Fig. 1.2B) (Guo & Ohlstein, 2015). Given that Notch signaling is an important player in establishing the adult midgut cell population, it may also be crucial in establishing the larval midgut cell population.
Figure 1.2 Bidirectional Notch signaling regulates ISC specification. (A) EEs maintain ISC stem cell identity by sending low levels of Delta ligand. (B) ECs are generated when ISCs send a strong Notch signal over to the enteroblast (EB) (Guo & Ohlstein, 2015).
1.2 Signaling Pathways

1.2.1 Notch Signaling

The Notch signaling pathway is evolutionarily conserved amongst all metazoans. Its functional role is to regulate cell fate and differentiation through local cell interactions. It is involved in various developmental and disease contexts where it regulates developmental patterning by establishing cell fates in developing tissues (Artavanis-Tsakonas, S., Rand, M.D. & Lake, 1999; Gazave et al., 2009). Notch is a transmembrane receptor with two distinct molecular ligands in Drosophila: Delta and Serrate. Receptor-ligand binding is followed by the proteolytic cleavage of the Notch intracellular domain (NICD) in the cytoplasm. The NICD enters the nucleus and binds to its designated transcriptional machinery. The transcriptional complex consists of a DNA-binding protein complex collectively called CSL [Centromere Binding Protein 1-Suppressor of Hairless (Su(H))- Longevity Assurance Gene 1]. The CSL is associated with its co-activator Mastermind (Mam). NICD binding to the CSL-Mam complex activates the transcriptional machinery and initiates transcription of downstream target genes (Fig. 1.3) (Bray, 2016). In some contexts, the gene hindsight, is reported as being of these downstream target genes.
Figure 1.3 Illustration of the Notch-Delta signaling pathway. Delta ligands are embedded in the plasma membrane of the neighboring cell. Receptor-ligand binding of Notch receptors on the neighboring cell initiates signaling. The Notch Intracellular Domain (NICD) is cleaved and released in the cytoplasm. It enters the nucleus where it binds and derepresses the CSL-MAM complex, allowing initiation of transcription of downstream targets (Bray, 2016).
Notch signaling has two primary communication mechanisms: inductive signaling and lateral inhibition. In inductive signaling, a positive feedback loop exists between the two communicating cells. The expression of the ligand is perpetuated and the Notch signal is intensified in a population of cells that are adopting the same fate. This is seen in the *Drosophila* wing disc where patches of progenitor cells create boundaries of different fates (de Celis & Bray, 1997).

In a situation where neighboring cells are adopting different cell fates, Notch signalling can involve lateral inhibition. Here, cells with a particular fate inhibits adjacent cells from achieving the same fate (Fig. 1.4). The mechanism of action involves a feedback loop where one cell has higher levels of Notch signaling and the neighbouring cell has lower levels, which then establishes the fate of each cell – the cell fates become mutually exclusive (Collier, J. R. *et al*., 1996). This bidirectional Notch signaling event is required in asymmetric cell divisions. Stem cells of the midgut and the germline require Notch signaling in order to maintain the pluripotency of the stem cell (Guo & Ohlstein, 2015, Song *et al*., 2007). In the *Drosophila* midgut for instance, a low level of Notch signaling is required to maintain the adult midgut stem cells. The signal is important to maintain gut homeostasis (see **1.4.2 Adult Midgut Development**).
**Figure 1.4 Lateral inhibition of Notch-Delta signaling in neighboring cells.** Activation of Notch signaling in the cell on the right-hand side creates a feedback loop that decreases the production of Delta in that same cell, which then decreases the level of Notch signaling in the cell on the left-hand side. The cell with lower Notch signaling will adopt a cell fate that is different from its neighbor (Collier, J. R. *et al.*, 1996).
1.2.2 Epidermal Growth Factor Receptor (EGFR) Signaling

Epidermal Growth Factor Receptor (EGFR) signaling is required for various developmental contexts in *Drosophila* embryogenesis – regulating cell proliferation and cell differentiation. For instance, it is involved in establishing the Anterior-Posterior and Dorsal-Ventral axis in the developing oocyte (Tian *et al.*, 2014). EGFR signaling is also implicated in morphogenetic processes such as maintaining the epithelium of the trachea, regulating cell differentiation of the photoreceptor cells in the eye, and regulating the development of the oenocytes, cells responsible for lipid processing (Cela & Llimargas, 2006, Elstob *et al.*, 2001, Lusk *et al.*, 2017).

In *Drosophila*, EGFR signaling activates the RAS/MAPK pathway. The pathway is initiated upon ligand binding, similar to Notch signaling. In the fruit fly, there are four distinct secreted EGFR ligands: Spitz, Keren, Gurken, and Vein. In the larval midgut Vein is secreted from the surrounding visceral muscle (Jiang & Edgar, 2009). The ligands bind to transmembrane EGFR receptors. Ligand binding induces receptor dimerization and activates the signaling pathway. First, two EGFR receptors autophosphorylate their tyrosine residues located on the intracellular portion of the receptors. The phosphate groups recruit docking proteins Grb2 and Sos. Sos activates Ras by removing GDP, via the GTP Exchange Factor, and allowing for GTP to bind to its site on the Ras protein. Activated Ras initiates the MAPK cascade that involves Ras-Raf-Mek-Erk, which ultimately leads to transcriptional regulation of target genes. Downstream targets of EGFR-MAPK signaling are involved in cell growth and cell fate determination (*Fig. 1.5*) (Katzel, Fanucchi, & Li, 2009; Krasinskas, 2011; Lusk, Lam, & Tolwinski, 2017).
Figure 1.5 The EGFR-RAS-MAPK signaling pathway. Ligands in *Drosophila* that bind to EGFR receptors are Gurken, Spitz, Keren, and Vein. Autophosphorylation of the tyrosine residues of the intracellular portion of EGFR activates the Ras guanine nucleotide exchange factors (Sos and Grb2) that activate Ras. Active-Ras then goes onto activate downstream targets. Ras-MAPK activation promotes gene expression involved cell growth and cell fate (Roberts & Der, 2007).
1.2.3 Ecdysone Signaling

Ecdysone, sometimes known as the molting hormone, is a steroid hormone that regulates larval development in metamorphosis in *Drosophila*. Ecdysone has an Ecdysone Receptor (EcR) belonging to the class of nuclear receptors that, once activated, initiates a signaling pathway to activate a class of genes essential for normal development (Buszczak *et al.*, 1999). One way the pathway regulates larval development is by establishing and maintaining a stem cell niche. In the early larval stages, gonadal somatic cell precursors and primordial germ cells proliferate to form niches. Later in larval development, Ecdysone signaling inhibits this proliferation and thereby inhibits niche formation. This allows the gonadal somatic cells to stop proliferating and also to enter the differentiation pathway (Belles & Piulachs, 2015). Ecdysone is regulated by various signaling pathways that positively and negatively regulate its secretion in various tissues. Ecdysone is primarily produced in the prothoracic gland, where several signaling pathways promote its expression in a time-specific manner (Yamanaka *et al.*, 2014). Ecdysone also plays an important role in regulating AMP expansion and ISC differentiation (see section 1.2). Converse to its effect in gonadal somatic cell precursors, ecdysone promotes proliferation of the AMPs during the larval stages of the midgut epithelium. Ecdysone also promotes AMP-to-ISC differentiation (Micchelli *et al.*, 2011, Zeng & Hou, 2012). All in all, ecdysone signaling is context dependent; its effects varies depending on the tissue and the cellular environment.
1.3 *Hindsight*

