Dissecting the Mechanisms and the Nutritional Requirements of the Expansion of Normal and Tumorigenic Intestinal Stem Cell Lineages in Drosophila melanogaster

Wen-Chih Lee
Washington University in St. Louis

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Dissecting the Mechanisms and the Nutritional Requirements of the Expansion of Normal and Tumorigenic Intestinal Stem Cell Lineages in *Drosophila melanogaster*

by

Wen-Chih Lee

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2013

St. Louis, Missouri
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<th>Description</th>
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<tbody>
<tr>
<td>Akt</td>
<td>Akt1; CG4006.</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>Apc1</td>
<td>APC-like; CG1451.</td>
</tr>
<tr>
<td>Apc2</td>
<td>APC homolog 2; CG6193.</td>
</tr>
<tr>
<td>Bsk</td>
<td>Basket, JNK homolog; CG5680.</td>
</tr>
<tr>
<td>CDF</td>
<td>chemically defined food</td>
</tr>
<tr>
<td>Ci</td>
<td>cubitus interruptus; CG2125.</td>
</tr>
<tr>
<td>CRC</td>
<td>colon and rectal cancer</td>
</tr>
<tr>
<td>DI</td>
<td>Delta; CG3619.</td>
</tr>
<tr>
<td>Dome</td>
<td>Domeless; CG14226.</td>
</tr>
<tr>
<td>Hop</td>
<td>Hopscotch, JAK-like; CG1594.</td>
</tr>
<tr>
<td>EC</td>
<td>enterocyte</td>
</tr>
<tr>
<td>EE</td>
<td>enteroendocrine cell</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Egfr</td>
<td>EGF receptor; CG10079.</td>
</tr>
<tr>
<td>Esg</td>
<td>Escargot; CG3758.</td>
</tr>
<tr>
<td>FLP</td>
<td>Flippase</td>
</tr>
<tr>
<td>FRT</td>
<td>Flippase recognition target</td>
</tr>
<tr>
<td>Gal4</td>
<td>a yeast transcriptional activator</td>
</tr>
<tr>
<td>Gal80</td>
<td>a yeast transcriptional suppressor</td>
</tr>
<tr>
<td>GBE</td>
<td>Grh protein binding element</td>
</tr>
<tr>
<td>GOI</td>
<td>gene-of-interest</td>
</tr>
<tr>
<td>Grh</td>
<td>Grainy head; CG42311</td>
</tr>
<tr>
<td>Inr</td>
<td>Insulin-like receptor; CG18402.</td>
</tr>
<tr>
<td>ISC</td>
<td>intestinal stem cells</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus Kinase/Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>lof</td>
<td>loss-of-function</td>
</tr>
<tr>
<td>MARCM</td>
<td>mosaic analysis with a repressible cell marker system</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin; CG5092</td>
</tr>
<tr>
<td>Myc</td>
<td>diminutive; CG10798.</td>
</tr>
<tr>
<td>N</td>
<td>Notch; CG3936.</td>
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<tr>
<td>Notum</td>
<td>CG13076.</td>
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<tr>
<td>pH3</td>
<td>phospho-histone H3</td>
</tr>
<tr>
<td>PI3K</td>
<td>PI3K92E, CG4141.</td>
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<tr>
<td>Pros</td>
<td>Prospero; CG17228.</td>
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<tr>
<td>Pten</td>
<td>CG5671.</td>
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<tr>
<td>Puc</td>
<td>Puckered, JNK phosphatase; CG7850.</td>
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<tr>
<td>Raptor</td>
<td>CG4320.</td>
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<tr>
<td>Ras</td>
<td>Ras oncogene at 85D; CG9375.</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras homolog enriched in brain ortholog; CG1081.</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>S6K</td>
<td>RPS6-p70-protein kinase; CG10539.</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Socs</td>
<td>Socs36E, Suppressor of cytokine signaling at 36E; CG15154.</td>
</tr>
<tr>
<td>Stat92E</td>
<td>Signal-transducer and activator of transcription protein at 92E; CG4257.</td>
</tr>
<tr>
<td>Su(H)</td>
<td>Suppressor of Hairless; CG3497.</td>
</tr>
<tr>
<td>TARGET</td>
<td>temporal and regional gene expression targeting system</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Tk</td>
<td>Tachykinin; CG14734.</td>
</tr>
<tr>
<td>Tkv</td>
<td>Thickveins, TGFβ type I receptor; CG14026.</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activation sequence</td>
</tr>
<tr>
<td>Upd1</td>
<td>Unpaired 1, outstretched; CG33542.</td>
</tr>
<tr>
<td>Upd2</td>
<td>Unpaired 2; CG5988.</td>
</tr>
<tr>
<td>Upd3</td>
<td>Unpaired 3; CG33542.</td>
</tr>
<tr>
<td>Yki</td>
<td>Yorkie; CG4005.</td>
</tr>
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Publications arising from this thesis


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ACKNOWLEDGMENTS

First and foremost I would like to thank Craig for giving me the opportunity to work in his lab, and for his investment in training me as a scientist. I would also like to thank all members of the Micchelli lab for their friendship and for creating a wonderful working environment for a foreigner without fluent language skills. I am and will always be grateful for their patience to listen to my thoughts and willingness to provide suggestions for my projects. Especially, I am truly grateful to my "PhD-lifetiem" labmate Kate for being a constant source of critical discussions and emotional support since the day we both joined the lab. Special thanks to Marie for feeding us with delicious but probably unhealthy sweet cookies, and to Jill, Micah, and Ryan for providing countless food vials to feed my innumerable fly stocks.

I am also indebted to the previous and current members of my thesis committee, Thomas Baranski, David Beebe, Shin-ichiro Imai, Raphael Kopan, Fanxin Long, Lilianna Solnica-Krezel, and James Skeath, for their insightful suggestions and valuable guidance during my PhD study. I would like to give special thanks for their guidance in initiating the nutritional study of ISCs, which ignites my interest in metabolism of stem cells. This project would not have been possible without the kindness of the Drosophila community in sharing many important materials. Special thanks to Brooke McCartney (Department of Biological Science, Carnegie Mellon University) for the most important stock for this project, the APC loss-of-function flies.

Finally and most importantly, I would like to thank my family for their unconditional love and invaluable trust in me for many years. My parents and my sister have always sent me incredible amounts of Taiwanese souvenirs to soothe my homesickness, which were often sent with lovely drawings from my sweet niece to her uncle. I would like to acknowledge many friends from WU and from Taiwan for their encouragement and support during my PhD study, especially Chun-Chen, Sidney, Annie, Justin, Jeannie, Mike, KC, EJ, and Waylon. Last but not least, I would like to dedicate this thesis to my beloved grandmother who died from colon and rectal cancer, for whom I hope my research can make contributions against colon and rectal cancer.
ABSTRACT OF THE DISSERTATION

Dissecting the Mechanisms and the Nutritional Requirements
of the Expansion of Normal and Tumorigenic Intestinal Stem Cell Lineages

in Drosophila melanogaster

by

Wen-Chih Lee

Doctor of Philosophy in Developmental, Regenerative and Stem Cell Biology
Washington University in St. Louis, 2013
Assistant Professor Craig A. Micchelli, Chair

Organisms are constantly challenged by injury and senescence. To replenish lost cells, adult tissue must contain a cellular reserve to maintain homeostasis. For example, adult stem cells exhibit two unique properties, self-renewal and potency, that make them well suited for this role. Tight regulatory control of stem cell properties is critical to maintain homeostatic balance; misregulation often leads to tumors or diseases. Recently, homeostasis of the intestinal epithelium has been shown to depend on a population of molecular defined intestinal stem cells (ISCs) in both Drosophila and mammals. However, the regulatory mechanisms controlling ISC behaviors remain poorly defined. We took advantage of the molecular genetic approaches available in Drosophila melanogaster to investigate how ISC properties are regulated by both intrinsic and extrinsic factors.

In the course of the thesis, we identified two conserved signaling pathways that regulate ISC properties intrinsically. First, we demonstrated that WNT/APC signaling is an important regulator of ISC proliferation and relatively dispensable for potency. Parallel studies in mammals also show that loss of APC (APC\textsuperscript{lof}) in the ISC lineage leads to hyperplasia, supporting a model where APC\textsuperscript{lof} ISCs can function as a cell-of-origin for intestinal tumors. In follow-up studies, we have taken a genetic approach to identify modifiers that control the expansion of tumorigenic APC\textsuperscript{lof} lineages. We have now identified Insulin signaling as a modifier that is necessary and sufficient
for the expansion of tumorigenic $APC^{lof}$ lineages. Future studies will define the precise cellular and molecular mechanisms underlying this phenomenon. A second key finding of our studies is that JAK/STAT signaling pathway coordinates ISC proliferation and regulates the competency for daughter cell differentiation. In summary, ISC properties are regulated by conserved WNT/APC and JAK/STAT signaling similar to what has been shown in other stem cell lineages.

The intestinal tract is a primary site of nutrient absorption. We hypothesized that dietary nutrients can extrinsically regulate ISC properties. However, the precise requirement of individual macronutrients or micronutrients could not be investigated because a chemically defined food (CDF) consisting entirely of purified compounds was not available. We developed a new CDF media that permits CDFs with different nutrient compositions at iso-caloric density to be generated. Using this CDF recipe, we defined the individual macronutrient requirements for development, reproduction and longevity of *Drosophila*. Ongoing experiments are focused on determining the role of macronutrients in regulating ISC properties. We also seek to determine if nutrients are differentially required for normal and tumorigenic ISC lineages. Findings from this nutritional study may identify new therapeutic targets for regenerative medicine or cancer therapy.

Visual abstract of the dissertation
Chapter 1

General introduction to intestinal biology and intestinal stem cell biology
1.1 Overview of the dissertation

The intestinal epithelium is known for its rapid renewal in both mammals and *Drosophila* (Barker et al., 2008; Wang and House, 2010), and its homeostasis being maintained by resident intestinal stem cells (Barker et al., 2007; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Sangiorgi and Capecchi, 2008). In a rapid and constantly renewing tissue, a dynamic but rigorous control of stem cell properties is essential to avoid accumulation of serial accidental misbehaviors that endanger tissue homeostasis. Mis-regulation of major signaling pathways including WNT, TGF-beta, BMP, RAS/RAF, LKB, Notch and Hedgehog has been shown to be correlated with intestinal cancers (Sancho et al., 2004). Although cancer stem cells have been shown to be the cell-of-origin of many cancers (Reya et al., 2001; Shackleton et al., 2009), it is unknown whether ISCs are the origin of intestinal lesions leading to cancers. Using *Drosophila* as a model, the first goal of this dissertation is focused on deciphering the mechanisms used by ISCs to maintain the homeostasis of the intestinal epithelium under baseline conditions and to test the cancer stem cell hypothesis. The mechanisms of WNT/APC and JAK/STAT pathways regulating ISC behaviors are described in Chapter 2 and 3, respectively. Importantly, our work (Lee et al., 2009) together with the study by Barker et al. (Barker et al., 2009) established the APC loss-of-function (*APC*\textsuperscript{lof}) intestinal stem cells as the cells-of-origin of cancer in both *Drosophila* and mouse. Thus, I chose the *APC*\textsuperscript{lof} ISCs as a model to study the tumorigenic lineage. In Chapter 5.2, I describe the identification of the Insulin pathway as an enhancer which promotes *APC*\textsuperscript{lof} ISC lineages, and the experiments aimed to establish the synergistic mechanisms by which Insulin and *APC*\textsuperscript{lof} regulates ISC behavior.

The intestinal epithelium functions as the primary tissue to absorb the nutrients and thus supply the energy and metabolic needs for the entire organism. Dietary nutrition is known to be an important stimuli for the growth of the intestinal epithelium (Bragg et al., 1991). However, how dietary nutrients influence the homeostasis of the intestinal epithelium is not well described. To dissect the effects of specific dietary nutrients on intestinal stem cells, we first generated a chemically defined food (CDF) for the nutritional studies in *Drosophila*. We designed and performed a series of measurements of overall performance (including longevity, reproduction,
and development) for both adult flies and larvae to demonstrate this CDF recipe is adequate for nutritional studies, and defined a nutritional composition at 400 K-cal/L for CDF with a similar overall performance as flies grown on regular medium. All the detailed measurements and statistics are described in Chapter 4. In Chapter 5.3, I describe the experiments aimed at establishment of the different nutritional requirements of both normal and tumorigenic ISC lineages using CDF.

1.2 The stem cell and its properties

Adult tissues are constantly under insult from the environment and influences from the aging process, referred to injury and senescence respectively. To maintain the tissue homeostasis, many adult tissues contain a population of stem/progenitor cells that are capable of producing new cells to replenish those lost from injury or senescence. Two classic properties are used to describe stem/progenitor cells that allow this specialized cell to maintain tissue homeostasis: self-renewal and potency (Barker et al., 2008; Bongso and Lee, 2005; Smith, 2006). Self-renewal describes the processes in which a stem/progenitor cell is able to produce at least one identical daughter cell retaining the “stemness” property, mimicking the mother cell. Potency describes the ability to produce a daughter cell having the potential to differentiate into other cell type(s) in a given tissue.

Although stem cells and progenitor cells share similar abilities to replenish cell loss, their capacity of self-renewal and the degree of potency are known to be different in different systems. For example, in the mammalian neuronal system, the neuronal stem cells are referred to the cells that have the lifetime self-renewal ability and the within-tissue multi-potency (Seaberg and van der Kooy, 2003), while the neuronal progenitor cells have a limited self-renewal capacity and are often uni-potent. In the mammalian blood system, the hematopoietic stem cells produce all cell types of the blood lineage but can be sub-grouped into long-term and short-term stem cells, while the hematopoietic progenitor cells are lineage restricted with limited self-renewal ability (Seaberg and van der Kooy, 2003). In the mammalian male reproductive system, the lifetime self-renewal ability is possessed by the spermatogonial stem cells but not the spermatogonial progenitor cells,
while both cells are uni-potent \textit{in vivo} (Conrad et al., 2008). In summary, a rigorous and consistent definition for the stem cell among different systems is a cell possessing both the ability of lifetime self-renewal and multi-potency, in contrast to a progenitor cell that has limited self-renewal ability and a varied degree of potency. Alternatively, in the mammalian intestinal stem cell system, these two stem cell properties are described as longevity and multi-potency (Barker et al., 2008). In this dissertation, lifetime self-renewal ability and multi-potency are used to describe the stem cell properties of \textit{Drosophila} ISCs.

1.3 The function and architecture of the mammalian Intestinal tract

The primary function of the mammalian intestinal tract is to absorb nutrients from pre-digested food processed in the stomach. To accomplish this goal, the intestinal tract maintains an epithelial barrier between the external luminal environment and the internal environment of the body, which allows the further digestion of food inside the intestinal lumen and the selective absorption of completely digested nutrients into the body (Ma and Anderson, 2006).

The mammalian intestinal barrier is composed of two major elements (Ma and Anderson, 2006; McGuckin et al., 2011): The intrinsic element of the intestinal barrier is composed of a continuous single layer of columnar epithelial cells connected by intercellular junctions; this intestinal epithelium plays a major role of nutrient absorption. The extrinsic element of the barrier, also called the mucus layer, is generated by secretory goblet cells of the intestinal epithelium, and is an adherent gel-like layer serving as an interface between the intestinal lumen and intestinal epithelium. These two elements of the intestinal barrier not only exclude the gastric acid, food pathogens, and colonic bacteria from entering the internal environment of the body, but also to function as the natural habitat for a large bacterial community, known as the microbiota (Guarner and Malagelada, 2003; Ma and Anderson, 2006). One of the major functions of the gut microbiota is to salvage the energy and absorbable nutrients, thus enhancing the absorption efficiency by enterocyte cells of the intestinal epithelium (Guarner and Malagelada, 2003).

The mammalian intestinal tract is divided into the small intestine and the large intestine (or colon), which have different functional structures created by the folding of the intestinal epithelium
(Gilbert, 2000). The luminal protrusions of the intestinal epithelium generate the finger-like compartments termed villi to increase the surface area for nutrient absorption, and the invaginations of the epithelium create cylindrical compartments known as crypt of Lieberkühn (or intestinal crypt) (Fig. 1.1A) (Pinto and Clevers, 2012). The small intestine is highly populated with villi and crypts, and functions as the primary tissue for the absorption of all major macronutrients. The large intestine is populated by wider crypts without villi, and functions primarily for the reabsorption of water and minerals.

1.4 Cell types of the mammalian small intestinal epithelium

On average, each villus is populated by 3500 differentiated cells from 6-10 surrounding crypts, and each crypt by 250 differentiated and undifferentiated cells (Potten and Loeffler, 1990). 1400 cells per villus per day are shed from the tip of villus and are replaced with new cells produced from the crypt. This rapid renewing ability of the intestinal epithelium is mainly fueled by 150-160 proliferative cells, known as transient amplifying cells, which reside in the middle third of the crypt (Fig. 1.1B) (Potten and Loeffler, 1990). Transient amplifying cells have a rapid cell cycle and divide twice a day to produce about 300 non-proliferative daughter cells per crypt per day (Potten and Loeffler, 1990). While migrating through the top-third of crypt, the non-proliferative daughter cells undergo differentiation into three differentiated cell types, including enterocytes (absorptive cells, 90% of the villus-associated cells), mucus-producing goblet cells (8-10%), and hormone secreting enteroendocrine cells (1%) (Pinto and Clevers, 2012; Umar, 2010). Once reaching the tip of the villi, these differentiated daughter cells are shed into the lumen, a migration process that takes place in 5-7 days (Marshman et al., 2002). Unlike the upward migrating differentiated daughter cells, antimicrobial peptide secreting Paneth cells (5-15 cells per crypt) migrate downward to the bottom-third of the crypt, where they reside for 18-23 days (Fig. 1.1B) (Porter et al., 2002).

Although transient amplifying cells can undergo rapid division to produce new cells, they only remain in the crypts for 2-3 days and undergo 4-6 rounds of cell division (Marshman et al., 2002;
Rao and Wang, 2010) before terminally differentiating. This result implies the existence of long-lived intestinal stem cells (ISCs) to sustain the renewing ability of the intestinal epithelium.

1.5 Identification and interrelationship of the two types of murine intestinal stem cells

Although missing the definitive biomarkers or isolation methods for direct evaluation of stem cell properties, studies from previous decades have reached a conclusion that 4-16 ISCs reside in a crypt (Potten and Loeffler, 1990). Two types of cells are found to possess some stem cell properties inside the crypt bottom, the +4 cells named by their position in relation to the crypt bottom (Potten, 1977; Potten et al., 1974) and crypt base columnar cells (CBCs) (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b; Cheng and Leblond, 1974a; Cheng and Leblond, 1974b) (Fig. 1.1B). +4 cells are known for their BrdU label-retaining ability (known as label-retaining cells; LRCs), and are highly sensitive to radiation as a protective function from genetic damage (Barker et al., 2008; Potten, 1977; Potten et al., 1974). CBCs are slowly cycling cells dividing once a day (Cheng and Leblond, 1974b), and are capable of producing all four differentiated daughter cell types demonstrated by Dlb-1 mutation based clonal analysis (Bjerknes and Cheng, 1999; Winton et al., 1988). Both models provide certain supporting evidence of stem cell properties, and therefore these two types of crypt-resided ISCs has been widely recognized in intestinal biology studies.

Recently, many molecular markers have been identified for the two functionally distinct ISC populations, commonly Lgr5 as a CBC marker and Bim1 as an LRC marker (Yan et al., 2012). Barker et al. (Barker et al., 2007) identified Lgr5 as a molecular maker for CBCs. Lgr5 is a WNT target gene that encodes an orphan G-protein-coupled receptor. Lineage tracing using Lgr5-Cre-ER transgenic mice, the authors demonstrated that Lgr5+ cells are capable of producing all cell types of the crypt, and that the labeled lineage can be retained in the intestinal epithelium for up to 60 days. In contrast to LRCs that are sensitive to both low-dose (1 Gy) and high-dose (10 Gy) radiation, CBCs are resistant to low-dose (1 Gy) radiation but sensitive to high-dose (10 Gy), suggesting that CBCs are more resistant to moderate injury and can be a driving force of cell production during the regeneration process. To further validate the stem cell identity of Lgr5+
cells, Sato et al. (Sato et al., 2009) isolated single Lgr5\(^+\) cells and cultured them into long-term growing epithelial organoids, which possess the crypt-villus-like structures in the absence of a mesenchymal niche. Further study by Snippert et al. (Snippert et al., 2010) showed 12-16 Lgr5\(^{hi}\) cells per crypt in the duodenum and that these Lgr5\(^{hi}\) cells divide symmetrically to maintain their population and produce transient amplifying cells to sustain the rapid renewal of the intestinal epithelium. Using a multi-color labeling method, Snippert et al. showed that these Lgr5\(^{hi}\) cells continuously undergo neutral competition, and the intestinal crypts progress toward 98% monoclonality after 30 weeks of labeling. This data implies that, at a population level, the Lgr5\(^{hi}\) cells are maintained throughout most of adult life. All together, these data demonstrate the Lgr5\(^{hi}\) cells / CBCs fulfill the requirements of multipotent ISCs sustaining the renewal ability of the intestinal epithelium.

Sangiogi et al. (Sangiorgi and Capecchi, 2008) identified Bmi1 as a molecular marker for LRCs. Bmi1 encodes a component of the Polycomb Repressing Complex 1, known to have an essential role in maintenance of chromatin silencing. The authors concluded that 4-5 Bmi1\(^+\) cells can be observed at position +4 and +5 above the base of the crypt, which fits the position profile of LRCs. The authors also performed lineage tracing experiments utilizing Bmi1-Cre-ER transgenic mice and demonstrated that after 20 hours the activated Cre reporter can be first observed at position +4 and +5, and the marked cells are capable of producing all cell types in a crypt within a few days after the start of lineage tracing. Although the number of labeled lineages / crypts slightly decreased 1 month after lineage tracing, many labeled cells are still observed after 9 months. Most importantly, the ablation of the Bmi1\(^+\) lineage causes the crypt loss. These data imply that Bmi1\(^+\) cells can live through the majority of murine adult life and are necessary and capable of producing all differentiated daughter cells, also fulfilling the requirements of multipotent ISCs. Nevertheless, the rapidly dividing ability of Bmi1\(^+\) cells to produce daughter cells in a non-stressed condition argues against the quiescent state of LRCs. Thus, further studies are required to elucidate if the Bmi1\(^+\) cells represent the LRCs.

Recent studies provide evidence for the possible interrelationship between the Lgr5\(^+\) and Bmi1\(^+\) populations. Tian et al. (Tian et al., 2011) showed that Lgr5\(^+\) cells are dispensable for
homeostasis of the intestinal crypt, and Bmi1+ cells can give rise to Lgr5+ cells under normal and injury conditions. Under culture conditions, constant ablation of Lgr5+ cells also does not affect the formation or maintenance of organoids. The authors concluded that the Bmi1+ cells are both a reserved stem cell pool for regeneration after injuries and a source of Lgr5+ cells under normal conditions. Interestingly, the authors mentioned that Lgr5+ cell independent regeneration is primarily restricted to the anterior part of the small intestine (duodenum and a portion of jejunum) due to the gradual reduction of Bmi1+ cells along the intestinal tract, suggesting the regeneration process in the posterior part of small intestine (a portion of jejunum and ileum) requires another stem cell populations. By transcriptional profiling of FACS-sorted Lgr5+ cells, Muñoz et al. (Muñoz et al., 2012) demonstrated that Lgr5+ cells also express many quiescent stem cell / +4 cell markers, including Bmi1, Tert (Montgomery et al., 2011), Hopx (Takeda et al., 2011) and Lrig1 (Powell et al., 2012). Similarly, Buczacki et al. (Buczacki et al., 2013) showed that LRCs are a subgroup of Lgr5+ cells. The LRCs are secretory precursors to Paneth cells and enteroendocrine cells under normal conditions, but can contribute to the stem-cell pool following injury. These data suggest that the CBCs represent an active ISC population and LRCs a quiescent ISC population, and that LRCs can contribute to the replenishment of CBCs during the regeneration process. However, as concluded by Muñoz et al. (Muñoz et al., 2012), the more detailed molecular signatures and cellular behaviors of these two stem cell types are necessary to draw a definitive conclusions regarding their interrelationship.

1.6 The architecture of the Drosophila intestinal epithelium

The adult Drosophila intestinal tract is comprised of the midgut and hindgut, which are related to the small intestine and large intestine (colon) in mammals (Fig. 1.2A,B) (Wang and House, 2010). In contrast to mammals, the Drosophila intestinal tract is covered by a pseudostratified epithelium instead of a columnar epithelium (Casali and Batlle, 2009), and has no villi nor crypt structures (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Shanbhag and Tripathi, 2009). However, the apical surface of the Drosophila midgut epithelium is folded with many cellular protrusions that likely increase the surface area for nutrient absorption (Shanbhag and
Tripathi, 2009). In both *Drosophila* and mammals, the apical surface of enterocytes possesses many cytoplasmic extensions termed microvilli. The microvilli form a continuous layer on the surface of the entire intestinal epithelium (midgut in *Drosophila*) to further increase the surface area for nutrient absorption (Baumann, 2001; Shanbhag and Tripathi, 2009). This continuous microvilli structure is termed the brush border.

Although no evidence for the existence of secretory goblet cells in the *Drosophila* midgut, a mucus layer, the extrinsic element of the intestinal barrier, is formed on top of the brush border to protect the *Drosophila* intestinal epithelium as in mammals. The intestinal mucins are secreted mainly by an organ located at the anterior end of the midgut termed the cardia (proventriculus), and released into the entire intestinal tract (Hegedus et al., 2009; Theopold et al., 1996). In *Drosophila*, an additional layer of type II peritrophic matrix forms a continuous sleeve lining the entire intestinal tract above the mucus layer and brush border (Lehane, 1997), which is composed of chitin and glycoproteins produced by the cardia (Hegedus et al., 2009; Kuraishi et al., 2011). This type II peritrophic matrix functions as a physical barrier to restrict the passage of pathogens or toxins, and also as a permeable barrier enclosing the ingested food and allowing digested food to pass through it along the intestinal tract (Hegedus et al., 2009; Kuraishi et al., 2011).

The *Drosophila* adult midgut contains approximately 1200 ISCs, 600 EBs, 5600 ECs and 1200 EEs throughout adult life (Sudmeier and Micchelli, unpublished data). According to the regional morphology, histology, and genetic properties, the *Drosophila* midgut can be further defined into 14 compartments (Buchon et al., 2013). Only a few studies describe the difference in the expression of digestive enzymes or the cell types regarding the discrete parts of the *Drosophila* adult midgut (Abraham and Doane, 1978; King, 1988; Singh et al., 2011; Strand and Micchelli, 2011; Veenstra et al., 2008). The function of each compartment and the cell behavior in each region remains at large.
1.7 Cell types of Drosophila intestinal epithelium

Four major types of cells have been identified in the Drosophila midgut epithelium thus far, including intestinal stem cells (ISCs), enteroblasts (EBs), enterocytes (ECs) and enteroendocrine cells (EEs) (Fig. 1.2C) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs are the only cells capable of proliferation in the Drosophila midgut under baseline conditions (Ohlstein and Spradling, 2006). Enteroblasts are the intermediate daughter cells of ISCs, which have the potential to differentiate into absorptive ECs and secretory EEs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs in Drosophila are characterized by the expression of the marker genes escargot (Micchelli and Perrimon, 2006), a zinc-finger transcription factor of the snail superfamily (Whiteley et al., 1992), and Delta (Ohlstein and Spradling, 2007), a ligand of the Notch receptor. EBs are marked by Su(H)Gbe-LacZ (Micchelli and Perrimon, 2006), a reporter of Notch signaling activity (Furriols and Bray, 2001); ECs by Pdm1 (Lee et al., 2009), a POU domain containing transcription factor (Billin et al., 1991); and EEs by Prospero (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), a homeodomain protein (Chu-Lagraff et al., 1991). In addition, at least six regulatory peptides are expressed in adult midgut enteroendocrine cells with regional expression patterns (Veenstra et al., 2008).

In addition to molecular markers, intestinal cell types can be also distinguished by morphology, nuclear size, and the location of the nuclei (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs have a pyramidal morphology adjacent to the basement membrane, with small nuclei (5.1 µm) and little cytoplasm. EBs also have a pyramidal morphology but with slightly bigger nuclei (5.6 µm) and slightly more cytoplasm than ISCs. EBs possess cellular contact with ISCs and usually form bow-tie like cell pairs with ISCs. ECs are polyploid (Ohlstein and Spradling, 2006), with large (9.3 µm) and centrally located nuclei. Cytoplasm of the EC is the major place for nutrient absorption, thus ECs also have the highest cytoplasm/nucleus ratio in comparison to other midgut cells. EEs have the smallest nuclei (3.8 µm) with varied cell morphology and nuclear location. (All nuclear sizes mentioned above are measured by Beebe and Micchelli; unpublished data.)
1.8 Genetic tools for studying signaling pathways that regulate stem cell properties of intestinal stem cells in *Drosophila*

The most common genetic tools for studying the lifetime self-renewal ability and multipotency of *Drosophila* intestinal stem cells are the temporal and regional gene expression targeting (TARGET) system (McGuire et al., 2003), the “flip-out” based clone labeling strategy (Ito et al., 1997), and the mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 1999). These systems are all derived from the Gal4/UAS system (Brand and Perrimon, 1993), thus ectopic expression of a gene-of-interest (GOI) driven by the upstream activation sequence (UAS) enhancer can be applied to these systems to alter the activity of signaling pathways.

The TARGET system uses the Gal4/UAS system in combination with a temperature-sensitive repressor of Gal4 (Gal80<sup>ts</sup>), allowing the gene-of-interest to be overexpressed in a spatial and temporal manner. Three transgenic constructs are required for the TARGET system, including a tissue or cell-type specific Gal4, a UAS driven GOI expression construct, and a ubiquitously expressed Gal80<sup>ts</sup> (Fig. 1.3A). After shifting flies to the non-permissive temperature, Gal80<sup>ts</sup> loses its ability to repress Gal4 activity, which in turns allows Gal4 to activate the expression of the GOI. For example, a GOI construct altering a signaling pathway can be driven by an ISC specific Gal4, such as Dl-Gal4 (Zeng et al., 2010), to assess the influence of the signaling pathway on ISC properties. To study the influence of signaling pathways on lifetime self-renewal ability of ISCs with the TARGET system, total ISC numbers have to be counted and compared throughout adult life. To study of the influence of signaling pathways on multi-potency of ISCs, one can combine a “flip-out” lineage tracing strategy (Struhl and Basler, 1993) with the TARGET system. However, due to the high number of labeled ISCs, this TARGET system based lineage tracing strategy may be not suitable for multi-potency studies in later experimental stages or in certain conditions when the intestinal epithelium undergoes rapid renewal. Therefore, two clonal labeling strategies can be used to reduce the labeling efficiency and increasing the resolution for multi-potency studies.