*Hindsight* (*hnt*) is a *Drosophila* gene that encodes a transcription factor with 14 C$_2$H$_2$-type zinc finger domains. There are two suggested *hnt* consensus binding sites, as determined through *in vitro* iterative PCR amplification and Hnt co-immunoprecipitation (SELEX): YGGWCCA and CAGCATCC (Ming *et al*., 2013). Neither motif, however, has been definitively defined as a bona fide *hnt* binding site. Interestingly, however, *Drosophila* labs at the University of Massachusetts Medical School created the Fly Factor Survey Database where they used a bacterial one-hybrid method to find DNA binding sites of transcription factors and created consensus sequences. The one reported for *hnt* is identical to the consensus CAGCATCC (“Fly Factor Survey”, Ming *et al*., 2013).

A relatively recent report suggests that Hnt transcriptionally regulates genes that are involved in the regulation of the cytoskeleton, including the *Drosophila* ortholog of filamin, a conserved actin binding protein encoded by the gene known as *jitterbug* (Oliva *et al*., 2015). Immunolocalization of the Hnt protein on salivary gland polytene chromosomes identifies more than 50 potential binding sites (Ming *et al*., 2013). Only 2 potential Hnt target genes have been examined in detail, one of which is *hnt* itself, which displays negative autoregulation in a tissue specific manner (Ming *et al*., 2013). In other cases, *hnt* can either transcriptionally activate or repress its downstream targets.

*hnt* expression is essential in various developmental contexts. First, it is required for germ band retraction. Loss-of-function (LOF) alleles of *hnt* result in the failure of the morphogenetic process of germ band retraction during embryogenesis. Hnt is also required to maintain epithelial integrity in the amnioserosa, the extraembryonic membrane that regulates retraction (Yip, Lamka, & Lipshitz, 1997).
*Hnt* expression in larval tissues includes the larval tracheal system, the peripheral nervous system (PNS), the larval oenocytes, the larval lymph, the crystal cells (a type of hemocyte), the salivary glands and the midgut (Ming *et al.*, 2013, Pitsouli & Perrimon, 2010). In the pupal stages, *hnt* is expressed in the sensory organ precursors (SOPs), myoblasts, and photoreceptor cells of the retina (Krejci *et al.*, 2009, Pickup *et al.*, 2002), Reeves & Posakony, 2005). Finally, in the adult fly, *hnt* is expressed in the midgut, in the follicle cells, in the border cells of the egg chambers, and in the central nervous system where it prevents axon degeneration and fragmentation (Baechler *et al.*, 2015, Farley *et al.*, 2018, Melani *et al.*, 2008, Oliva & Sierralta, 2010).

The pattern of expression of *hnt* is dynamic across different cell types. For instance, *hnt* expression is highest in the adult ECs, then the ISCs and is non-existent in the EEs. In fact, *hnt* overexpression in the adult midgut forces all of the ISCs/EBs to differentiate into ECs. Conversely, a LOF mutation of *hnt* prevents EC differentiation in adult flies (Baechler *et al.*, 2015). Furthermore, *hnt* is upregulated in the SOPs in the pupa compared to the epithelial cells (Reeves & Posakony, 2005). Conversely, in the amnioserosa, the downregulation of *hnt* expression is permissive to the programmed cell death of this extraembryonic tissue (Mohseni *et al.*, 2009).

*Hnt* has three Notch-responsive enhancer elements (NREs) that are linked to its expression in the following tissues: in larval lymph glands, in the myoblasts of the pupa, and the follicle cells of the ovaries (Krejci *et al.*, 2009, Terriente-Felix *et al.*, 2013, Sun & Deng, 2007). These NREs are located upstream of the *hnt* transcription start site, ranging from 38 to 5 kb upstream – each NRE is about 1kb long (Terriente-Felix *et al.*, 2013). In other tissues, *hnt* expression is EGFR-dependent rather than Notch-dependent. The ISCs of the adult midgut, for instance,
express hnt in an EGFR-dependent manner and do not require Notch signaling (Baechler et al., 2015). Finally, there are tissues like the developing retinal cells in the pupa that express hnt in a Notch-independent and EGFR-independent manner. In this particular case, expression of hnt is driven by Jun Kinase signaling (Pickup et al., 2009).

Altogether, the role of hnt expression is very context dependent. Depending on the time of expression and the tissue in which it is expressed, Hnt can have various effects as a transcriptional activator or repressor on cell differentiation as well as cell survival. For this reason, the function of Hnt remains largely unknown and is subject to further investigation.
1.3.1 Ras-Responsive Binding Element 1 (RREB-1)

Hnt is functionally conserved with the human Ras-responsive binding element 1 (RREB-1) gene. Clusters of zinc fingers show 54 to 95% similarity in the DNA sequences (Ming et al., 2013). Human RREB-1 binds to the same chromosomal regions as hnt in Drosophila polytene chromosomes and rescues the hnt LOF embryonic lethality (Ming et al., 2013). Recently it was discovered that RREB-1 can also rescue the axon death phenotype associated with the loss of hnt expression. In other words, it can functionally substitute for Hnt (Farley et al., 2018).

RREB-1 protein was originally isolated as a protein that binds to a ras-responsive transcriptional element located at the promoter region of the calcitonin gene (Thiagalingam et al., 1996). Calcitonin transcriptionally regulates Ras and Raf in the EGFR pathway and mutations have created tumors (Thiagalingam et al., 1996). RREB-1 directly binds to and activates gene expression of calcitonin, p53, secretin (Liu et al., 2009, Ray et al., 2003, & Thiagalingam et al., 1996). It was also found to transcriptionally repress p16INK4a, a tumor suppressor gene (Zhang et al., 2003). Gain-Of-Function (GOF) mutations in RREB-1 are linked to colorectal cancer, pancreatic and thyroid cancers (Kent, Fox-Talbot, & Halushka, 2013; Zhang et al., 2003). The literature suggests that RREB-1 regulates EGFR – RAS - MAPK signaling pathway (Kent et al., 2013; Thiagalingam et al., 1996).
1.4 Programmed Cell Death

Programmed cell death (PCD) is a biological process that can result in apoptosis, which is a morphology of dying cells. PCD can be caspase-dependent or caspase-independent. Caspases are proteases - zymogens (inactive enzyme that is activated by another enzyme) – that target cysteine residues on proteins in response to cell death signals (Kumar, S. & Doumanis, J., 2000). Caspases can be activated either by extrinsic or intrinsic cell death signaling pathways. In the extrinsic pathway, the activation of transmembrane receptors leads to caspase activation.