The “flip-out” based clone labeling strategy uses the Gal4/UAS system (Brand and Perrimon, 1993) in conjunction with the FLP/FRT system (Golic and Lindquist, 1989). This system utilizes a
ubiquitous promoter-Gal4 fusion construct interrupted by an FRT (flippase recognition target) cassette containing transcriptional termination signals, and an inducible recombinase (flippase; FLP) construct (Fig. 1.3B). Upon induction of the flippase, the FRT cassette is excised, and Gal4 expression is driven by the upstream ubiquitous promoter. In cells where the flip-out event occurs, induced Gal4 expression can active the GOI construct driven by a UAS enhancer. When the flip-out event occurs in an ISC, it should produce a stable clone throughout the experimental period, if the ectopically expressed gene product does not affect the self-renewal activity. The number and the size of stable clones can be determined at different ages of the animal to assess the influence of signaling pathways on lifetime self-renewal ability and multi-potency of ISCs. The labeling event is equally likely in all cell types using this system. Given that the majority of cells are not ISCs in the Drosophila midgut, most of the clones induced by the flip-out strategy are transient clones produced from the labeling events in the differentiating and differentiated daughters. Because the normal turn-over rate of the midgut epithelium is 7-10 days (Buchon et al., 2010), ISC derived stable clones can only be easily quantified after 10 days when transient clones are lost from the midgut. Thus this flip-out strategy is not an ideal tools to study the influence of signaling pathways on stem cell properties during early experimental stages.

The MARCM system is generated by a combination of the Gal4/UAS system (Brand and Perrimon, 1993), the FLP/FRT system (Golic and Lindquist, 1989), and a constitutively expressed repressor of Gal4 (Gal80). The labeling events are initiated by an induced in trans recombination at FRT sites on homologous chromosomes during mitosis. With the exception of the inducible recombinase, all other components are placed at positions relative to the induced recombination site (Fig. 1.3C). The ubiquitous Gal4 and UAS-reporter (i.e. GFP, LacZ) constructs are inserted outside the induced recombination area, and the Gal80 construct has to be inside the area (Fig. 1.3C). After an induced recombination and chromosomal segregation event occurs during mitosis, the mitotic daughter cell that inherits 2 copies of the GOI is also the cell that does not inherit the Gal80 repressor. Because these homozygous GOI cells do not inherit the Gal80 repressor, Gal4 can activate the UAS-reporter thus generating the clonal labeling events. All labeled clones in MARCM system are homozygous for the GOI, whereas the surrounding tissue
is heterozygous. Thus the MARCM system can be used to study loss-of-heterozygosity events in a mitotic tissue. Moreover, the MARCM system also allows the ectopic expression of a GOI driven by UAS to alter the activity of signaling pathways (Fig. 1.3D). Because ISCs are the only cells undergoing mitosis in the *Drosophila* midgut (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), the MARCM system can be used to specifically label and trace ISCs to study lifetime self-renewal ability. The relatively lower labeling efficiency compared to the TARGET system and the “flip-out” based clone labeling strategy makes the MARCM system more suitable for multi-potency studies in later experimental stages or in certain conditions when the intestinal epithelium undergoes rapid renewal. One caveat of the MARCM system is the perdurance of Gal80 proteins and the delay of reporter protein expression. It takes 3 days at 25°C to detect the labeled clones in an adult midgut. Thus the MARCM system is not suitable for studying the acute responses of ISCs following clone induction.

Among these systems, only the MARCM system enables the analysis of loss-of-heterozygosity in a mosaic tissue when the desired mutation is located in the FRT GOI chromosomal arm. Thus, I chose the MARCM system as a platform to study intestinal related lesions caused by loss-of-heterozygosity in ISCs. Furthermore, due to better resolution of clonal analysis, I chose the MARCM system as the major tool for this thesis to study the lifetime self-renewal ability and the multi-potency of ISCs under the influences of signaling pathways or nutrients.
Figure 1.1: The architecture of the mammalian intestinal epithelium. (A) The mammalian intestine is lined by a simple columnar epithelium. The luminal protrusions of the intestinal epithelium generate the finger-like compartments termed villi to increase the surface area for nutrient absorption, and the invaginations of the epithelium create cylindrical compartments known as crypt of Lieberkühn (or intestinal crypt). (B) Diagram of the intestinal crypt. Two types of intestinal stem cells, crypt base columnar cells and label-retaining cells, are located at the bottom of crypt, and surrounded by niche Paneth cells.
Figure 1.2: The *Drosophila* midgut. (A) Diagram of the adult digestive system in lateral view. Anterior to the left. The gastrointestinal tract is highlighted in red; salivary gland in blue; crop in pink, malpighian tubules in green. (B) Diagram of the adult gastrointestinal tract in ventral view (see (Miller, 1994)). The adult midgut extends the length between the cardia and the pylorus (red area). (C) The lining of the midgut epithelium. Intestinal stem cells (ISCs), enteroblasts (EBs), enterocytes (ECs) and enteroendocrine cells (EEs) are the four major cell types that resided in the *Drosophila* midgut.
Figure 1.3: Genetic tools for studying stem cell properties in the Drosophila midgut. 

(A) The temporal and regional gene expression targeting (TARGET) system (McGuire et al., 2003).

(B) The “flip-out” based clone labeling strategy (Ito et al., 1997).

(C) The mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 1999).

Chapter 2

Adenomatous polyposis coli regulates Drosophila intestinal stem cell proliferation

The research work in this Chapter is published in Development. 2009 Jul;136(13):2255-64. Wen-Chih Lee is the first author in this publication.
2.1 Summary

Adult stem cells define a cellular reserve with the unique capacity to replenish differentiated cells of a tissue throughout an organism’s lifetime. Previous analysis has demonstrated that the adult *Drosophila* midgut is maintained by a population of multi-potent intestinal stem cells (ISCs) that resides in epithelial niches. *Adenomatous polyposis coli* (*Apc*), a tumor suppressor gene conserved in both invertebrates and vertebrates, is known to play a role in multiple developmental processes in *Drosophila*. Here, we examine the consequences of eliminating *Apc* function on adult midgut homeostasis. Our analysis shows that loss of *Apc* results in the disruption of midgut homeostasis and is associated with hyperplasia and multi-layering of the midgut epithelium. A mosaic analysis of marked ISC cell lineages demonstrates that *Apc* is required specifically in ISCs to regulate proliferation, but is not required for ISC self-renewal or the specification of cell fate within the lineage. Cell autonomous activation of Wnt signaling in the ISC lineage phenocopied *Apc* loss and *Apc* mutants were suppressed in an allele-specific manner by abrogating Wnt signaling, suggesting that the effects of *Apc* are mediated in part by the Wnt pathway. Together, these data underscore the essential requirement of *Apc* in exerting regulatory control over stem cell activity, as well as the consequences that disrupting this regulation can have on tissue homeostasis.

2.2 Introduction

Many adult tissues require the activity of tissue-specific stem cell populations to maintain homeostasis throughout the course of an organism’s lifetime. The dual characteristics of self-renewal and multi-potency make stem cells ideally suited for this central role. Adult stem cells reside in specialized microenvironments called niches, which regulate stem cell behavior at baseline homeostasis and dynamically respond to changing environmental stimuli by modulating lineage output (reviewed in (Jones and Wagers, 2008)). Tight control of stem cell proliferation is essential to ensure that homeostatic balance is maintained; disruption of stem cell proliferation can lead to homeostatic imbalance, compromised wound healing, and disease. However, little is currently known about the factors that constrain stem cell proliferation.
The precise location and cellular architecture of the adult stem cell niche has been defined with high resolution in only a small number of tissues (reviewed in (Morrison and Spradling, 2008)). The ability to identify, manipulate and mark individual stem cell lineages has made *Drosophila* an excellent model system with which to dissect stem cell regulation. For example, the adult *Drosophila* midgut contains approximately 1200 intestinal stem cells (ISCs) distributed along the anteroposterior (AP) axis of the organ (Fig. 2.1A,B) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs have a pyramidal morphology and are located in an epithelial niche distant from the midgut lumen, adjacent to the basement membrane and the visceral musculature surrounding the midgut. ISCs are multi-potent and give rise to a lineage that consists of two types of differentiated daughters, the enteroendocrine (ee) cells and the enterocytes (ECs), which together form a cellular monolayer lining the length of the adult midgut.

*Adenomatous polyposis coli* (*Apc*) encodes an evolutionarily conserved protein, which was first identified by positional cloning as one of the genes commonly deleted in the hereditary colon cancer syndrome familial adenomatous polyposis (FAP) (Groden et al., 1991; Kinzler et al., 1991). Mutations in both copies of *Apc* are also detected in many spontaneous colorectal adenomas (Ichii et al., 1992; Miyoshi et al., 1992; Powell et al., 1992). As the majority of *Apc* mutations are loss of function, *Apc* is thought to function as a tumor suppressor gene. Early insight into the molecular function of *Apc* came from the identification of β-catenin, a Wnt pathway effector, as a binding partner for *Apc* (Rubinfeld et al., 1997; Su et al., 1993). The requirement for *Apc* in the Wnt signaling pathway is now well characterized and current models suggest that *Apc* together with Axin, Glycogen Synthase Kinase 3 and Casein Kinase 1 comprise a multi-protein β-catenin destruction complex. In the absence of Wnt ligands, the destruction complex phosphorylates β-catenin, targeting it for proteasomal degradation.

The *Drosophila* genome, like that of both mouse and human, contains two *Apc* family members, *Apc1* (also known as *Apc* – FlyBase) and *Apc2* (Ahmed et al., 1998; Hamada et al., 1999a; Hayashi et al., 1997; McCartney et al., 1999). Previous analysis in *Drosophila* has implicated *Apc* in a number of developmental processes, including cell survival, cell fate specification and proliferation. Yet, the role of *Apc* in maintaining adult *Drosophila* midgut
homeostasis has not been examined. In this study, we investigate the consequences of eliminating Apc function on adult midgut homeostasis and ISC behavior.

2.3 Material and methods

2.3.1 Drosophila strains and culture

Apc2\textsuperscript{C9} / TM6 Tb, Hu (Apc2\textsuperscript{C9}) is a temperature-sensitive allele with a permissive temperature of 18°C and non-permissive temperature of 27°C; gift from B. McCartney, Carnegie Mellon University, Pittsburgh, PA, USA). w; FRT\textsuperscript{82B} Apc2\textsuperscript{g10}, Apc1\textsuperscript{Q8} / TM6C (Apc2\textsuperscript{g10} is null for Armadillo degradation but retains the ability to promote Wnt signaling in the eye; Apc1\textsuperscript{Q8} is a null allele; gift from B. McCartney). w; FRT\textsuperscript{82B} Apc2\textsuperscript{33}, Apc1\textsuperscript{Q8} / TM6C (Apc2\textsuperscript{33} is a strong hypomorph resulting from a deletion; gift from Y. Ahmed, Dartmouth College, Hanover, NH, USA). w; FRT\textsuperscript{82B} Axins\textsuperscript{044230} / TM3 Sb (Axins\textsuperscript{044230} is a loss-of-function allele; gift from Y. Ahmed). y, w, UAS-GFP, hsFLP; tub-Gal4, FRT\textsuperscript{82B}, tub-Gal80 / TM6B (gift from N. Perrimon, Harvard Medical School, Boston, MA, USA). y, w; esg-Gal4 / Cyo (gift from S. Hayashi, RIKEN, Kobe, Japan). w, UAS-\textsuperscript{\textit{N}}\textsuperscript{RNAi}. UAS-GFP. y, w, esg\textsuperscript{K606} / Cyo (esg-lacZ). w; FRT\textsuperscript{82B} hsrM (this stock was used as a wild-type control in the mosaic analysis performed in this study). y, w, UAS-arm\textsuperscript{510} (activated arm). y, w; UAS-pan.dTCF\Delta N4 (dominant-negative allele). Unless indicated, all strains were obtained from the Bloomington Stock Center. Crosses were cultured on standard dextrose media and were transferred to fresh food augmented with yeast paste every 1-2 days during the experimental period. Crosses were reared at 18, 25, or 29°C, in passively illuminated and humidified incubators. In this study, only adult female flies of the following genotypes were analyzed.

Figure 2.2:

+; esg\textsuperscript{K606} / +; FRT\textsuperscript{82B} Apc2\textsuperscript{g10}, Apc1\textsuperscript{Q8} / Apc2\textsuperscript{C9}.

+ / w; FRT\textsuperscript{82B} Apc2\textsuperscript{g10}, Apc1\textsuperscript{Q8} / Apc2\textsuperscript{C9}.

Figures 2.3, 2.4 and S2.2:

y, w, hsFLP, UAS-GFP / w; +; FRT\textsuperscript{82B} hsrM / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.

y, w, hsFLP, UAS-GFP / +; +; FRT\textsuperscript{82B} Apc2\textsuperscript{g10}, Apc1\textsuperscript{Q8} / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
2.3.2 Mosaic analysis

The MARCM system was used to generate marked ISC lineages or ‘clones’. MARCM was used to produce both marked wild-type and mutant lineages; in addition, all manipulations...
involving the misexpression of UAS transgenes were also performed using the MARCM system to ensure that only ISC lineages were analyzed. To induce clones, experimental animals were subjected to a 37°C heat pulse for 35-45 minutes within the first week of adulthood. Induction protocols varied from one to three heat pulses within a 24-hour period, depending on the desired rate of mitotic recombination.

2.3.3 Temperature shift experiments

Crosses were established and cultured at 18°C until adulthood. F1 progeny were divided into two equal pools; controls were cultured at 18°C and the experimental group was shifted to 29°C for 10 days. BrdU was administered ad libitum in Drosophila food media (200 µl of 6 mg/ml BrdU in 20% sucrose per vial) for the 24-hour period immediately preceding the 10-day time point.

2.3.4 Histology

Adult flies were dissected in 1x PBS (Sigma, USA). The gastrointestinal tract was removed and fixed in a final solution of 0.5x PBS (Sigma, USA) and 4% electron microscopy grade formaldehyde (Polysciences, USA) for a minimum of 30 minutes. Samples were washed in 1x PBS with 0.1% Triton X-100 (PBST) for 2 hours and then incubated with primary antibodies overnight. Samples were washed in PBST for 2 hours and then incubated with secondary antibodies for 3 hours. Finally, samples were washed in PBST overnight. Mounting media containing DAPI (Vectashield, USA) was added and samples were allowed to clear for 1 hour prior to mounting. All steps were completed at 4°C, with no mechanical agitation.

2.3.5 Antisera

Primary antibodies: Chicken anti-GFP (Abcam, USA) used at a dilution of 1:10,000; rabbit anti-β-Gal (Cappel, USA), 1:2000; mouse anti-Pros (Developmental Studies Hybridoma Bank; DSHB), 1:100; mouse anti-Dl (DSHB), 1:10; mouse anti-BrdU (Becton Dickinson, USA), 1:100; rabbit anti-Pdm1 (gift of W. Chia (Yeo et al., 1995)), 1:1000; rabbit anti-Tachykinin (gift of D. Nässel (Siviter et al., 2000)), 1:1000.

Dyes and mounting media: Alexa 594-conjugated Phalloidin (Molecular Probes, USA) diluted 1:500; Vectashield+DAPI mounting media (Vector, USA).

2.3.6 Microscopy and imaging

Samples were examined on a Leica DM5000 upright fluorescent microscope. Confocal images were collected using a Leica TCS SP5 confocal microscope system. Images were processed for brightness and contrast, and assembled in Photoshop CS (Adobe, USA).