Conversely, in the intrinsic pathway, signaling is initiated from intracellular signals such as mitochondrial-initiated events including the release of cytochrome C (Elmore, 2007). In Drosophila, the regulation of PCD involves the activation of the Reaper-Hid-Grim (RHG) protein complex. When expressed, these proteins promote PCD by inhibiting the Drosophila Inhibitor-of-Apoptosis-Protein 1 (DIAP-1). DIAP-1 is known to inhibit caspases (Wing et al., 2001). By activating RHG, DIAP-1 is inhibited, which ultimately relieves the inhibition of caspases and results in PCD (Fig. 1.6).

\[
\text{RHG} \quad \text{-------} \quad \text{DIAP-1} \quad \text{-------} \quad \text{Caspases} \quad \rightarrow \quad \text{PCD}
\]

Figure 1.6 Programmed Cell Death via RHG DIAP-1 inhibition. RHG inhibits DIAP-1 activity. DIAP-1 activity blocks caspase activity, which is required for PCD. Blocking DIAP-1, derepresses the caspases inhibition, thereby allowing PCD to occur.
1.4.1 Inhibition of caspases by p35

As an experimental approach, apoptosis can be prevented by the use of caspases inhibitors. One such an inhibitor is p35, a protein that was originally discovered in baculovirus (Martin, F.A., et al., 2009). P35, which functions as a suicide inhibitor, can inhibit caspases and subsequent apoptosis. P35 is available as a UAS transgene where it under GAL4 control (see Materials & Methods, section 2.2). When the system is activated, the cell that is destined to undergo PCD, would be prevented by doing so under UAS-p35 activation.

1.5 Research Goals and Objectives

The cell biology of the transient stem cell niche in the larval midgut of Drosophila remains largely unknown. It is curious to examine the cell biology of PC formation with respect to Notch signaling. I am interested in analysing the functional role of hnt expression in PC formation. I would like to examine the effect of a hnt LOF allele on the production of a PC. If hnt expression is required for the production of PCs, the next goal is to examine whether it is a failure of differentiation or whether the hnt LOF cell is undergoing programmed cell death. Findings in this research project would provide more information on the functional role of hnt in the cellular biology of a stem cell niche.
Chapter 2: Materials & Methods

2.1 Drosophila Stocks and Fly Husbandry

In Appendix 1 is a list of all the genetic stocks used and their sources. All *Drosophila* stocks were acquired from the following institutions: the Bloomington *Drosophila* Stock Center in the Department of Biology at Indiana University, the Kyoto *Drosophila* Genetic Resource Center at the Kyoto Institute of Technology in Kyoto, Japan, Dr. Eduardo Moreno’s Laboratory at the University of Bern in Bern, Switzerland, and Dr. Bruce Reed’s Laboratory at the University of Waterloo in Waterloo, Canada.

2.2 GAL4/UAS System

The GAL4/UAS system is a standard technique for inducible gene expression in *Drosophila*. GAL4 is a transcription factor that was identified in *Saccharomyces cerevisiae* and induced by galactose. It transcriptionally activates genes by binding to their Upstream Activating Sequence (UAS). This system was extrapolated to inducible gene expression *Drosophila*. It rapidly generates individual strains where the gene of interest can be either reported for or ectopically expressed. The GAL4 gene is engineered into the promoter region of a gene of interest that will drive expression of GAL4 when the endogenous gene is naturally expressed in a specific tissue. The UAS is inserted in a second fly with either a reporter gene or another gene of interest. The two fly strains are crossed and the progeny will display directed GAL4/UAS gene expression. If the UAS is tagged to a reporter gene such as GFP, then the expression pattern of the gene tagged to GAL4 will be visible under fluorescent microscopy (Fig 2.1). In the case where UAS is tagged to another gene, then ectopic gene expression is induced under the control of the gene driving GAL4 expression (Duffy, 2002) (Fig. 2.1).
**Figure 2.1 GAL4/UAS System.** A transgenic male fly contains a GAL4 construct downstream of the promoter of a gene of interest. The male is crossed to a transgenic female with a UAS-Marker construct. The F1 progeny will be able to activate the GAL4/UAS system, which will mark the expression of the gene of interest in a spatial and temporal manner.
2.2.1 GAL80

The GAL4/UAS system can be controlled using a GAL4 repressor called GAL80. GAL80 is an inhibitor protein which binds to the *GAL4* promoter and blocks transcription (Duffy, 2002). A GAL4/UAS system can then be regulated by inactivating the GAL80 repression.

2.3 Mosaic Analysis with a Repressible Cell Marker (MARCM)

Mosaic Analysis with a Repressible Cell Marker (MARCM) is a genetic technique used to label cells that have acquired a specific genotype at a specific point in time in *Drosophila*. The genotype of the labeled cell is usually a homozygous lethal mutant. MARCM generates a homozygous mutant cell in an otherwise heterozygous individual fly. The system is controlled so that labeling is induced at a given time in, in a specific tissue of the fly. There are two main components to MARCM: a controlled GAL4/UAS – GAL80 system and the FLP/FRT system. In a MARCM analysis the GAL80 must be separated from the GAL4 insertion using a FLP/FRT system.

This system involves a genetic cross between a male and a female fly where the gene of interest is one and the components of the GAL4/UAS system are on the other (*Fig. 2.2A*). An enzyme called flippase (FLP) catalyzes the homologous recombination of two Flippase Recognition Targets (FRTs). The gene encoding for flippase in a MARCM system is heat-shock sensitive, meaning that it will only be transcribed after a heat-shock induction. Flippase will only catalyze the recombination at FRT sites in chromosomes that have undergone the S phase, producing 2 sister chromatids per chromosome (*Fig. 2.2B*). The production of twin spots will depend on the orientation of the chromosomes at the metaphase plate. The chromatids containing *GAL80* must segregate from the gene of interest in order for the GAL4/UAS system to activate
(Fig. 2.2C). If the correct orientation is set at the metaphase plate, then twin spots will be produced where one daughter is reporting for the gene of interest via GAL4/UAS and the other has no visible expression due to the system repression by GAL8 (Wu & Luo, 2006).
Figure 2.2 Mosaic Analysis with a Repressible Cell Marker (MARCM) (A) Parental genotypes: Male – tubGal80 hsFLP FRT19A/Y; Su(H)GAL4+UAS-GFPnls/CyO. Female – hntXE81 FRT19A/FM7. The male and female parental lines are crossed, where the wanted progeny have the following genotype: tubGAL80 hsFLP FRT19A/hntXE81 FRT19A; Su(H)GAL4+UAS-GFPnls/+ . (B) Flp-mediated recombination. The progeny are heatshocked at the second instar stage and only chromosomes that have duplicated their DNA content will undergo flp-mediated homologous recombination, resulting in conformation ii. (C) Production of twin spots. The chromosomes that have undergone flp-mediated recombination will have a 50% chance at orienting themselves appropriately - orientation of the chromatids at the metaphase plate (dashed lined). Twin spots are formed only when Gal80 is segregated from hntXE81.
2.4 Perma-Twin