2.3.7 Cell counts, measurements and statistical analysis

In the temperature-shift analyses, entire midguts were scored. BrdU+ esg-lacZ+ positive pairs of cells were scored as a single event; clusters of three cells were scored as two events. In those TS experiments lacking esg-lacZ only small BrdU+ clusters were scored (Fig. 2.2D). Midgut area was determined by first acquiring digital images of the posterior midgut on a compound microscope; Leica application suite (LAS) software was then used to determine the area of the posterior region. Next, the maximal number of nuclear layers and maximal epithelial height was determined based on confocal micrographs taken from the same posterior midguts. Only those regions of the epithelium from the outer face of posterior midgut, which has a larger circumference, were analyzed to minimize secondary distortion of the epithelium due to the coiled morphology of the midgut (Fig. 2.3E,F). In our mosaic analysis, the number of cells per clone was scored in either anterior and/or posterior midgut frames 5 days following induction (Fig. 2.4A; see also Supplemental Fig. S2.1; Fig. 2.4D,F; Fig. 2.6G). Data from the anterior and posterior midgut are combined in Fig. 2.4D, but separated by region in Fig. 2.4F. Unless indicated, counts were collected from posterior frames. Clones within selected frames were defined as clusters of contiguous cells, as assessed at 40x magnification on compound or confocal microscopes. For a
clone to be scored it had to lie completely in the field of view; clones that partially wrapped around the ‘edge’ of the midgut sample were excluded from the analysis to minimize counting inaccuracy. To determine the number of dividing cells per frame, GFP⁺ pHH3⁺ cells were counted from the middle frame of the posterior midgut 5 days after heatshock induction (Fig. 2.4E). To assay ISC self-renewal, the number of clones per entire midgut was scored 5 and 10 days following heat-shock induction (Fig. 2.4G). Mitotic index was calculated by dividing the number of pHH3⁺ pros⁻ small nuclei by the total number of pros⁻ small nuclei from marked lineages in posterior midgut frames 5 days following induction (Fig. 2.6H; see also Supplemental Fig. S2.4). All t-tests were performed using Prism (GraphPad Software, USA).

2.4 Results

2.4.1 Apc regulates adult midgut homeostasis

Previous studies of Apc have demonstrated that functional redundancy exists between Apc1 and Apc2 in a number of Drosophila tissues, including the embryonic epidermis, the wing and eye imaginal discs, and the larval brain (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b; McCartney et al., 2006). We therefore reasoned that simultaneous reduction of both Apc1 and Apc2 would be a direct means by which to initially assess the requirement for Apc in the adult midgut. To globally reduce Apc function in a conditional manner, we used a temperature-sensitive allelic combination and measured the extent of 5-bromo-2-deoxyuridine (BrdU) incorporation in the adult midgut (Fig. 2.2A-D). Here, we employed three well-characterized alleles of Apc to establish the temperature-sensitive genotype Apc2²¹⁰, Apc1⁰⁸/Apc2⁰⁸ [subsequently referred to as ApcTS (Ahmed et al., 1998; McCartney et al., 2006). Unshifted control animals grown at the permissive temperature were compared with experimental animals shifted to the non-permissive temperature for 10 days during adulthood. In these experiments, we observed an increase in the number of BrdU⁺ cells in experimental samples that often appeared as clusters of two to three small cells (Fig. 2.2A-C). Previous analysis has demonstrated that the transcriptional repressor encoded by escargot (esg) can be used to identify ISCs and their nascent daughters, called enteroblasts (EBs) (Fig. 2.2A,B) (Micchelli and Perrimon, 2006). To quantify the ApcTS phenotype,
we counted the number of small BrdU+ clusters in the midgut following a 24-hour BrdU pulse that immediately preceded the 10-day time point (BrdU administered ad libitum in food media). Global reduction in the levels of Apc1 and Apc2 resulted in a significant increase in the number of BrdU+ small cell clusters (Fig. 2.2D; n=11). This analysis suggested that Apc is necessary for the maintenance of midgut homeostasis in the adult.

2.4.2 Apc is required in ISC lineages to maintain homeostasis

Although the TS analysis suggested a role for Apc in regulating midgut homeostasis, it did not directly establish a requirement for Apc in the midgut. To test the requirement for Apc specifically in the midgut, we next conducted a mosaic analysis of Apc double mutants (Apc2Q70, Apc1Q8; subsequently referred to as Apc clones, except where indicated). Positively marked ISC lineages lacking Apc function were generated in the adult using the MARCM system (Lee and Luo, 1999) and identified on the basis of GFP expression. At 20 days after induction, midguts containing Apc clones were associated with gross anatomical changes, including midgut hyperplasia and multi-layered cellular masses that distorted the luminal surface of the midgut (Fig. 2.3A-D). Plotting the maximal number of nuclear layers as a function of midgut area revealed an inverse correlation in Apc mosaics and an increase in each parameter compared with wild-type (Fig. 2.3E; wild-type, n=11; Apc, n=12). At 20 days after induction, it was often difficult to unambiguously identify individual marked Apc mutant lineages because of the changes in overall midgut morphology. However, by plotting the maximal number of nuclear layers as a function of maximal epithelial height, it was evident that up to five layers could be detected in mosaic Apc midguts (Fig. 2.3F; wild-type, n=11; Apc, n=12). Finally, in those cases in which individual clones could be definitively identified, Apc mutant lineages were found to produce multi-layered masses more frequently than were wild-type (Apc, 31.5%, n=181; wild-type, 1.3%, n=227).

The analysis of Apc clones was extended to determine the number of labeled cells in individual ISC lineages. As we observed little or no distortion of the midgut due to multi-layering at early time points, a 5-day post-induction time point was selected to quantify the number of cells...
per ISC clone. Cell counts were performed by scoring clones at two defined regions within each midgut analyzed (Fig. 2.4A; see Materials and methods and Supplemental Fig. S2.1 for experimental criteria). Mosaic analysis revealed a significant increase in the number of cells per clone in Apc lineages compared with in marked wild-type controls (Fig. 2.4B-D; n=76). This increase in clone size suggested that Apc loss leads to an increase in proliferation. Consistently, we observed that Apc mutant lineages were associated with a significant increase in the number of phosphohistone H3 positive (pHH3+) cells when compared with wild-type cell lineages (Fig. 2.4E; wild-type, n=18; Apc, n=17).

An analysis of Apc clone size along the AP axis of the gastrointestinal tract was performed to determine whether the requirement for Apc was dependent on midgut region. This analysis revealed a significant increase in Apc clone size in both the anterior and posterior midgut compared with in wild-type controls (Fig. 2.4F). Although the trend towards increased clone sizes in ISCs lacking Apc was observed throughout the midgut, the average size of Apc clones was found to be greater in the posterior. Taken together, our mosaic analysis demonstrates that Apc is required in the ISC cell lineage throughout the midgut to maintain homeostasis.

2.4.3 Apc loss does not affect ISC self-renewal

One possible explanation for the hyperplasia associated with Apc loss is that Apc affects ISC self-renewal. Early studies of Drosophila germ line stem cells employed a lineage-tracing assay to measure stem cell self-renewal (Margolis and Spradling, 1995). A pulse/chase experiment was used to determine the number of marked stem cell lineages retained in the tissue at defined intervals following induction. To determine whether Apc loss affected ISC self-renewal in the midgut, we generated labeled ISCs and counted the number of marked ISC lineages per midgut at 5 and 10 days after induction. When the number of ISC lineages per gut lacking Apc was compared with wild-type, no significant differences were detected (Fig. 2.4G). These data show that Apc loss does not detectably affect the fidelity of ISC self-renewal.
2.4.4 ISCs lacking Apc generate the differentiated cells of the lineage

A second possible explanation for the hyperplasia associated with Apc loss is that Apc is required for differentiation in the ISC lineage. Inspection of Apc clone morphology initially suggested that cell fate in the lineage was correctly specified. To more rigorously analyze cell fate in Apc lineages, we examined a panel of molecular markers to label distinct cell types in the ISC lineage. The transcriptional repressor encoded by esg is expressed in ISCs and their undifferentiated EB daughters, but esg is not expressed in either of the two differentiated cell types of the midgut, the ee cells or the ECs (Fig. 2.5A) (Micchelli and Perrimon, 2006). Examination of esg-lacZ expression in Apc clones revealed the presence of both esg+ and esg- cell populations (Fig. 2.5B). Similarly, elevated levels of Delta (Dl), which marks a subset of ISCs (Ohlstein and Spradling, 2007), were also detected in Apc clones (Fig. 2.5C). We note that in some instances levels of Dl appeared to be higher in certain ISC/EB pairs (Fig. 2.5C; see also Supplemental Fig. S2.2B). Thus, undifferentiated cells of the ISC lineage can be detected in the absence of Apc.

The presence of esg- cell populations in Apc lineages suggested that ee cell and EC fates had been specified in the absence of Apc. To test this directly, we examined Apc lineages for the presence of ee cells and ECs using molecular markers. To determine whether ee cell fate is specified in lineages lacking Apc, we examined the expression of Prospero (Pros), a marker of the ee cell population (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The presence of pros+ cells was detected in Apc clones (Fig. 2.5D), as observed in wild-type cell lineages (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Similarly, examination of Tachykinin (Tk) expression, which marks a specific subset of ee cells (Ohlstein and Spradling, 2006), demonstrated that Apc is not necessary for this ee cell subtype (Fig. 2.5E). Finally, differentiated ECs are distinguished by their large, polyploid nuclei (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), and by the expression of Pdm1 (Nubbin - FlyBase). Inspection of Apc clones revealed the presence of large Pdm1+ nuclei within mutant lineages (Fig. 2.5F). Taken together, this analysis demonstrates that ISCs lacking Apc are capable of producing both ee cell and EC fates.
2.4.5  Apc is required in ISCs to regulate proliferation

Our experiments show that a reduction or loss of Apc leads to ISC lineages of increased size, as well as an increase in the number of both S-phase and M-phase markers. Over time, this leads to hyperplasia and multi-layering of the midgut. Yet, no alteration in ISC self-renewal or cell fate specification was detected using lineage-tracing analysis. Collectively, these findings raised the possibility that Apc is required specifically in ISCs to regulate proliferation. Previous analyses demonstrated that targeted knockdown of Notch (N) in ISCs leads to an expansion of esg* cell number in the midgut (Micchelli and Perrimon, 2006). Similarly, the generation of ISC lineages completely lacking N function leads to an expanded clonal population of undifferentiated cells (Ohlstein and Spradling, 2006). Thus, reduction of N function generates a population of ectopic cells that exhibit the characteristics of midgut ISCs.

If Apc functions specifically in ISCs to limit proliferation, then we predict that the loss of Apc should enhance the severity of the N loss-of-function phenotype. To directly test this possibility, we compared the number of cells per clone generated by ISCs lacking N with the number generated by ISCs lacking both N and Apc. Using the MARCM system, we created Apc mosaics that simultaneously expressed a N\textsuperscript{RNAi} transgene (Presente et al., 2002) (N\textsuperscript{RNAi}, Apc2\textsuperscript{g10}, Apc1\textsuperscript{Q8}; subsequently referred to as N\textsuperscript{RNAi}, Apc clones). At 10 days after induction, the cell proliferation and multi-layering observed in N\textsuperscript{RNAi}, Apc clones was often extensive (Fig. 2.6A-D; see also Supplemental Fig. S2.3), suggesting that Apc enhances the N\textsuperscript{RNAi} phenotype.

A quantitative analysis was performed to compare the number of ISCs generated in N\textsuperscript{RNAi}, Apc clones with the number of ISCs in marked N\textsuperscript{RNAi} lineages 5 days after induction (Fig. 2.6E-G; see also Supplemental Fig. S2.4A,B). As in previous experiments, a 5-day time point was selected for this analysis to minimize the distortion of clone size due to multi-layering. This comparison revealed a significant increase in the number of ISCs present in N\textsuperscript{RNAi}, Apc clones compared with in N\textsuperscript{RNAi} lineages alone (Fig. 2.6G; N\textsuperscript{RNAi}, n=116; N\textsuperscript{RNAi}, Apc, n=110; also compare with Fig. 2.4D for Apc alone). Thus, loss of Apc is sufficient to enhance the N\textsuperscript{RNAi} phenotype.

One explanation for the increased clone size in ISCs lacking both N\textsuperscript{RNAi} and Apc is that ISC proliferation has increased. If loss of Apc specifically affects stem cell proliferation, then we would
expect to see an associated increase in the ISC mitotic index. Mitotic index was determined by counting the number of ISCs in M-phase as a function of the total number of ISCs (see Supplemental Fig. S2.4C,D). This analysis revealed a significant increase in the mitotic index of ISCs lacking both N and Apc compared with a lack of N alone (Fig. 2.6H; N<sup>RNAi</sup>, Apc, n=16; N<sup>RNAi</sup>, n=18). This finding, together with the increased size of N<sup>RNAi</sup>, Apc mutant clones, demonstrates that the loss of Apc can affect ISC proliferation in the midgut independently of N-mediated cell fate specification.

### 2.4.6 Wnt signaling regulates homeostasis in ISC lineages

The requirement for Apc in the β-catenin destruction complex suggested that the Wnt signaling pathway might function to regulate the ISC lineage. To investigate this possibility, we first examined the effects of activating Wnt signaling on the size of marked ISC lineages. Wnt signaling was activated by generating mosaic animals expressing a constitutively active form of β-catenin, arm<sup>S10</sup> (Pai et al., 1997). As in the case of Apc loss, arm<sup>S10</sup> clones appeared abnormally large (see Supplemental Fig. S2.5A-C).

To quantify the armS10 phenotype, we compared the number of cells labeled in ISC lineages expressing armS10 to the number in marked wild-type lineages 5 days after induction. This analysis revealed a significant effect of arm<sup>S10</sup> on ISC clone size compared with wild-type controls (see Supplemental Fig. S2.5D). A comparison of the Apc and arm<sup>S10</sup> phenotypes revealed that in both cases there was a significant increase in clone size relative to wild-type. Nevertheless, the magnitude of the increase was greater following Apc loss than in the presence of arm<sup>S10</sup>, as has been observed in other contexts (e.g. (Hayden et al., 2007; Pai et al., 1997)) (see Supplemental Fig. S2.5D). Thus, Wnt pathway activation leads to an increase in the size of marked ISC lineages.

### 2.4.7 Apc hyperplasia is suppressed by reductions in Wnt signaling

The finding that constitutive Wnt activation resembled Apc loss raised the possibility that hyperplasia observed in ISC lineages lacking Apc resulted from Wnt activation. To test this
directly, we examined the effect of blocking Wnt signaling in Apc mutants. In these studies, Wnt signaling was reduced by generating mosaic animals expressing a dominant-negative form of pangolin (pan), panΔN, a transcription factor necessary for Wnt signaling (Brunner et al., 1997; van de Wetering et al., 1997). Control experiments showed first that, in contrast to wild-type lineages, mosaic expression of panΔN resulted in reduced clone size 5 days after induction (Fig. 2.7A,B), as has previously been reported for the loss of other Wnt pathway components upstream of pan (Lin et al., 2008). Second, loss of a negative regulator in the Wnt pathway, Axin (Hamada et al., 1999b; Willert et al., 1999) phenocopied both Apc loss and arm<sup>S10</sup> expression (Fig. 2.7C). Third, ISC lineages simultaneously expressing panΔN and lacking Axin led to complete suppression of the Axin loss-of-function phenotype (Fig. 2.7D). Together, these control experiments established that panΔN can suppress robust activation of the Wnt signaling pathway resulting from Axin loss.