The Perma-Twin method allows both cells of a twin spot to be marked. The system is a mitotic recombination-dependent lineage-labeling method that is derived from the MARCM system. The system is regulated by a temperature sensitive GAL80 repressor that inhibits GAL4 activity, just like in the MARCM system. Under 18°C, the Perma-Twin system is inactive due to activity of the GAL80 repressor. A temperature shift to 29°C derepresses the system by inactivating GAL80, which allows the GAL4/UAS system to be turned on – the system allows conditional activation of twin spot induction. The GAL4 driver is ubiquitous, Actin-GAL4, which drives expression of UAS-Flp, that will drive FLP-mediate mitotic recombination just like in the MARCM system. The labeling of the cells depends on two fusion reporter lines and two inhibitors (Fig. 2.3). The two fusion reporters are under UAS control: UAS-CD8-GFP and UAS-CD2-RFP. The nature of the two inhibitors is a micro-RNA and they too are under UAS control: UAS-CD2-miRNA and UAS-GFP-miRNA (Fernandez-Hernandez et al., 2013). The inhibitors are designed to suppress expression of the reporters so that the twin spots are marked by one reporter resulting in differential labeling. The crossing scheme of the Perma-Twin method is described in Appendix 1, Table 2.
**Figure 2.3 Perma-Twin method.** There are two fusion reporters: UAS-CD8-GFP and UAS-CD2-RFP. They also have corresponding inhibitors driven by micro-RNAs: UAS-CD2-miRNA and UAS-GFP-miRNA. A temperature sensitive GAL80 repressor allows the GAL4 system to be regulated. The GAL4 driver is ubiquitous (Actin-GAL4) and its activation is required to turn on flippase, which is under UAS control. The flippase enzyme is required to catalyze the mitotic recombination at the FRT sites, just like in the MARCM system. The system is turned on when a temperature shift is made from 18°C to 29°C. Flp-mediated mitotic recombination allows differential labeling in the daughter cells with the activation of the reporter genes and the micro-RNA lines ([https://bdsc.indiana.edu/stocks/misc/twinspot_marcm.html](https://bdsc.indiana.edu/stocks/misc/twinspot_marcm.html), Fernandez-Hernandez *et al.*, 2013).
2.5 Confocal Microscopy

All midguts were imaged using a Nikon Eclipse 90i microscope fitted with a Nikon D-eclipse C1 scan head, and a 20x objective Nikon CFI Plan Apo VC lens. Images were captured using the Nikon EZ-C1 software. Z-stack scans of the midguts were taken in slices of 2.00 µm. Live imaging was performed as described previously.

2.6 Time-Lapse Video

Midguts were dissected and mounted in a water-based solution of Schneider’s Insect Medium from Sigma Aldrich. Z-stack scans of midgut epithelia were taken in slices of 2.00 µm every 15 minutes for a total of 9 hours. A projection of the 36 z-stacks was produced to make a time-lapse video of larval midgut development.

2.7 Live Image Mount

Confocal imaging midguts were dissected and mounted in a 50/50 mix of halocarbon oil 27 and halocarbon oil 700. Midguts dissected for time-lapse videos were mounted in Schneider’s insect medium solution.

2.8 Cell Counts

Cell counts were made in the MARCM experiments where the number of GFP-positive cells were compared between control groups and experimental groups. GFP-positive cells were found to be spread randomly along the length of the midgut epithelium, with most of the GFP expression found near the anterior larval midgut. Since each midgut length and width varied, cell counts were made within a frame of 50 cells, maximum.
Chapter 3: Results

3.1 Peripheral Cell Specification and Differentiation

The timing of PC differentiation is essential to know in order to examine the biology and the genetics of the transient stem cell niche. Confocal microscopy was the method used to find the time at which PCs formed in the larval midgut. The genotype that was produced for this experiment was the following:

\[ \text{esg-GAL4} > \text{UAS-GFP}^{\text{NLS}} \]

The GAL4 driver, \textit{esg}, encodes a transcription factor that is expressed in both AMPs and PCs. Expression is higher in the PCs and this is apparent with the higher expression of the GFP marker, which in this case includes a nuclear localisation signal (NLS). Images were taken after 96 hours and before 120 hours After Egg Deposition (AED) in attempts to pinpoint the time of PC differentiation. This time frame was chosen based on the paper published by Jiang & Edgar in 2009 where they showed confocal images of AMP clusters at 120 hours versus at 96 hours AED. My observations showed that at 100 hours AED AMPs had undergone a second round of symmetric division where they were in groups of three to four in a linear formation shaped like peas in a pod (Fig. 3.1, 3.2A). At 115 hours AED, AMPs were in clusters with a visible PC that is apparent with the higher level of GFP expression. Clusters were small, with a maximum of 2 AMPs and 1 PC per niche (Fig. 3.2B). Finally, at 125 hours AED, it was curious to look at the growth of AMP clusters after PC formation. At 125 hours AED, I had found clusters with 2 and at times 3 PCs (Fig. 3.2C). This observation begged the question of whether or not there was a second wave of PC formation. The sample size of larval midguts dissected for each of the three time points in this experiment was 65, 70, and 68, respectively.
Figure 3.1 Timeline of AMP cluster formation. AMPs start off as single solitary cells. As development progresses, they undergo up to two rounds of symmetric division until one AMP is summoned to undergo a round of asymmetric division to form a PC (in green) at the mid-third instar stage. *The enterocytes (in pink) undergo endoreplication, hence the enlargement of the nuclei (Zielke, Edgar, & DePamphilis, 2013).
A similar experiment was performed to mark the PCs differently from the AMPs. Here, the genotype that was selected for live imaging was the following (see Appendix 1, Table A1.1 for crossing scheme):

\[
\text{Su(H)GAL4}\text/>UAS-GFP\text{\textsuperscript{NLS}}, \text{His2Av-RFP}
\]

\[
+ \quad +
\]

\text{Su(H)GAL4} is a PC-specific GAL4 driver that is coupled with a UAS-GFP\text{\textsuperscript{NLS}} marker. The Histone-2Av-RFP fusion that is located on the third chromosome serves as a background marker that labels the AMPs, PCs, and it also marks the larval enterocytes. A sample size of 91 larval midgut dissections showed that PCs would be labeled via \text{Su(H)GAL4} expression at 115 AED – just before the larvae enter the wandering stage (Fig. 3.3B). Midguts dissected at a 110 AED showed no signs of \text{Su(H)GAL4}>UAS-GFP\text{\textsuperscript{NLS}} expression (Fig. 3.3A). In other words, there was no GFP expression when the AMPs were in the ‘peas in a pod’ morphology.
Figure 3.2 Confocal images of larval midguts. Genotype: \textit{esgGAL4} > \textit{UAS-GFP^{NLS}}

Taken at (A) 100 hours, (B) 115 hours, and (C) 125 hours AED. \textit{EsgGAL4} is driving expression of \textit{UAS-GFP^{NLS}}. \textit{Esg} is expressed in AMPs and in PCs with higher expression in the PCs, which can be seen with the higher level of GFP expression.

Scale bars: 20\(\mu\text{m}\).
Figure 3.3 Confocal images of larval midguts.
Genotype: \textit{Su(H)GAL4+UAS-GFP}^{nls} : \textit{His2Av-RFP}

Taken at (A) 110 hours, (B) 115 hours, and (C) 125 hours AED. \textit{Su(H)GAL4} is driving expression of \textit{UAS-GFP}^{nls} (in yellow). The His2Av-RFP (in blue) fusion protein is serving as a background, labeling AMPs, PCs, and larval ECs in the background. Scale bars: 20µm.
3.2 AMP Cluster Lineage Analysis

Determining the origin of the PC and the lineage of the AMPs within a cluster was of interest. In attempt to answer these questions, the Perma-Twin system was used. The goal of using this system was to examine the lineage of the AMPs and PCs. Theoretically, the PC and the AMP from which it asymmetrically divided would be differentially labeled. I hypothesized that the AMP and PC clusters are established from two cells (Fig. 3.4A). In order to label the two cells differentially, the system was activated – a temperature shift was made – just before PC production. The development of Drosophila is slower at 18°C. The time of PC formation, which is 115 hours AED under 25°C, is approximately doubled to about 9.5 days instead of 4.5 days. The larvae were subsequently shifted to 29°C just before PCs were expected at 18°C. The frequency of PC-AMP twin spots differently labeled was not as prevalent as expected. The red arrow is pointing to an AMP and a PC that supports the hypothesis (Fig. 3.4B). However, the white arrow is pointing to a cluster that must have been established from 3 or more AMPs since two cells in that cluster are labeled with RFP (Fig. 3.4B). In a sample size of 48 larval midgut dissections, differentially labeled PC-AMPs duos were observed on average 2 out of 50 AMP clusters. Most of the AMP clusters, including their respective PCs, were labeled with just one fluorescent protein (Fig. 3.4B). There seems to be a pattern of expression where patches of clusters are labeled with either RFP or GFP. Further studies must be done with controls to show that the Perma-Twin system is not leaky.

It is still unclear if there is a second wave of PC formation based on the results produced from the Perma-Twin experiment. This is again due to the observation that there was no mosaic pattern of labeling within the AMP clusters. Temperature shifts were also made early on in larval development in later on, after PC formation, and the results were the same as Figure 3B.
In general, this analysis was not pursued further, but a live imaging approach was developed instead. As will be explained, the results and interpretations of live imaging suggest this type of lineage analysis is not tractable in this system.
*Figure legend on next page*
Figure 3.4 Perma-Twin Confocal Images. (A) Diagram of expected result between an AMP and a PC that are differently labelled using the Perma-Twin system. (B) Confocal images of Perma-Twin larval midguts, and the separated channels to show the GFP (in yellow) and RFP (in blue) expression. The arrow is pointing to a twin spot that is showing a PC marked with GFP and its associate AMP cluster marked in RFP. Scale Bars: 20µm.
3.3 Adult Midgut Precursor Movement

A similar attempt to examine AMP lineage and the possibility of a second wave of PC differentiation, a time lapse video technique was developed. Two sets of imaging were designed: the first to capture AMP proliferation before PC formation and the second to examine the events subsequent to PC differentiation. Unfortunately, due to the instability of the midguts, before PC formation, live imaging was only successful on older larvae, after PCs differentiated. The cross to generate the genotype listed below allowed me to differentially label PCs and AMPs (see Appendix 1, Table A1.2 for crossing scheme):

\[
\text{brGAL4}^{+}\text{UAS-H2B-RFP} \quad \text{NRE-GFP} \quad \text{(III)}
\]

*Broad (br)* is a gene that encodes a zinc finger transcription factor and it is expressed in both AMPs and PCs. *BrGAL4* drives expression of a *UAS* line tagged to the histone *H2B-RFP* encoding fusion protein. This *GAL4/UAS* line is crossed to a *Notch Responsive Element (NRE) – GFP* reporter gene. The *NRE* is expressed exclusively in PCs in the larval midgut epithelium – GFP expression is hence exclusive to the PCs. Midguts were mounted for live imaging shortly after PC formation. Over the course of 9 hours, AMP clusters merged forming islands that went from groups of 15 cells to 30 cell islands. The merging of clusters also created larger islands with 2 to 3 PCs (Fig. 3.5). These observations explained the confocal images taken of the Perma-Twin system and also negated the entire experiment. The merging of clusters explains why there were clusters in the Perma-Twin system labeled either with GFP or RFP. It negates the system as well because AMPs are dynamic with migratory ability. The Perma-Twin lineage tracing system could only work if the cells examined are stationary. In this case, no lineage could be traced if the cells are moving around and merging with each other, especially in a system with only two
different reporter lines.

Another observation from the time-lapse video showed that certain PCs ‘jumped’ from one cluster to another. PCs are therefore, according to these observations, dynamic and not limited to just one cluster. This observation also negates the hypothesis of having a second wave of PC formation. PCs are formed once but the appearance of multiple PCs in one cluster is due to the migration of the clusters and the dynamic behavior of the PCs (Fig. 3.6). It is also important to note a caveat with these time-lapse videos. The fact that live imaging is performed on the midgut ex vivo can have an effect on the organ and may steer the cell biology away from its normal course of action. A total of 4 time-lapse videos were performed to show the dynamics of AMP clusters.
Figure 3.5 Live imaging of AMP cluster movement. Confocal snap shots of time-lapse video that shows two AMP clusters merging after 8 hours and 30 minutes. *NRE-GFP* (in yellow) expression marks the PCs and *brGAL4>UAS-H2B-RFP* (in blue) labels the AMPs. Scale bar: 20µm.
Figure 3.6 Movement of Peripheral Cells. PCs are dynamic and they are not limited to one AMP cluster. Confocal snap shots of time-lapse video. The two PCs that are jumping around are labeled with a yellow and white asterisk. NRE-GFP fusion protein (in yellow) labels the PCs and brGAL4>UAS-H2B-RFP (in blue) labels the AMPs and PCs. Scale bar: 20µm.
3.4 The role of Ecdysone in AMP cluster growth

It has been previously shown that AMPs requires Ecdysone signaling but the question of PCs requiring Ecdysone signaling has not been addressed. We used a temperature-sensitive LOF ecdysone allele to examine mutant phenotypes. The genotype below was produced for this experiment:

\[NRE-GFP; \text{ecd st ca}\]

18°C is the permissive temperature whereas 29°C is the restrictive temperature – the larvae are viable at 18°C. The genotype above has an \(NRE-GFP\) reporter that marks PCs in the larval midgut. A temperature shift was performed before PC differentiation. Shifted larvae remained at the third instar stage for up to 12 days. During this prolonged third larval stage, AMP clusters did not proliferate but there is \(NRE-GFP\) reporter gene expression. This supports the interpretation that in the absence of Ecdysone signaling there is no AMP proliferation but there is PC differentiation (Fig. 3.7). The GFP is encasing the AMPs that appear as black holes surrounded by the \(NRE-GFP\) reporter.
Figure 3.7 Confocal images of the LOF ecdysone midguts at the late third instar stage. NRE-GFP is reporting the PCs. (A) The vertical band on the left-hand side is the hindgut-midgut junction. At this stage in development (the late wandering third instar stage), the AMP clusters should hold up to 30 cell islands. A LOF allele of ecdysone has severely inhibited the proliferation of AMPs within their clusters and it has also inhibited the generation of clusters with more than 1 PC. Control group of NRE-GFP late wandering third instar larval midguts. A total of 36 larval midguts were examined for each group.