We next investigated whether panΔN was sufficient to suppress the Apc double mutant phenotype. Simultaneous expression of panΔN in ISC lineages lacking Apc<sup>233</sup> and Apc<sup>1Q8</sup> led to a complete suppression of Apc hyperplasia (Fig. 2.7E,F). However, simultaneous expression of panΔN in ISC lineages lacking Apc<sup>2g10</sup> and Apc<sup>1Q8</sup> resulted only in a partial suppression of the Apc phenotype (Fig. 2.7G,H). One potential explanation for these differences is that Apc<sup>2g10</sup>, in contrast to Apc<sup>233</sup>, encodes a protein that retains an amino-terminal fragment, which has been shown to have an activating role in the Wnt signaling pathway (Takacs et al., 2008). Similarly truncated alleles of Apc are known to be associated with human adenomas (Albuquerque et al., 2002; Cheadle et al., 2002; Lamlum et al., 1999; Rowan et al., 2000). Taken together, our studies show that the abrogation of Wnt signaling in ISC lineages is sufficient to suppress the effect of Apc loss in an allele-specific manner.

2.5 Discussion

In the current study, we report that loss of Apc results in a disruption of midgut homeostasis and is associated with hyperplasia and multi-layering of the midgut epithelium. Our mosaic analyses show that Apc is required specifically in ISCs to regulate stem cell proliferation. By contrast, loss of Apc did not detectably affect self-renewal or cell fate specification in the ISC
lineage. Activation of Wnt signaling in the ISC lineage phenocopied Apc loss and Apc mutants were suppressed in an allele-specific manner by abrogating Wnt signaling, suggesting that the effects of Apc are mediated in part by the Wnt pathway. The finding that Apc differentially affects ISC proliferation without obviously altering self-renewal or multi-potency highlights the ability of the stem cell to fine-tune lineage output to meet homeostatic need; loss of Apc appears to short-circuit this regulation, providing increased cellular output in the absence of true physiological demand for new cells.

Previous analysis of Wnt signaling in the midgut has led to the assertion that Wnt functions as the primary maintenance signal for ISCs; cell-autonomous loss of Wnt transduction components results in a failure of ISC maintenance, while ectopic expression of Wnt ligand leads to an increase in the number of Dl-expressing cells (Lin et al., 2008). These observations led to the following model: transduction of the Wnt signaling pathway in ISCs adjacent to a Wnt source maintains the stem cell population by preventing lineage differentiation. A central prediction of the model is that cell-autonomous activation of the Wnt signaling pathway in ISCs should lead to the production of daughter cells, which constitutively transduce the Wnt signal and, as such, remain undifferentiated. The predicted consequence of this manipulation is an expansion in the number of ISCs at the expense of differentiated cells within the lineage, as has been observed in the case of N loss (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). In this study, we directly tested this prediction by analyzing marked ISC lineages. Our experiments clearly demonstrate that in contrast to N loss, ISCs lacking Apc generate both differentiated cell types of the adult midgut: ee cells and ECs. Importantly, these findings suggest that ISCs and their daughters are not distinguished solely on the basis of Wnt signal transduction.

Several explanations could account for these apparent disparities. First, it is worth noting that although Dl might be a reliable marker for certain stem cells in wild-type midguts, this might not be the case in every mutant background examined. For example, a previous analysis in Drosophila has demonstrated that Wnt activation is sufficient to stimulate high levels of Dl expression in a cell-autonomous manner (Micchelli et al., 1997). Thus, it is possible that Wnt pathway activation can uncouple Dl expression from stem cell identity, thereby diminishing the
utility of DI as a reliable stem cell marker. A second possible explanation is methodological; the use of genetic mosaic analyses to analyze individual ISC lineages, as in this study, might provide a different view of the Wnt pathway activation phenotype to that seen following the use of Gal4 driver lines. Third, it is possible that Wnt can affect stem cell maintenance upstream of Apc via a noncanonical pathway. For example, studies have demonstrated that Wnt signaling can act directly via dishevelled (dsh) to inhibit the N signaling pathway (Axelrod et al., 1996; Rulifson et al., 1996). Such effects might not be detected in the Apc, Axin or activated arm\textsuperscript{S10} ISC lineages analyzed here.

In this study, we have demonstrated an increase in the number of dividing cells following Apc loss, which is consistent with what has previously been reported for mutations that activate the Wnt pathway (Lin et al., 2008). And yet, as discussed above, the presence of differentiated cells within marked Apc mutant lineages strongly suggested that the increase in dividing cells could not be explained solely by a N-dependent change in cell fate within the ISC lineage as was proposed by Lin et al. Based on our analysis of Apc, we hypothesized that the increased proliferation following Apc loss reflects a cell-autonomous requirement for Apc specifically in ISCs. This view is further supported by the observation that Apc loss can lead to an increase in mitotic index when N and Apc are simultaneously removed from the ISC lineage. Thus, there is a requirement for Apc specifically in ISCs to regulate proliferation that is separable from N-dependent cell fate specification. Taken together, we conclude that a primary requirement for Apc in the ISC lineage is to autonomously restrict the proliferation of ISCs, and not to regulate the choice of cell fate.

On the basis of our findings we propose that ISC activity is regulated by the level of Wnt signal transduction (Fig. 2.8). In this model, Wnt functions as a permissive signal for ISC self-renewal, as we show that constitutive Wnt activation is not a sufficient criterion to convert all ISC progeny to stem cells, nor is activation sufficient to alter the fidelity of ISC self-renewal. Intermediate levels of Wnt define an adaptive homeostatic range, which permits the midgut to respond to environmental changes that the organism encounters. ISCs transducing Wnt at levels outside this range appear refractory to homeostatic input, as low levels of Wnt are associated with ISC loss, whereas Wnt activation leads to hyperplasia.
Nevertheless, our data do not rule out additional roles for Apc in the ISC lineage. For example, it is possible that the hyperplasia observed in Apc mutants reflects the combined requirement of Apc to regulate proliferation both in the ISCs and in nascent EB daughters. Similarly, Apc might also play a role in regulating cell turnover in the midgut. Indeed, several of our observations support the view that Apc might, in fact, be required for EC differentiation. First, mosaic analysis of Apc shows that cells of the lineage contribute to the multi-layering phenotype, which suggests that mutant cells might have failed to properly establish appropriate adhesive contacts with the monolayer and/or the surrounding extracellular matrix (Fig. 2.3D,F). Second, although ECs lacking Apc appear to have large, polyploid nuclei and express molecular markers such as Pdm1, their nuclei are often detectably smaller than those of wild-type ECs (Fig. 2.4B,C). Furthermore, ECs lacking Apc often exhibit a restricted basal profile, failing to develop the tiled morphology that is characteristic of wild-type ECs (Fig. 2.4B,C). Finally, in the absence of Apc, ECs often display reduced cytoplasm, suggesting a requirement for cellular growth, a requirement that is not observed in either Axin mutant or armS10-expressing ICS lineages (Fig. 2.7; see also Supplemental Fig. S2.5).

The discovery that Apc is somatically mutated in cells of the smallest human adenomas (Miyoshi et al., 1992; Powell et al., 1992) and that the frequency of Apc mutations detected among early adenomas is roughly the same as the frequency of Apc mutations in more advanced carcinomas (Powell et al., 1992) were among the seminal observations that established Apc as the rate-limiting step for gastrointestinal tumor initiation (reviewed in (Clevers, 2006; Kinzler and Vogelstein, 1996)). Subsequently, a number of Apc models have been established in both mouse and zebrafish to study gastrointestinal tumorigenesis (Fodde et al., 1994; Haramis et al., 2006; Oshima et al., 1995; Su et al., 1992). Yet, the precise cell(s) in which Apc is required remained unknown. Recently, through the use of refined genetic cell lineage tracing methodologies in the mouse, specific subpopulations of cells in the intestinal mucosa have been identified (e.g. Lgr5+ and Bmi1+), which display the ability to self-renew and undergo multi-lineage differentiation (Barker et al., 2007; Sangiorgi and Capecchi, 2008).
Subsequent studies have demonstrated that deletion of Apc specifically within the Lgr5+ cell population leads to the formation of rapidly proliferating cells in both the large and small intestine (Barker et al., 2009). Thus, in the case of both Drosophila ISCs and mouse Lgr5+ cells, loss of Apc leads to a disruption of homeostasis in the intestinal stem cell lineage. The remarkable parallels that exist between the dipteran and mammalian gastrointestinal tract suggest that the Drosophila midgut will continue to be a powerful genetic model system with which to dissect the molecular mechanisms underlying tumor initiation.
Figure 2.1: The adult *Drosophila* midgut is maintained by a population of multi-potent intestinal stem cells (ISCs). (A) Diagram of the adult midgut in cross section. ISCs (green) occupy a basal position in a niche adjacent to the basement membrane and the visceral muscle (red). ISCs give rise to two types of differentiated daughters, enteroendocrine (ee) cells (blue) and enterocytes (ECs; orange). (B) A cross section of the adult midgut showing ISCs marked by *esg-Gal4, UAS-GFP* (green). ECs have large polyploid nuclei (blue, DAPI) and form a polarized cellular monolayer with an actin-rich (red, phalloidin) brush border on their luminal surface; ee cells are not marked.
Figure 2.2: Apc is required to maintain adult midgut homeostasis. (A-D) Global reduction of Apc family genes leads to an increase in BrdU incorporation in esg-lacZ cells (anti-BrdU, red; anti-βgal, green; DAPI, blue). Superficial views of midgut. (A) Apc\textsuperscript{TS} unshifted. Note that large polyploid EC nuclei incorporate BrdU. (B,C) Apc\textsuperscript{TS} shifted. Asterisks indicate esg\textsuperscript{*} BrdU\textsuperscript{*} cells. (D) Quantitation of BrdU\textsuperscript{*} cells (unshifted, \(n=11\); shifted, \(n=11\)). Error bars denote s.e.m. **\(P=0.0002\). Scale bar: 50 \(\mu m\).
Figure 2.3: Loss of Apo in the midgut leads to hyperplasia and multi-layering. (A-D) The MARCM system was used to positively identify ISC lineages with GFP 20 days after induction (anti-GFP, green; DAPI, blue). Midgut viewed in cross section; dashed red line indicates midgut outline. (A) Wild-type ISC lineages. Marked cells define a single cellular layer. (B) ISC lineages lacking Apo. Loss of Apo leads to midgut hyperplasia and extensive multi-layering. (C) High magnification view of A. Frame corresponds to the region on the outer face of the midgut indicated by the asterisk (A). (D) High magnification view of B. Frame corresponds to the region on the outer face of the midgut indicated by the asterisk (B). (E) Maximal number of midgut layers plotted as a function of midgut area in wild-type and Apo mosaic midguts. Note the multi-layering
and increased area in Apc mosaic midguts. (F) Maximal number of midgut layers plotted as a function of maximal epithelial height in wild-type and Apc mosaic midguts. Scale bars: 50 µm.
Figure 2.4: Loss of Apc in ISCs leads to an increase in clone size. (A) Diagram of the adult Drosophila gastrointestinal tract (see Miller, 1950); midgut in heavy black outline. Data were collected from two morphologically defined regions: one in the middle of the anterior midgut (green frame) and one in the middle of the posterior midgut (red frame). (B-G) The MARCM system was used to positively identify ISC lineages with GFP (anti-GFP, green; DAPI, blue). (B) Wild-type ISC lineages in posterior midgut 5 days after induction, superficial view. (C) ISC lineages lacking Apc in posterior midgut 5 days after induction, superficial view. (D) Apc loss leads to an increase in the number of cells per clone (wild-type, \( n=76 \); Apc, \( n=76 \)). (E) Mosaic midguts lacking Apc display an increase in the number of pHH3+ cells (wild-type, \( n=18 \); Apc, \( n=17 \)). (F) Analysis of Apc clones along the anteroposterior axis of the midgut. Compared with wild-type, Apc clones are larger in both the anterior (wild-type, \( n=38 \); Apc, \( n=37 \)) and posterior (wild-type, \( n=38 \); Apc, \( n=39 \)) regions. (G) Self-renewal is not detectably altered in ISCs lacking Apc at 5 days (wild-type, \( n=6 \); Apc, \( n=6 \)) or 10 days (wild-type, \( n=8 \); Apc, \( n=9 \)) after induction. Error bars denote s.e.m. Scale bar: 50 µm.
Figure 2.5: Cell fate in the ISC lineage is correctly specified in the absence of Apc. (A-F) The MARCM system was used to positively identify ISC cell lineages with GFP 5 days after induction (anti-GFP, green; DAPI, blue). (A) Wild-type ISC lineages. esg-lacZ (red) marks the undifferentiated cells in the ISC lineage: stem cells and their undifferentiated EB daughters. (B) ISC lineages lacking Apc; esg-lacZ shown in red. Note the presence of both esg+ and esg− cells in marked Apc lineages. (C) ISC lineages lacking Apc (anti-DI, red). (D) ISC lineages lacking Apc.
Anti-Pro (red) marks ee cells in the clone. (E) ISC lineages lacking Apc. Anti-Tachykinin (red) marks a subset of ee cells in the clone. (F) ISC lineages lacking Apc. Anti-Pdm1 (red) marks ECs. Scale bar: 50 µm.
Figure 2.6: Apc is required in ISCs to regulate proliferation. (A-F) The MARCM system was used to positively identify ISC lineages with GFP. (A,B) Clone outlines indicated in red, nuclei in blue (DAPI), and dividing cells in white (phospho-histone H3). (A) ISC clones expressing \( N^{\text{RNAi}} \) 10 days after induction. (B) ISC clones expressing \( N^{\text{RNAi}} \) and lacking Apc, 10 days after induction. (C,D) ISC lineages marked with GFP (anti-GFP, green; DAPI, blue); dashed red line indicates midgut outline. (C) ISC clones expressing \( N^{\text{RNAi}} \) 10 days after induction. (D) ISC clones expressing \( N^{\text{RNAi}} \) and lacking Apc, 10 days after induction. Extensive multi-layering is evident in cross section. Asterisk indicates hyperplastic cells in lower focal planes extending up into the luminal space. (E-H) Quantitation of ISC proliferation phenotype. (E) ISC clones expressing \( N^{\text{RNAi}} \) 5 days after induction. (F) ISC clones expressing \( N^{\text{RNAi}} \) and lacking Apc, 5 days after induction. (G) Comparison of \( N^{\text{RNAi}} \) and \( N^{\text{RNAi}}, \) Apc clone size 5 days after induction. \( N^{\text{RNAi}}, \) Apc loss leads to an increase in number of cells per clone (\( N^{\text{RNAi}}, n=116; N^{\text{RNAi}}, \) Apc, \( n=110 \)). (H) Comparison of \( N^{\text{RNAi}} \) and \( N^{\text{RNAi}}, \) Apc mitotic index 5 days after induction. Apc loss leads to an increase in the ISC mitotic index (\( N^{\text{RNAi}}, n=18; N^{\text{RNAi}}, \) Apc, \( n=16 \)). Error bars denote s.e.m. Scale bars: 50 \( \mu \)m.
Figure 2.7: Apc hyperplasia is suppressed by reducing Wnt signaling. (A-H) The MARCM system was used to positively identify posterior ISC cell lineages with GFP 5 days after induction (anti-GFP, green; DAPI, blue). (A) Wild-type ISC lineages. (B) ISC lineages expressing panΔN (dominant negative). (C) ISC lineages lacking Axin. (D) ISC lineages lacking Axin and expressing panΔN. (E) ISC lineages lacking Apc2^{33} and Apc1^{Q8}. (F) ISC lineages lacking Apc2^{33} and Apc1^{Q8}, and expressing panΔN. Note that hyperplasia is suppressed. (G) ISC lineages lacking Apc2^{g10} and Apc1^{Q8}. (H) ISC lineages lacking Apc2^{g10} and Apc1^{Q8}, and expressing panΔN. Scale bar: 50 µm.
Figure 2.8: Wnt regulation of midgut ISCs. Wnt functions as a permissive signal, and at intermediate levels of signal transduction defines an adaptive homeostatic range for ISCs activity (green). In this model, niche modulation fine-tunes ISC activity within this adaptive homeostatic range.
Supplemental Figure S2.1: The standard quantitative method used in analyzing mosaic midguts. (A) Diagram of the adult *Drosophila* gastrointestinal tract (see (Miller, 1994)). The adult midgut extends the length between the cardia and the pylorus (heavy black outline). Data were collected from two defined regions of each midgut analyzed: the middle of the anterior midgut and the middle of the posterior midgut. To reproducibly determine the middle of both the anterior and
posterior midgut, each segment was first independently divided into a series of non-overlapping 40x fields of view or frames using the confocal microscope (green and red asterisks, respectively). The anterior segment extends from the narrowest region following the cardia to the anterior limit of the copper cells; the anterior-most frame we designated a1. The posterior segment extends from the posterior limit of the copper cells to the anterior limit of the pylorus where the malpighian tubules enter the tract; the anterior-most frame of the posterior midgut we designated p1. Under our experimental conditions, we found that the anterior and posterior segments of the midgut can each be covered in the span of 5 (or 4) frames (e.g. a1-a5, p1-p4). (B) In a 10-frame midgut, the middle of the anterior is therefore centered at a3 (green frame), and the middle of the posterior is centered at p3 (red frame). (C) In an 8-frame midgut the middle of the anterior is centered at a2.5, and the middle of the posterior is centered at p2.5. Midguts were measured twice before selecting the final frames used for analysis.
Supplemental Figure S2.2: Apc loss leads to increased DI levels. (A,B) The MARCM system was used to positively identify ISC lineages with GFP 5 days after induction (anti-GFP, green; anti-Pros and anti-DI, red; DAPI, blue). Note that anti-DI and anti-Pros are both shown in red; DI staining appears punctate and membrane localized, whereas Pros expression is saturated and nuclear. (A’,B’) Clone boundary indicated by green outline, anti-DI and anti-Pros are both shown in white. (A, A’) Wild-type ISC lineages. (B, B’) ISC lineages lacking Apc. Note that, compared with wild type, DI appears at higher levels in some ISC/EB pairs both within and outside of marked Apc lineages. Scale bar: 50 µm.
Supplemental Figure S2.3: Loss of Apc in ISCs enhances hyperplasia and multi-layering of the \( N^{\text{RNAi}} \) phenotype. (A, B) The MARCM system was used to positively identify ISC lineages with GFP 10 days after induction (anti-GFP, green; anti-Pros, red; DAPI, blue). A series of progressively deeper optical sections from the surface of the midgut is shown at 10 \( \mu \text{m} \), 30 \( \mu \text{m} \) and 50 \( \mu \text{m} \) from the basement membrane. (A-A”) ISC lineages expressing \( N^{\text{RNAi}} \). (B-B”) ISC
lineages expressing $N^{\text{RNAi}}$ but lacking $Apc$. Hyperplasia, multi-layering and disruption of midgut organization are evident. Scale bar: 50 µm.
Supplemental Figure S2.4: Quantification of clone size and mitotic index in $N^{RNAi}$, $Apc$
lineages. (A-D) The MARCM system was used to positively identify ISC lineages with GFP 5
days after induction (anti-GFP, green; DAPI, blue). (A’-D’) Clone boundary indicated by green
outline. (A’, B’) Quantification of the number of cells per clone. To quantify the phenotype only
esg$^+$ pros$^-$ cells were scored (esg-lacZ, red; anti-Pros; blue). (A’) $N^{RNAi}$; (B’) $N^{RNAi}$, $Apc$. (C’,D’)
Quantification of mitotic index. To quantify the phenotype, only small pH3$^+$ pros$^-$ cells were
scored (anti-Pros, red; anti-phospho-histone H3, white; DAPI, blue). (C’) $N^{RNAi}$; (D’) $N^{RNAi}$, $Apc$.
Scale bar: 50 µm.
Supplemental Figure S2.5: Wnt activation in the ISC lineage leads to an increase in clone size. (A-D) The MARCM system was used to positively identify posterior ISC lineages with GFP 5 days after induction (anti-GFP, green; DAPI, blue). (A) Wild-type ISC lineages. (B) ISC lineages expressing armS10. (C) ISC lineages lacking Apc. (D) Quantitation of the number of cells per clone in armS10 and Apc mutant lineages. armS10 expression leads to a significant increase in clone size compared with wild type, although not as great as that caused by Apc loss. (wild-type, n=41; armS10, n=144; Apc, n=157). Error bars denote s.e.m. Scale bar: 50 µm.
Chapter 3

JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the *Drosophila* intestinal stem cell lineage

The research work in this Chapter is published in Developmental Biology. 2010 Feb;338(1):28-37. Wen-Chih Lee is the second author in this publication.
3.1 Summary

Adult stem cells are the most primitive cells of a lineage and are distinguished by the properties of self-renewal and multi-potency. Coordinated control of stem cell proliferation and multi-lineage differentiation is essential to ensure a steady output of differentiated daughter cells necessary to maintain tissue homeostasis. However, little is known about the signals that coordinate stem cell proliferation and daughter cell differentiation. Here we investigate the role of the conserved JAK/STAT signaling pathway in the Drosophila intestinal stem cell (ISC) lineage. We show first, that JAK/STAT signaling is normally active in both ISCs and their newly formed daughters, but not in terminally differentiated enteroendocrine (ee) cells or enterocyte (EC) cells. Second, analysis of ISC lineages shows that JAK/STAT signaling is necessary but not sufficient for daughter cell differentiation, indicating that competence to undergo multi-lineage differentiation depends upon JAK/STAT. Finally, our analysis reveals JAK/STAT signaling to be a potent regulator of ISC proliferation, but not ISC self-renewal. On the basis of these findings, we suggest a model in which JAK/STAT signaling coordinates the processes of stem cell proliferation with the competence of daughter cells to undergo multi-lineage differentiation, ensuring a robust cellular output in the lineage.

3.2 Introduction

Adult stem cell populations are present in a variety of tissues and function throughout the lifetime of an organism to maintain homeostasis. The dual characteristics of self-renewal and multipotency make stem cells ideally suited for this central role. In a variety of tissue systems, adult stem cells have been shown to reside in specialized microenvironments called niches, which regulate stem cell behavior at baseline homeostasis and dynamically respond to changing environmental stimuli by modulating lineage output (reviewed in (Jones and Wagers, 2008)). The coordinated control of stem cell proliferation and multi-lineage differentiation is essential to ensure a steady output of differentiated daughter cells at baseline homeostasis and in response to changing environmental conditions. However, little is currently known about the factors that coordinate these processes.
The ability to identify, mark and manipulate individual stem cell lineages has made *Drosophila* an excellent model system with which to dissect stem cell regulation. In the adult *Drosophila* midgut, for example, the stem cell compartment is comprised of individual intestinal stem cells (ISCs) that are dispersed throughout the entirety of the tissue (Fig. S3.1A-D; (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006)). ISCs have a pyramidal morphology and are located in an epithelial niche distant from the midgut lumen and adjacent to both the basement membrane and surrounding visceral musculature of the midgut. ISCs are multipotent and give rise to a lineage that consists of two types of differentiated daughters, the enteroendocrine (ee) cells and the enterocyte (EC) cells. Together, these cell populations form a cellular monolayer lining the length of adult midgut.

The *Janus Kinase/Signal Transducer and Activator of Transcription* (JAK/STAT) pathway is a conserved signal transduction pathway that has been implicated in a number of distinct developmental and disease processes (reviewed in (Arbouzova and Zeidler, 2006)). In *Drosophila*, the JAK/STAT pathway utilizes a set of core signaling components: a transmembrane receptor encoded by *domeless* (*dome*), a single JAK tyrosine kinase encoded by *hopscotch* (*hop*), the transcription factor *stat92E*, and *unpaired* (*upd*), as well as two related ligands encoded by *upd2*, *upd3*. Binding of Upd ligands to the Dome receptor leads to activation of Hop, a receptor-associated kinase, which has at least two substrates, Hop and Dome. Cytoplasmic Stat92E can bind to phosphorylated Dome/Hop complexes via SH2 domains. Once bound to the Dome/Hop complex, Stat molecules are also phosphorylated and form Stat dimers, which translocate to the nucleus and activate downstream transcriptional targets.

Phenotypic analysis in *Drosophila* has revealed that the JAK/STAT signaling pathway is a versatile regulator of stem cell populations and their cell lineages (reviewed in (Fuller and Spradling, 2007; Gregory et al., 2008)). Evidence suggests that JAK/STAT is necessary for the maintenance of germline and somatic stem cells and functions as a powerful signal promoting stem cell proliferation (Decotto and Spradling, 2005; Kiger et al., 2001; Singh et al., 2007; Tulina and Matunis, 2001). JAK/STAT signaling is also required in the ovary for subsequent differentiation and maintenance of specific cell types (Baksa et al., 2002; Ghiglione et al., 2002;
McGregor et al., 2002; Silver et al., 2005). Recent studies in the adult midgut indicate that JAK/STAT signaling is a central mediator of adaptive homeostasis following a variety of experimental challenges including bacterial infection, directed cell ablation or stress signaling (Buchon et al., 2009; Cronin et al., 2009; Jiang et al., 2009). Collectively, these studies support a model in which JAK/STAT ligands are induced in response to challenge and stimulate ISC activity.

In this study, we investigate the role of the JAK/STAT pathway within the ISC lineage under conditions of baseline homeostasis. We show that JAK/STAT signaling is normally active in both ISCs and their newly formed daughters, but not in terminally differentiated enteroendocrine (ee) cells or enterocyte (EC) cells. We also show that cell autonomous loss of JAK/STAT signaling from individual ISC lineages results in a failure of ee and EC cell fate specification. Previous studies indicate that Notch signaling is a critical regulator of cell fate in the ISC lineage (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). Genetic analysis demonstrates that the transcription factor, stat92E, is epistatic to Delta/Notch (Dl/N) signaling in cell fate specification, suggesting that the JAK/STAT pathway functions downstream or in parallel to Dl/N signaling in newly formed ISC daughters. Finally, our analysis shows that JAK/STAT signaling is a potent regulator of ISC proliferation; tests revealed little evidence that JAK/STAT is required for ISC self-renewal. Thus, JAK/STAT signaling has dual, yet separable requirements in the undifferentiated cells of the ISC lineage. On the basis of these findings, we suggest a model in which JAK/STAT signaling coordinates the processes of stem cell proliferation with the competence of daughter cells to undergo multi-lineage differentiation.

3.3 Material and methods

3.3.1 Drosophila strains and culture

w; 10xSTAT92E-GFP and w; 10xSTAT92E-GFP\textsuperscript{destabilized} (transcriptional reporters of JAK/STAT activity; (Bach et al., 2007)). upd1-Gal4 (Tsai and Sun, 2004). w; upd3-Gal4 (Agaisse et al., 2003). stat92\textsuperscript{E} (temperature sensitive allele; (Baksa et al., 2002)). w; FRT\textsuperscript{82B} stat92\textsuperscript{E}\textsuperscript{85C9} / TM6B-GFP (missense mutation generated by EMS; (Silver and Montell, 2001)). FRT\textsuperscript{82B}
stat92E^{65346} / TM3 (also called stat92E^{P1581}, null allele; (Hou et al., 1996)). hop^{C111} FRT^{19A} / FM7 (null allele; (Binari and Perrimon, 1994)). y, w; UAS-hop^{Tum} / Cyo-GFP (activated form of hop; (Harrison et al., 1995)). w; UAS-Upd / Cyo-GFP (Harrison et al., 1998). w; UAS-N^{FNC} (A dominant negative form of N; (Rebay et al., 1993)). w, N^{65eff1} FRT^{19A} / FM7 (null allele; (Kidd et al., 1986)). ry^{506}, p[GBE + Su(H) m8, ry+, lacZ] (Su(H)GBE-lacZ; (Furriols and Bray, 2001)). w; UAS-N^{intra} / Cyo (Struhl et al., 1993). ry^{506}, Dl^{5151} (Dl-lacZ; (Röttgen et al., 1998)). w; UAS-Dl.H (Jönsson and Knust, 1996). vkg-GFP (collagen IV-GFP protein trap; (Morin et al., 2001)). w, tub-GAL80, hsFLP FRT^{19A}; UAS-lacZ^{2uc}, UAS-mCD8GFP; tub-Gal4 (Bello et al., 2003). y, w, UAS-GFP, hsFLP; tubGal4, FRT^{92b} tub-Gal80 / TM6B (Lee and Luo, 1999). w; FRT^{19A} and w; FRT^{92b} hsM (these stocks were used as a wild-type control in the mosaic analysis performed in this study; (Xu and Rubin, 1993)). w; esg-Gal4 / Cyo (Goto and Hayashi, 1999). w; esg-Gal4, UAS-GFP (Micchelli and Perrimon, 2006). w; esg-Gal4, UAS-GFP, tub-Gal80^{TS} (Micchelli and Perrimon, 2006). y, w; esg^{K606} / Cyo (esg-lacZ; (Samakovlis et al., 1996)). y, w, hsFLP: AyGal4, UAS-GFP (Flip-out clones; (Ito et al., 1997)). Crosses were cultured on standard dextrose media and transferred to fresh food supplemented with yeast paste every 1-2 days during the experimental period. Crosses were reared at 18, 25, or 29 °C, in passively illuminated and humidified incubators. In this study adult female flies of the following genotypes were analyzed:

**Figure 3.1:**

w; esg-Gal4, UAS-GFP

vkg-GFP / esg^{K606}

**Figure 3.2 and S3.1:**

y, w / w; esg^{K606} / 10xSTAT92E-GFP.

w / +; 10xSTAT92E-GFP / +; ry^{506}, Dl^{5151} / +.

w; 10xSTAT92E-GFP.

w / +; 10xSTAT92E-GFP^{D} / +.

w / +; 10XSTAT92E GFP^{D} / +; ry^{506}, p[GBE + Su(H) m8, ry+, lacZ] / +.

w / y, w, UAS-mCD8GFP; upd1-Gal4 / esg^{K606}.

w; upd3-Gal4, UAS-GFP.
Figure 3.3, S3.2, S3.3, S3.5, and S3.6:

\[ \text{stat92E}^F / \text{FRT}^{82B} \text{stat92E}^{06346} \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{hs\pi M} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ w, \text{tub-Gal80, hsFLP FRT}^{19A} / \text{hop}^{C_{111}} \text{FRT}^{19A}; \text{UAS-lacZ}^{\text{fluc}}, \text{UAS-mCD8GFP} / +; \text{tub-Gal4} / +. \]

\[ y, w, \text{hsFLP, UAS-GFP} / +; \text{FRT}^{82B} \text{stat92E}^{06346} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / +; \text{UAS-DI} / +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

Figure 3.4:

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{esg}^{K_{606}} / +; \text{FRT}^{82B} \text{hs\pi M} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{esg}^{K_{606}} / +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ \text{ry}^{506}, p[\text{GBE}\text{+Su(H)m8,ry+}, \text{lacZ}] \]

Figure 3.5 and S3.4:

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{hs\pi M} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{UAS-DI} / +; \text{FRT}^{82B} \text{hs\pi M} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{UAS-DI} / +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{UAS-N}^{\text{RNAi}} / +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ \text{ry}^{506}, \text{p[GBE}\text{+Su(H)m8,ry+}, \text{lacZ}] \]

Figure 3.6:

\[ w, \text{tub-GAL80, hsFLP FRT}^{19A} / N^{55c11} \text{FRT}^{19A}; \text{UAS-lacZ}^{\text{fluc}}, \text{UAS-mCD8GFP} / +; \text{tub-Gal4} / +. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{UAS-N}^{\text{RNAi}} / +; \text{FRT}^{82B} \text{hs\pi M} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; \text{UAS-N}^{\text{RNAi}} / +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

Figure 3.7:

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{hs\pi M} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

\[ w, \text{tub-Gal80, hsFLP FRT}^{19A} / \text{hop}^{C_{111}} \text{FRT}^{19A}; \text{UAS-lacZ}^{\text{fluc}}, \text{UAS-mCD8GFP} / +; \text{tub-Gal4} / +. \]

\[ y, w, \text{hsFLP, UAS-GFP} / w; +; \text{FRT}^{82B} \text{stat92E}^{85C9} / \text{tub-Gal4}, \text{FRT}^{82B} \text{tub-Gal80}. \]

Figure 3.8:
w; esg-Gal4, UAS-GFP, tub-Gal80\textsuperscript{TS} / +.

w; esg-Gal4, UAS-GFP, tub-Gal80\textsuperscript{TS} / UAS-upd.

w; esg-Gal4, UAS-GFP, tub-Gal80\textsuperscript{TS} / +; UAS-N_{ECN} / +.

w; esg-Gal4, UAS-GFP, tub-Gal80\textsuperscript{TS} / UAS-upd; UAS-N_{ECN} / +.

y, w, hsFLP / w; AyGal4, UAS-GFP / UAS-upd.

y, w, hsFLP, UAS-GFP / y, w; UAS-hop\textsuperscript{tum} / +; FRT\textsuperscript{82B} hs\pi M / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.