Scale Bars: 20µm.
3.5 The Role of hnt Expression in Peripheral Cell Formation

The effect of a hnt LOF mutation on the production of PCs and hence the establishment of a transient stem cell niche was a particular research question of interest. The MARCM system was used to produce mosaic clones specific to PCs. Two hnt LOF alleles were used: \( hnt^{XE81} \) and \( hnt^{FG47} \). The following genotype was produced to induce mosaic clones (see Appendix 1, Table 1.2 for crossing scheme):

\[
\begin{align*}
tubGAL80 \hspace{0.2cm} & \hspace{0.2cm} hsFLP \hspace{0.2cm} & \hspace{0.2cm} FRT19A \hspace{0.2cm} & \hspace{0.2cm} Su(H)GAL4+UAS-GFP^{nls} \hspace{0.2cm} & \hspace{0.2cm} His2Av-RFP \\
& \hspace{0.2cm} hnt^{XE81} & \hspace{0.2cm} FRT19A & + & + \\
or & \hspace{0.2cm} hnt^{FG47} & \hspace{0.2cm} FRT19A \\
or & \hspace{0.2cm} y w & FRT19A \ (control) \\
\end{align*}
\]

For all three groups, clones were induced 48 hours AED, before PC differentiation. Larvae were dissected for confocal imaging 72 hours post-heat shock. Clones were consistently present in the \( yw \ FRT19A \) control group (Fig. 3.8A). Conversely, the hnt LOF alleles – \( hnt^{XE81} \) and \( hnt^{FG47} \) – expressed 0 clones along the length of the midgut epithelium (Fig. 3.8B). To ascertain that this result was not due to a technical failure of the MARCM system, different tissues in the same individual larva were examined for clones. Indeed, plenty of hnt LOF clones were present in the imaginal discs where hnt is not required (Fig. 3.8C). A total of 63 midguts were dissected for clonal analysis in the three groups – the two hnt LOF groups and the control. The average number of clones expressed along the length of the midgut epithelium in the control was five (Fig. 3.9).

Characterization of both LOF hnt alleles was also done via immunostaining with monoclonal and polyclonal anti-hnt antibodies. Interestingly, both alleles came out polyclonal
positive. The monoclonal antibody stain for \( hnt^{XE81} \) was negative whereas as the monoclonal stain in \( hnt^{FG47} \) had a punctate pattern of expression (see Appendix 2, Figure 1). These results suggested that the LOF alleles of \( hnt \) are still producing protein. Although dysfunctional protein, the proteins were being recognized by the anti-hnt antibodies and therefore could not be used in further experiments to demonstrate LOF phenotypes.
Figure 3.8 MARCM clonal analysis of hnt expression in the Peripheral Cells of the larval midgut. (A) Control group showing larval midgut epithelium with the PC-specific Su(H)GAL4>UAS-GFP^{nls} (in yellow) expressing clones with His2Av-RFP (in blue) expression in the background. (A’) GFP-positive cells are His2av-RFP positive. (B) hnt^{XE81} larval midgut – no clones are present. (C) same hnt^{FG47} larva form (B) showing hnt LOF function clones in the imaginal disc.
3.6 Examination of \textit{hnt} LOF Mutations on Peripheral Cell Formation and Inhibition of Cell Death

The absence of MARCM clones in a \textit{hnt} LOF background led to two plausible hypotheses:

1. The PCs are signaled to differentiate but quickly undergo cell death
2. The PCs are not specified to differentiate by their respective AMP

3.6.1 Programmed Cell Death

An experiment was designed to show whether or not the \textit{hnt} null PCs were undergoing cell death. The cross set up had two generations of progeny, where the F2 generation would have provided the genotype of interest to study the effects of programmed cell death (see Appendix 1, Table 2 for crossing scheme). The genotypes of the F1 and F2 generation that were of interest are:

\[
\text{F1 } \delta \ y \ w \ hnt^{XE81} \ FRT19A; \ Su(H)-GAL4+UAS-GFP^{nls} \ Y \ Dp(1;2) \ 4FR \ Dup \\
\text{F2 } \♀ \ \text{tubGAL80 hsFLP FRT19A; Su(H)-GAL4+UAS-GFP^{nls}} \ hnt^{XE81} \ FRT19A \ UAS-p35
\]

This cross was designed to mark \textit{hnt}^{-/-} MARCM PCs. If it is true that \textit{hnt}^{-/-} PCs undergo apoptosis, then inhibiting their death via p35 caspase inhibition would force them to remain alive. This would then allow the PCs to be marked by \textit{Su(H)}GAL4>UAS-GFP^{nls}.

However, the experiment was not generating progeny successfully. The F1 generation was undergoing embryonic lethality and larvae were not developing. The chromosome containing the \textit{Dp(1;2) 4FR Dup} transgene was likely the cause of embryonic lethality. This duplication contains a wildtype copy of \textit{hnt} but also contains a wildtype copy of \textit{Notch}. It is
hypothesized that excessive activation of Su(H)GAL4 was resulting in cellular toxicity – possibly the excessive expression of GFP (see Discussion section 4.3.1).
Chapter 4: Discussion

4.1 A Model for Peripheral Cell Differentiation

The mechanism by which one cell at the 2 to 3 AMP cluster stage, is specified to become a PC is unknown. My observations showed that AMP clusters initiate from a ‘peas in a pod’ type morphology. It is possible that the cell in the middle is specified to become a PC. One hypothesis would be that because of the linear arrangement of the cells, the middle cells could be receiving a high level of Notch activation. The idea being that the cell in the middle has increased surface area to receive Notch signaling (Fig. 4.1).

Figure 4.1 Proposed model of PC differentiation with respect to Notch signaling. The first model suggests an obvious mechanism by which Notch signaling would select the PC. The second model is much less likely to occur given the direction of Notch signaling and the positioning of the AMP that would differentiate into a PC.
4.2 Merging of AMP Clusters and Peripheral Cell Behaviour

The merging of AMP clusters was first suggested as a Notch LOF phenotype. It was hypothesized that PCs inhibit the merging of clusters (Mathur et al., 2010). Contrary to this observation, I showed through live imaging that normal larval midguts show AMP clusters that merge.

Jiang & Edgar published in 2009 that AMPs disperse as solitary cells in early development as a result of maintaining a low level of EGFR signaling. An increase in EGFR signaling decreases the dispersion of AMPs, early in larval development. EGFR activity and the mobility of AMPs are hence inversely related. A high level EGFR activity promotes the expression of adhesion molecules like Shotgun (Drosophila epithelial cadherin, de-cadherin) that limit cell migration and promotes the formation of clusters (Jiang & Edgar, 2009). That being said, it is possible that a decrease in EGFR signaling occurs in the late third instar stage, just before pupal formation, that allows the AMPs to regain their dispersal behavior. This would be a plausible explanation for the merging observation of the clusters in the wandering third larval stage. Nonetheless, further experiments must be performed to address this question.