### 3.3.2 Mosaic analysis

Positively marked ISC lineages were generated using the MARCM system. MARCM clones were induced by placing experimental fly vials in a 37 °C water bath for 35-45 min. Induction protocols consisted of 2-3 heat pulses within a 24-h period and were performed within the first 5-10 days of adulthood. Expression of UAS-upd using the Flip-out technique was performed bysubjecting experimental flies to a single 30 min heat shock within the first 5-10 days of adulthood (Fig. 3.8C,D).

### 3.3.3 Temperature shift experiments

Two different temperature shift analyses were performed in this study (Fig. 3.3A,B and Fig. 3.8A,B). In both cases, we established and cultured crosses at 18 °C until collection of F1 progeny. In the stat92E\textsuperscript{TS} analysis (Fig. 3.3A,B), we divided F1 progeny into two equal pools, maintaining controls at 18 °C and shifting the experimental group to 29 °C for 14 days. For the series of experiments shown in Fig. 3.8A and B, all F1 adult progeny were aged 4-7 days at 18 °C and were then shifted to 29 °C for 48 h prior to dissection.

### 3.3.4 Histology

Adult flies were dissected in 1x PBS (Sigma, USA). The gastrointestinal tract was removed and fixed in a final solution of 0.5x PBS (Sigma, USA) and 4% electron microscopy grade formaldehyde (Polysciences, USA) for a minimum of 30 min. Samples were washed in 1x PBS + 0.1% Triton-X100 (PBST) for 2 h, then incubated with primary antibodies overnight. Samples
were washed in PBST for 2 h then incubated with secondary antibodies for 3 h. Finally, samples were washed in PBST overnight. Mounting media containing DAPI (Vectashield, USA) was added and samples were allowed to clear for 1 h prior to mounting. All steps were completed at 4 °C with no mechanical agitation.

3.3.5 BrdU incorporation experiments

Flies were aged for 4 weeks on standard media following clone induction and then transferred to BrdU media for 1 week. BrdU was administered *ad libitum in Drosophila* food media (200μl of 6 mg/ml BrdU in 20% sucrose per fresh vial). Dissected samples were fixed for 30 min at room temperature and then washed for 30 min at room temperature. DNA was denatured by applying a 2.2N solution of HCl to samples for 30 min at room temperature followed by neutralization in Borax (100 mM) for 5 min and a final 30 min PBST wash. Samples were then stained as described above.

3.3.6 Antiseras

Primary antibodies: Chicken anti-GFP (Abcam, USA) used at a dilution of 1:10,000; rabbit anti-β-Gal (Cappel, USA), 1:2000; mouse anti-β-Gal (Developmental Studies Hybridoma Bank; DSHB), 1:100; mouse anti-Pros (DSHB) 1:100; mouse anti-Dl (DSHB), 1:100; mouse anti-BrdU, 1:100; rabbit anti-Pdm-1 (gift of W. Chia (Yeo et al., 1995)),1:1000; rabbit anti-pHH3 (Upstate), 1:1000.


Mounting media: Vectashield + DAPI mounting media (Vector, USA).
3.3.7 Microscopy and imaging

Samples were examined on a Leica DM5000 upright fluorescent microscope. Confocal images were collected using a Leica TCS SP5 confocal microscope system. Images were processed for brightness and contrast and assembled in Photoshop CS (Adobe, USA).

3.3.8 Measurements, cell counts and statistical analysis

Maximum nuclear diameter was measured in both anterior and posterior midgut frames at 40x magnification (A2.5 and P2.5; see (Lee et al., 2009) for additional information on midgut nomenclature) using the Leica Application Suite (LAS; Fig. 3.3E). We scored the number of Pros\(^+\) or Pdm1\(^+\) cells in both anterior and posterior midgut frames 5 days following clone induction (Fig. 3.4, Fig. 3.5 and Fig. 3.6). The number of GFP\(^+\) cells per frame was quantified in both the anterior and posterior midgut at 5, 10 and 20 days following clone induction (Fig. 3.7G and Supplemental Fig. S3.5E). In the experiments described above, we counted cells on the top surface of the midgut and excluded cells along the side and bottom surfaces. Combined data for anterior and posterior regions are displayed (Fig. 3.3, Fig. 3.7 and Supplemental Fig. S3.5E). To determine the number of dividing cells following UAS transgene activation, we counted the number of GFP\(^+\) pHH3\(^+\) cells along the entire length of the midgut 48 h after temperature shift (Fig. 3.8B). The number of GFP\(^-\), Pros\(^-\) small cells was quantified in both anterior and posterior 40x frames and combined (Supplemental Figs. 3.5E,B). The number of GFP\(^-\), pHH3\(^+\) small cells was counted for the entire length of the midgut (Supplemental Fig. S3.2C). All \(t\)-tests were performed using Prism (GraphPad Software, USA); significance was tested at the 95% CI.

3.4 Results

3.4.1 JAK/STAT signaling is dynamically regulated in the midgut

JAK/STAT activation in the midgut was examined using a transcriptional reporter for pathway activation (Bach et al., 2007). The reporter, 10xSTAT92E-GFP, is composed of multimerized Stat92E consensus binding sites fused to GFP, which is expressed in a manner that recapitulates the tissue specific distribution of Stat92E protein. Moreover, JAK/STAT signaling is both
necessary and sufficient for the activation of this reporter (Bach et al., 2007). Thus, $10x$STAT92E-GFP functions as a high fidelity reporter for JAK/STAT activity in vivo.

Examination of the adult midgut revealed the presence of numerous $10x$STAT92E-GFP positive cells with small nuclei distributed along the entire anterior-posterior axis of the tissue (52/52 midguts analyzed). Previous analysis has shown that esg marks both individual ISCs and their undifferentiated EB daughters, which can often be detected as pairs of cells or “doublets” in the midgut (Micchelli and Perrimon, 2006). Thus, esg distinguishes the undifferentiated cells of the ISC lineage from differentiated ee and EC cells. To determine the precise identity of the cells expressing the JAK/STAT reporter, we double labeled the midgut to reveal the distribution of esg-lacZ and the $10x$STAT92E-GFP reporter. Double staining showed complete marker coexpression, suggesting that the JAK/STAT signal is transduced in both the ISC and EB (Fig. 3.2A). It has also been shown that elevated Dl protein levels mark a subset of midgut ISCs (Ohlstein and Spradling, 2007). Consistently, double staining of Dl-lacZ and $10x$STAT92E-GFP also showed colocalization of these reporters in ISCs (Fig. 3.2B).

We next asked if JAK/STAT pathway activation could be detected in the differentiated cells of the ISC lineage. Staining for Pros protein, a marker for differentiated ee cells, revealed an inverse correlation with the $10x$STAT92E-GFP reporter (Fig. 3.2C). Similarly, EC cells characterized by their large polyploid nuclei and positive staining for Pdm1 also did not detectably express $10x$STAT92E-GFP (Fig. 3.2D). Taken together these expression studies suggest that JAK/STAT signaling is transduced primarily in the ISCs and EB cells.

The observation that $10x$STAT92E-GFP is expressed at elevated levels in both the ISCs and EB cells raised the possibility that the EB signal detected is due to GFP perdurance. To investigate this possibility, we examined a more sensitive reporter of JAK/STAT activity, $10X$STAT92E-GFP$^{\text{destabilized}}$ (Bach et al., 2007). Previous analysis has suggested that Su(H)GBE-lacZ marks nascent EB cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007); EBs can often be identified morphologically by the presence of a flared cytoplasmic “foot” (e.g. Fig. 3.2A). To determine if $10x$STAT92E-GFP$^D$ is detectable in EB cells we crossed the reporter into a genetic background containing Su(H)GBE-lacZ. Double labeling revealed that
10xSTAT92E-GFP and Su(H)GBE-lacZ are both expressed at elevated levels in EB cells (Fig. 3.2E,F). Importantly, detectable levels of 10xSTAT92E-GFP reporter could also be observed in Su(H)GBE-lacZ cells adjacent to ISCs with no detectable expression (Fig. 3.2F, inset). These findings suggest that the JAK/STAT reporter is active in the EB cell.

In marked contrast to the stable reporter line, lower levels of expression and a great degree of heterogeneity existed in 10xSTAT92E-GFP reporter. We observed regional differences along the anterioposterior axis of the midgut as well as differences among ISC/EB pairs (Supplemental Fig. S3.1A,B; 50/50 midguts analyzed). In ISC/EB pairs that both expressed the reporter, ISC expression levels were often detectably lower than in the EB (Supplemental Fig. 3.1C,D). These patterns of activity differed among individual midguts analyzed and were observed with two independent destabilized transgenic strains indicating that variation was not due to transgene insertion site. Consistent with these findings, examination of upd1 and upd3 reporters revealed detectable levels of both JAK/STAT ligands distributed throughout the midgut, although the extent of expression varied widely even among age-matched samples (Supplemental Fig. S3.1E-H). Together, these observations suggest that at baseline homeostasis, JAK/STAT signaling is dynamically regulated in the midgut at both the regional and cellular level.

3.4.2 JAK/STAT signaling is required in the ISC lineage

Characterization of 10xSTAT92E-GFP transcriptional reporters suggested that the JAK/STAT pathway might have a function in the midgut. To directly test the functional requirement of JAK/STAT, the effects of partially reducing JAK/STAT signaling were examined using a temperature sensitive, hypomorphic allelic combination of stat92E, stat92E06346 (referred to subsequently as statTS). The distribution of cell nuclei in statTS adults was examined in DAPI stained samples following a 14-day shift to the non-permissive temperature. In contrast to unshifted controls, statTS adults exhibited an aberrant midgut organization characterized by the presence of distinct multicellular clusters most prominent in the posterior midgut (Fig. 3.3A,B; 6/8 midguts analyzed). Moreover, cells within the cluster typically displayed a small nuclear morphology unlike that of differentiated EC cells. Such clusters appeared to be the product of
individual ISCs, although this could not be verified due to the absence of a genetic lineage marker.

To examine the consequence of reducing JAK/STAT signaling from individual ISC lineages we conducted a mosaic analysis of the stat92E. Positively marked ISC lineages were generated in the adult midgut using the MARCM system (Lee and Luo, 1999) and identified on the basis of GFP expression 5 days after induction (Fig. 3.3C-E). In contrast to wild-type cell lineages, ISCs lacking stat92E produced lineages consisting of smaller cells with the clones themselves often appearing fragmented (compare Fig. 3.3C,D). Measurements of nuclear diameter indicated that daughter cells failed to attain the maximal nuclear size detected in wild-type lineages (Fig. 3.3E). ISC lineages lacking hop produced a similar phenotype (Fig. 3.3F). Together, this analysis suggests that JAK/STAT signaling is required for normal differentiation in the ISC lineage.

In our mosaic analysis, we occasionally observed that the overall organization of the midgut epithelium was disrupted. For example, regions of increased small cell number could be detected in heterozygous tissue (e.g. GFP− cells in Fig. 3.3D) raising the possibility that an increased number of small cells exist in the genetic background used to generate clones. To address this possibility we quantified the number of small cells in uninduced wild-type and stat92E85C9 midguts used in our mosaic analysis however, no significant difference was observed (Supplemental Fig. S3.2A). A second possible explanation is that stat92E85C9 clones can exert a non-autonomous effect on the surrounding tissue. To examine this possibility we quantified the number of both GFP− small cells and pHH3+ cells in induced wild-type and stat92E85C9 midguts and observed a significant increase in each measure (Supplemental Fig. S3.2B,C). Thus, the variation in the midgut epithelium we observe is not a result of genetic background but is consistent with a non-autonomous effect of stat92E clone induction.

3.4.3 JAK/STAT signaling is required for differentiation in the ISC lineage

Clone morphology suggested that ISC lineages unable to transduce the JAK/STAT signal fail to undergo normal multi-lineage differentiation. To more rigorously analyze cell fate in JAK/STAT mutant lineages, we examined a panel of molecular markers. In wild-type lineages esg is
expressed in ISCs and EBs, but not in either of the two differentiated cell types of the midgut, the ee or EC cells (Fig. 3.4A; (Micchelli and Perrimon, 2006)). Examination of esg-lacZ expression in stat92E^{85C9} lineages revealed that almost every cell present was esg^{+} (Fig. 3.4B; 18/18 midguts analyzed), suggesting that ee and EC cells had not differentiated. To test this directly, we examined the expression of prospero (pros) a marker of the ee cell population. Pros positive cells were rarely detectable within stat92E^{85C9} mutant lineages (Fig. 3.4C,D; 47/47 midguts examined; wild-type, n=8; stat92E^{85C9}, n=8). Differentiated EC cells are distinguished by their large, polyploid nuclei and are often Pdm1^{+}. Inspection of stat92E^{85C9} lineages revealed a reduction or absence of Pdm1 staining (Fig. 3.4E,F; 21/21 midguts examined; wild-type, n=8; stat92E^{85C9}, n=8). Taken together, these observations suggest that stat92E is required for differentiation of both ee and EC cells.

Analysis of marker gene expression suggested that multi-lineage differentiation does not occur in the absence of stat92E; to determine if ISCs were present in lineages lacking JAK/STAT signaling we examined the distribution of DI protein. As in the case of wild-type lineages, DI protein could be detected, suggesting that ISCs are present in the absence of JAK/STAT, however the level of expression often appeared to be reduced.