Ecdysone signaling may also affect the merging of AMP clusters. A LOF mutation in ecdysone created a severe loss of AMP proliferation but the PCs seemed to be intact. Clusters were only 2 to 3 cells large with only 1 PC encasing them (see Results, Fig. 3.7). Ecdysone and EGFR signalling regulate the development of follicle cells. A high-titer ecdysone pulse occurs at the end of the third instar larval stage, around the same time as the AMP clusters merge (Fig. 4.2). Ecdysone signaling in male gonads of Drosophila was shown to inhibit the differentiation of the cyst stem cells – the somatic cell lineage in the tissue. Simultaneously, EGFR activity increased and was shown to antagonize ecdysone signaling, promoting the differentiation of the
cyst stem cells (Qian et al., 2014). In addition, ecdysone activity has an inverse relationship with the expression of De-cadherin, the *Drosophila* epithelial adhesion molecule that regulates cellular migration. In follicle cells, the increase in ecdysone activity leads to a decrease in levels of De-cadherin, which decreases cell migration and vice versa (Hackney et al., 2007). It is plausible that there may be a requirement for a specific level of ecdysone activity that limits EGFR signaling to prevent premature differentiation of the stem cell niche and that regulates de-cadherin activity to allow the PCs to migrate away from their original AMP clusters and form the transient pupal midgut (Hackney et al., 2007, Mathur et al., 2010, Qian et al., 2014).

My observations also showed that once clusters merged, PCs were dynamic and jumped from one cluster to the next. Some PCs would leave clusters and place themselves in between the stem cell islands forming the transient larval midgut (Mathur et al., 2010). It is reasonable to state that the movement of the PCs is regulated by the waves of ecdysone activity experiments need to be done to support this hypothesis. Finally, it is important to ascertain that the number of PCs remained constant throughout development – this would demonstrate that there really is no second wave of PC differentiation. Further calculations need to be performed beyond this point in order to support this hypothesis.
Figure 4.2 Ecdysone titre throughout Drosophila development. Levels of ecdysone activity are lowest in the third instar. There is a sharp rise in the late third instar stage, just before the larva becomes a prepupa. [http://www.biology.ualberta.ca/king-jones_lab/KKJ_lab/steroids.html](http://www.biology.ualberta.ca/king-jones_lab/KKJ_lab/steroids.html)
4.3 The Role of Hindsight in Peripheral Cell Formation

Mosaic analysis of hnt mutant clones in the larval midgut showed that AMP clusters failed to produce $hnt^{+/} Su(H)GAL4>UAS-GFP^{nls}$ positive cells. Morphological analysis of these midguts also showed an absence of a crescent shaped cell surrounding the periphery of an AMP cluster, which is distinctive to the PCs. Loss of function $hnt$ in the AMPs could have caused the AMPs undergoing asymmetric division to form a PC to fail in specifying the asymmetric daughter as a PC. There may be $hnt$ downstream target genes required for this specification to take place. Hnt has over 50 downstream targets in the salivary glands alone and even more in other tissues (Wilk et al., 2000).

It is plausible to state that $hnt$ may be necessary for PC specification. Previous work has shown that $hnt$ expression is required for the ISC-to-EC differentiation. A $hnt$ LOF mutation prevents ISCs from differentiating into ECs and conversely, an overexpression of $hnt$ forced all ISCs to become ECs (Baechler et al., 2015). Since $hnt$ encodes a transcription factor, some of its downstream targets could be cell fate-specific genes that are activated and/or repressed to turn on the PC-differentiation pathway. Overexpression of $hnt$ forced all of the AMPs to differentiate into adult ECs with none left to differentiate into adult ISCs (Baechler et al., 2015).

Further analysis has to be done in order to establish with certainty that $hnt$ is required for PC formation – an anti-$Su(H)$ immunostaining can be done to mark the PCs in a $hnt$ mutant background. Anti-$hnt$ immunostaining was rendered ineffective after the two $hnt$ mutants, $hnt^{XE81}$ and $hnt^{FG47}$, were detected in the amnioserosa of the Drosophila with the anti-$hnt$ monoclonal and polyclonal antibodies (see Appendix 2, Figure A2.1).

It is also unlikely that $hnt$ expression is required for the asymmetric division of an AMP and PC. $Hnt$ is a downstream target of Notch signaling where the cell destined to become a PC
receives the signal. This means that the PC-to-be cell could possibly have an upregulation of \textit{hnt} expression. The asymmetric division will already have occurred in this situation and therefore it is unlikely that a \textit{hnt} LOF would prevent the AMP form undergoing asymmetric division.

Furthermore, the function of \textit{hnt} could be similar to its function in cone-cell induction. Hnt could induce PC formation by regulating the levels of Delta ligand in the AMPs. In cone cell precursors, \textit{hnt} was shown to elevate the levels of Delta ligand in order to achieve cone-cell induction (A. T. Pickup, Ming, & Lipshitz, 2009).

It remains unknown whether a LOF of \textit{hnt} causes PCs to undergo programmed cell death. \textit{Hnt} expression is required for germ band retraction – a LOF mutation causes embryonic lethality. It was suggested that the potential role of \textit{hnt} was to prevent premature apoptosis and thereby promoting survival of the tissue (M. L. R. Yip, Lamka, & Lipshitz, 1997). A similar role is possible in the case of PC survival, where the cell may require \textit{hnt} expression in order to survive. However, no inferences can be made until further experiments can support the hypothesis that \textit{hnt} is involved in preventing programmed cell death in PCs.
4.3.1 Programmed Cell Death

The experiment designed to examine potential PCD of the PC in a $hnt^{cr}$ background failed to give off progeny with the correct genotype. The extra copy of the Notch gene could have been the cause of death of the F1 progeny. With an additional copy of Notch, overexpression leads to hyperactivation of Notch signaling. This leads to an overexpression of $Su(H)$, a downstream target of Notch signaling, which in this case also overactivates the GAL4/UAS system. Overexpression of $Su(H)$ drives overexpression of $UAS-GFP^{nls}$, resulting in a higher than normal level of GFP that is toxic to the embryo (Liu H. et al., 1999). It is plausible that the cause of lethality of the F1 progeny was due to GFP toxicity (see Results 3.4).

Overall, although this analysis has indicated that $hnt$ is required for PC differentiation, the absence of PC cells may be attributable to a failure of PC specification or onset of cell death in the $hnt$ mutant PCs. Further studies need to be done in order to ascertain these unknowns.
Chapter 5: Future Directions

The cell biology of the merging of AMP clusters remains an unresolved matter. Examining the mechanism of action of how AMP clusters merge and why they do so during development will provide information about the behavior of these stem cell niche populations and how they contribute to morphogenesis.

Furthermore, it is paramount to this project to examine the reason why \textit{hnt}-null PCs are not found in mosaic clones. The first line of experiments to do will provide supporting evidence that the observed phenotype is due to a failure of specification of PCs – \textit{hnt} expression is required to induce PC differentiation. The second line of experiments, although it is stated that this hypothesis is less likely to occur, is to establish whether or not programmed cell death is killing the \textit{hnt}-null PCs in the MARCM analysis. These experiments will provide more information on the functional role of \textit{hnt} in morphogenesis of the \textit{Drosophila} midgut and on its role in establishing a stem cell niche.
References


## Appendix 1: Stocks and Crossing Schemes

### Table A1.1: Genetic Stocks

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drivers and Reporters</strong></td>
<td></td>
</tr>
<tr>
<td>( Su(\text{H})\text{GBE-GAL4} + \text{UAS-GFP}^{\text{NLS}} )\text{CyO}</td>
<td>Bruce H. Reed Laboratory</td>
</tr>
<tr>
<td>tub\text{GAL80 hsFLP FRT19A; }( Su(\text{H})\text{GBE-GAL4+UAS-GFP}^{\text{NLS}} )\text{CyO}</td>
<td>Bruce H. Reed Laboratory</td>
</tr>
<tr>
<td>( \text{w; (UAS-CD8-GFP)*; brGAL4+UAS-H2b-RFP} )/\text{TM6B}</td>
<td>Bruce H. Reed Laboratory</td>
</tr>
<tr>
<td><strong>Hnt Alleles</strong></td>
<td></td>
</tr>
<tr>
<td>( \text{hnt}^{\text{XE81}} ) FRT19A/ FM7, \text{Kr}</td>
<td>Bruce H. Reed Laboratory</td>
</tr>
<tr>
<td>( \text{hnt}^{\text{FG47}} ) FRT19A/FM7c, \text{sn}</td>
<td>Trudi Schupbach Laboratory</td>
</tr>
<tr>
<td><strong>Fusion Protein</strong></td>
<td></td>
</tr>
<tr>
<td>( \text{w; } \text{His2Av-RFP} )</td>
<td>Bloomington <em>Drosophila</em> Stock Center</td>
</tr>
<tr>
<td><strong>Reporter Line</strong></td>
<td></td>
</tr>
<tr>
<td>( \text{w;; } \text{NRE-GFP} )</td>
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</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>( \text{y w FRT19A} )</td>
<td>Bloomington <em>Drosophila</em> Stock Center</td>
</tr>
</tbody>
</table>

*(UAS-CD8-GFP) is floating on the 2\text{nd} chromosome
**Appendix 1: Stocks and Crossing Schemes**

**Table A1.2:** Crossing schemes and the progeny genotypes of interest.

<table>
<thead>
<tr>
<th>Figure #</th>
<th>Cross</th>
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<tbody>
<tr>
<td>1</td>
<td>$♀ \text{Su(H)GBE-GAL4+UAS-GFP}^{\text{nls}}<em>{\text{CyO}} \times ♂ w; \text{His2Av-RFP}</em>{\text{Y}}$</td>
</tr>
<tr>
<td></td>
<td>$\text{*floats}$</td>
</tr>
<tr>
<td>2 &amp; 4</td>
<td>$♀ w; (\text{UAS-CD8-GFP})<em>{\text{TM6B}}^{\text{*}} \times ♂ w; \text{NRE-GFP}</em>{\text{Y}}$</td>
</tr>
<tr>
<td></td>
<td>$\text{F1 brGAL4+UAS-H2B-RFP} \text{ (III)}_{\text{NRE-GFP}}$</td>
</tr>
<tr>
<td>3</td>
<td>$♀ w; \text{FRT40A UAS-CD8-GFP UAS-CD2-miR; Act-GAL4 UAS-Flp}_{\text{CyO}} \text{TM6B} \times$</td>
</tr>
<tr>
<td></td>
<td>$♀ w; \text{FRT40A UAS-CD2-RFP UAS-GFP-miR; tubGAL80}^{\text{TS}}_{\text{CyO}} \text{TM6B}$</td>
</tr>
<tr>
<td></td>
<td>(since the genes of interest are located on autosomal chromosomes (2\text{\textsuperscript{nd}} and 3\text{\textsuperscript{rd}})) both males and females from 1 and 2 can be used to set up a Perma-Twin cross)</td>
</tr>
<tr>
<td>4A</td>
<td>4B &amp; C</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------</td>
</tr>
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</table>
| ♂ tubGAL80 hsFLP FRT19A; Su(H)GAL4+UAS-GFP<sub>nls</sub>  
            CyO  
            x  
            ♂ w; ; His2Av-RFP  
| F1 ♂ tubGAL80 hsFLP FRT19A; Su(H)GAL4+UAS-GFP<sub>nls</sub>; His2Av-RFP  
            Y  
            +  
            +  
            x  
            ♂ y w FRT19A  
| F2 ♂ tubGAL80 hsFLP FRT19A; Su(H)GAL4+UAS-GFP<sub>nls</sub>; His2Av-RFP  
            y w  
            FRT19A;  
            +  
            +  

| F1 ♂ tubGAL80 hsFLP FRT19A; Su(H)GAL4+UAS-GFP<sub>nls</sub>; His2Av-RFP  
            Y  
            +  
            +  
            x  
            ♂ hnt<sup>XE81</sup> FRT19A  
            FM7, Kr  
| F2 ♂ tubGAL80 hsFLP FRT19A; Su(H)GAL4+UAS-GFP<sub>nls</sub>; His2Av-RFP  
            hnt<sup>XE81</sup>  
            FRT19A  
            +  
            +  

### Results 3.4.1

<table>
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<th>Crossbreeding Details</th>
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<tr>
<td>F1</td>
<td>♀ y w hnt&lt;sup&gt;XE81&lt;/sup&gt; FRT19A; Dp(1;2) 4FR Dup x ♂ Su(H)-GAL4+UAS-GFP&lt;sup&gt;nls&lt;/sup&gt; CyO</td>
</tr>
<tr>
<td></td>
<td>♀ w hnt&lt;sup&gt;XE81&lt;/sup&gt; FRT19A; Su(H)-GAL4+UAS-GFP&lt;sup&gt;nls&lt;/sup&gt; Y Dp(1;2) 4FR Dup</td>
</tr>
<tr>
<td>F2</td>
<td>♀ tubGAL80 hsFLP FRT19A; UAS-p35 x F1 ♂ hnt&lt;sup&gt;XE81&lt;/sup&gt; FRT19A; Su(H)-GAL4+UAS-GFP&lt;sup&gt;nls&lt;/sup&gt; Y Dp (1;2) 4FR Dup</td>
</tr>
<tr>
<td></td>
<td>♀ tubGAL80 hsFLP FRT19A; Su(H)-GAL4+UAS-GFP&lt;sup&gt;nls&lt;/sup&gt; hnt&lt;sup&gt;XE81&lt;/sup&gt; FRT19A UAS-p35</td>
</tr>
</tbody>
</table>
Appendix 2: Anti-hnt immunostains

Figure A2.1: Immunostains of hindsight mutants in the amnioserosa of Drosophila. (A) hnt$^{XE81}$ mutant embryo double immunostain: anti-hnt polyclonal in blue (TRITC) and anti-hnt monoclonal in yellow (FITC). There is no detection of the monoclonal antibody in the hnt$^{XE81}$ mutant. (B) hnt$^{FG47}$ mutant embryo double immunostain – same as hnt$^{XE81}$. The monoclonal antibody in the hnt$^{FG47}$ is detected in a punctate form and the polyclonal is detected the same as hnt$^{XE81}$ polyclonal.