3.4.4 DI is sufficient to specify EC cell fate

Prior studies indicate that DI/N signaling is critical to correctly specify cell fate within the ISC lineage (Supplemental Fig. S3.4A; (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006)). Therefore, we next performed an epistasis analysis to determine the relationship between JAK/STAT and DI/N signaling in the ISC lineage. We first examined the consequences of expressing a wild-type DI transgene (DI^{wt}) in ISC lineages and followed cell fate in the resultant lineage. Here, we employ the MARCM system to trace the lineages of individual ISCs expressing DI^{wt} for 5 days following induction. To assay cell fate, Pdm1 and nuclear size were used to identify EC cells, while Pros was used to identify ee cells. Interestingly, even in wild-type controls ISC lineages could frequently be observed that consisted entirely of EC cells and lacked Pros^{+} cells (Supplemental Fig. S3.4B). This trend is most pronounced in the anterior midgut; in the
posterior, the majority of wild-type clones contained one or more Pros\(^+\) cells in addition to ECs (Supplemental Fig. S3.4D). These observations suggest that there are significant regional differences affecting cell fate in the ISC lineage.

To quantify the effect of Dl\(^{\text{wt}}\) expression in ISC lineages we scored the number of Pros\(^+\) cells in the lineage. In contrast to wild-type, ISC lineages expressing Dl\(^{\text{wt}}\) resulted in a significant decrease in the number of clones containing Pros\(^+\) cells within the lineage (Supplemental Figs. S3.4C,D; wild-type, n=12; Dl\(^{\text{wt}}\), n=12). In addition, staining with the EC marker Pdm1 suggested that ISCs expressing Dl\(^{\text{wt}}\) produce lineages consisting almost entirely of ECs (Fig. 3.5A,B; Supplemental Fig. 3.4E). These effects on cell fate were also observed in Dl\(^{\text{wt}}\) expressing clones analyzed 10 days after induction, suggesting that the reduction in Pros\(^+\) cells was not simply due to a delay in ee cell differentiation (Supplemental Fig. S3.4F). We note that at later time points, ISCs expressing Dl\(^{\text{wt}}\) also produced large clones that appeared to contain many undifferentiated cells, as they were negative for both Pros and Pdm1 (Supplemental Fig. S3.4F). Such cells appeared to most closely resemble ectopic ISCs or EBs, raising the possibility that Dl has an additional role in regulating ISC proliferation. Taken together, these findings demonstrate that Dl\(^{\text{wt}}\) is sufficient to specify EC cell fate at the expense of ee cells and supports a model in which ISCs signal non-autonomously to nascent EB cells to specify the EC cell fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007).

### 3.4.5 JAK/STAT is epistatic to Dl/N signaling in multilineage differentiation

To determine if stat92E is required for Dl-mediated EC cell fate specification we conducted a mosaic analysis of ISC lineages expressing Dl\(^{\text{wt}}\) but lacking stat92E\(^{85C9}\) function. In contrast to Dl\(^{\text{wt}}\) expressing lineages alone, Dl\(^{\text{wt}}\), stat92E\(^{85C9}\) lineages appeared to lack differentiated EC cells based on the reduced number of Pdm1\(^+\) cells and were virtually indistinguishable from stat92E\(^{85C9}\)-mutants (compare Fig. 3.5B,C and Fig. 3.4E). Similar results were also obtained by analyzing a second allele, stat92E\(^{65346}\) (Supplemental Fig. S3.5C).

A quantitative analysis was performed to determine the effect of stat92E\(^{85C9}\) loss on Dl\(^{\text{wt}}\) expressing lineages by comparing the number of Pdm1\(^+\) cells present in Dl\(^{\text{wt}}\) lineages with the
number of Pdm1\(^+\) cells present in \(Dl^{wt}\), \(stat92E^{85C9}\) lineages. This analysis revealed a significant decrease in the number of Pdm1\(^+\) GFP\(^+\) cells in \(Dl^{wt}\), \(stat92E^{85C9}\) lineages compared to \(Dl^{wt}\) alone (Fig. 3.5D; \(Dl^{wt}, n=12\); \(Dl^{wt}, stat92E^{85C9}, n=12\)). Consistent with this finding, we observe that differentiated ECs induced by cell autonomous N activation were also blocked by the loss of \(stat92E\) (Fig. 3.5E,F; 29/29 midguts analyzed). Thus, although \(stat92E\) is required in the ISC for normal levels of DI expression (Supplemental Fig. S3.3), our epistasis analysis shows that \(stat92E\) is also necessary for EC differentiation in the presence of DI/N signaling.

We next sought to extend the epistasis analysis to determine the requirement for JAK/STAT in differentiation of ee cells. Previous analysis has demonstrated that ISC lineages lacking \(N\) generate two phenotypic classes, clones that appear to be comprised of ectopic ISCs and clones that appear to be comprised of ectopic ee cells (Fig. 3.6A; (Ohlstein and Spradling, 2006)). To test whether JAK/STAT signaling is required for ee differentiation, we asked if the production of Pros\(^+\) ee cells present in N mutant lineages depends on the JAK/STAT signaling pathway. To test this requirement, genetic mosaics were created in which both \(N\) and \(stat92E\) functions were reduced. In contrast to \(N^{RNAi}\), mosaic analysis of \(N^{RNAi}, stat92E^{85C9}\) clones revealed a reduction in ee cells (Fig. 3.6B,C; 30/30 midguts examined). Similar results were obtained by analyzing a second hypomorphic allele, \(stat92E^{06346}\) (Supplemental Fig. S3.5D).

A quantitative analysis was performed to determine the effect of \(stat92E^{85C9}\) loss on \(N^{RNAi}\) expressing lineages. This analysis revealed a significant decrease in the number of Pros\(^+\) GFP\(^+\) cells in \(N^{RNAi}, stat92E^{85C9}\) lineages compared to \(N^{RNAi}\) alone (Fig. 3.6D; \(N^{RNAi}, n=8\); \(N^{RNAi, stat92E^{85C9}}, n=8\)). Thus, the transcription factor encoded by \(stat92E\) is required downstream or in parallel to \(N\), suggesting that \(stat92E\) is also required in EB cells for ee differentiation.

### 3.4.6 Little evidence that JAK/STAT signaling is required for ISC self-renewal

Analysis of the 10xSTAT92E-GFP transcriptional reporters revealed detectable expression in ISCs (Fig. 3.2A,B), suggesting that JAK/STAT signaling might also have a function in the ISC. Studies of \textit{Drosophila} germline stem cells first employed a genetic lineage-tracing assay to measure stem cell self-renewal (Margolis and Spradling, 1995). A pulse/chase design was used
to determine the number of marked stem cell lineages retained in the midgut at defined times following induction. To determine if JAK/STAT signaling is required for ISC self-renewal we generated labeled ISCs lacking components of the JAK/STAT signaling pathway and analyzed the lineages at 5, 10, and 20 days after induction. In controls, wild-type lineages could be detected in the midgut throughout the chase interval. Similarly, in hop\textsuperscript{C111} and stat92E\textsuperscript{85C9} mutants, marked lineages were detectable in the midgut 20 days after induction (Fig. 3.7A-F).

A quantitative analysis was performed to examine the effect of JAK/STAT loss on ISC self-renewal. As noted above, loss of JAK/STAT signaling often results in a distinct clonal morphology and the inability to unambiguously identify individual clones (e.g. Fig. 3.3D,F). As such, clone number is not a suitable measure of ISC number in the JAK/STAT mutants we examined. Therefore, to approximate the number of ISCs we scored the number of GFP\textsuperscript{+} daughter cells in defined regions of each sample. Quantification of the pulse/chase studies showed that reductions in hop\textsuperscript{C111}, stat92E\textsuperscript{85C9}, or stat92E\textsuperscript{06346} were associated with the retention of 67%, 22% or 31% of labeled cells, respectively, at 20 days after induction (n=12; Fig. 3.7G, Supplemental Fig. 3.5E). In addition, we observed that hop\textsuperscript{C111} and stat92E\textsuperscript{85C9} lineages could be identified at late time points that stained positively for both pHH3 and BrdU, suggesting that JAK/STAT is not absolutely required for ISC proliferation (Fig. 3.7B,D,F and Supplemental Fig. S3.6D,E). We also note that hyperplastic stat92E clones could be detected at late time points suggesting that JAK/STAT may be a necessary modulator of ISC proliferation (Supplemental Fig. S3.6A-C). Taken together these data suggest that ISC self-renewal is not grossly disrupted in the absence of JAK/STAT signaling.

3.4.7 The JAK/STAT pathway promotes ISC proliferation

To determine if the levels of JAK/STAT signaling affect the ISC lineage, we examined the consequences of pathway activation in the midgut. Using conditional Gal4 induction in esg\textsuperscript{+} cells (esg\textsuperscript{TS}), we first examined the effect of expressing the JAK/STAT ligand upd on the number of pHH3\textsuperscript{+} cells 2 days after induction. Quantification revealed a significant increase in the number of pHH3\textsuperscript{+} cells following upd expression, compared to both wild-type controls and the expression of
a dominant negative form of $N$ ($n=18$; Fig. 3.8A,B). The effect of $upd$ expression on proliferation was enhanced by simultaneous expression of a dominant negative form of $N$, as would be predicted if Upd acts directly on ISCs to stimulate proliferation ($n=18$; Fig. 3.8B). Similar increases in pHH3$^+$ were obtained when $upd$ was expressed using the Flip-out (F/O) technique (27/27 midguts examined; Fig. 3.8C). However, in addition to the increase in pHH3$^+$ cells, we also observed many cells that appeared to be in the process of delaminating from the midgut epithelium (Fig. 3.8D). Finally, using the MARCM system we generated ISC lineages expressing constitutively active hop$^{Tum}$ (Harrison et al., 1995); such clones were rapidly lost from the midgut (7/7 midguts examined; Fig. 3.8E,F). Thus, activation of JAK/STAT signaling promotes ISC proliferation in the midgut, although this response may depend on the precise level and cell types in which the pathway is activated.

3.5 Discussion

3.5.1 Stem cells, JAK/STAT and self-renewal

A series of studies have addressed the requirement of JAK/STAT signaling for stem cell self-renewal in distinct Drosophila stem cell populations. For instance, male germline stem cells (GSCs) are arrayed in rosette like pattern around a focus of “hub” cells situated at the apical tip of the testis. Hub cells are a source of Upd ligand; GSCs lacking the ability to transduce the JAK/STAT signal are detectably reduced by 5 days and completely lost after 9 days. In contrast, misexpression of the Upd ligand increases the number of GSC like cells in the testis and mitigates GSC loss at the hub (Boyle et al., 2007; Kiger et al., 2001; Tulina and Matunis, 2001). Thus, JAK/STAT functions as a signal for GSC self-renewal. The rapid failure of stem cell self-renewal and concomitant depletion of the stem cell compartment following loss of JAK/STAT has subsequently been reported for female GSCs, cyst progenitor cells (CPCs), follicle stem cells (FSCs), and renal stem cells (RNSCs) (Decotto and Spradling, 2005; Kiger et al., 2001; Singh et al., 2007; Tulina and Matunis, 2001). Thus, the theme that has emerged is that JAK/STAT signaling is generally required for Drosophila stem cell self-renewal.
Our studies have revealed an exception to this generalization. Here, we examined the consequences of JAK/STAT loss in the midgut up to 20 days following induction (~40% of the fly lifetime), at which point marked ISC lineages were still detected. Consistently, BrdU and pH3 staining suggested that ISCs were still capable of dividing up to 5 weeks in the absence of JAK/STAT signaling. Finally, very large stat92E clones could occasionally be detected in the midgut. Taken together, these findings are not consistent with a requirement for JAK/STAT in ISC self-renewal. We note that our studies differed from the aforementioned in one respect, experimental limitations prevented us from unambiguously identifying individual ISC lineages. Therefore the number of stem cell progeny at each time point was scored, to infer the presence of stem cells. While these experiments do not rule out a requirement for JAK/STAT in long term ISC maintenance, they provide little evidence that JAK/STAT signaling is required for ISC self-renewal.

3.5.2 JAK/STAT coordinates competence to undergo multi-lineage differentiation and ISC proliferation

Two lines of evidence support a role for JAK/STAT signaling in multi-lineage differentiation of ISC daughters. First, a synthetic JAK/STAT transcriptional reporter is expressed throughout the midgut; coexpression studies demonstrate that JAK/STAT signaling occurs in Su(H)GBE-lacZ* cells, a marker for undifferentiated EB daughter cells. In addition, we observe JAK/STAT reporter expression in daughters adjacent to ISCs, which have no detectable JAK/STAT reporter expression themselves, suggesting this is not due to GFP perdurance. Second, reductions or loss of the JAK/STAT signaling pathway in marked ISC lineages leads to morphologically aberrant clones with reduced nuclear diameter, and the production of cells that lack molecular markers of either differentiated ee or EC cell types. Given this requirement of JAK/STAT signaling for both ee and EC cell differentiation, we might predict that activation of the JAK/STAT pathway would not be sufficient to affect cell fate. Consistently, analysis of ISC lineages in which JAK/STAT signaling is constitutively active was found to have no discernible effect on fate. Thus, JAK/STAT signaling is necessary but not sufficient for cell fate specification of ISC daughters. As such, we conclude
that JAK/STAT signaling is necessary to establish competence for EBs to undergo multi-lineage differentiation.

In addition to the requirement for JAK/STAT in ISC daughters, our studies demonstrate that JAK/STAT signaling is a potent regulator of ISC proliferation. First, co-labeling studies with transcriptional reporters indicated that JAK/STAT signaling is activated in cells that express ISC marker genes. Second, ectopic expression of Upd ligand resulted in a rapid increase in proliferation throughout the midgut. Third, the effect of Upd on proliferation was enhanced under conditions in which N signaling was simultaneously reduced, suggesting that upd is sufficient to directly stimulate ISC proliferation. Our analysis suggests that JAK/STAT signaling may also be necessary to regulate the ISC proliferation, perhaps via both canonical and non-canonical pathways (e.g. Fig. 3.7A,C,E; Supplemental Fig. S3.6A-C). Thus, JAK/STAT signaling has dual, yet separable roles in the ISCs and their immediate daughters. On the basis of these findings, we suggest a model in which JAK/STAT signaling coordinates the processes of stem cell proliferation with the competence of daughter cells to undergo multi-lineage differentiation, ensuring a rapid and robust cellular output in the lineage (Supplemental Fig. S3.7). JAK/STAT mediated gating of proliferation and differentiation may be particularly important in the midgut where ISCs and their undifferentiated daughters are typically found in a poised, “doublet” configuration. These conclusions are in general agreement with recent reports (Jiang et al., 2009).

3.5.3 N and JAK/STAT signaling pathways interact to regulate the ISC lineage

We have conducted genetic epistasis experiments to determine the nature of the interaction between Dl/N signaling and the JAK/STAT signaling pathway in the ISC lineage. Our experiments show that while JAK/STAT is required for wild-type levels of Dl expression in the ISC, the requirement for JAK/STAT in EB differentiation is independent of Dl/N signaling. This observation suggests that JAK/STAT functions downstream or in parallel with Dl to establish competence of EBs to undergo differentiation. Recent studies, however, suggest a different relationship. Studies of midgut regeneration show that Dl ligand is strongly induced in ISCs following Upd ligand expression, directed epithelial cell ablation or bacterial infection (Buchon et al., 2009; Jiang et al.,
These studies suggest that under conditions of adaptive homeostasis, Upd ligands are produced in gut epithelial cells and activate Dl in ISCs to promote new cell production. Consistent with this model, we observe that expression of Dl leads to an increase in the size of ISC lineages, suggesting a potential role for Dl in promoting ISC proliferation. Thus, there is evidence that JAK/STAT signaling functions both upstream and downstream of Dl/N in the ISC lineage.

### 3.5.4 Regulation of Upd ligands at baseline and adaptive homeostasis

At baseline homeostasis, the most distinctive feature of Upd ligand expression that we observe is heterogeneity among age-matched samples. Prior studies show that Upd ligands are strongly induced following bacterial infection, directed cell ablation, or stress signaling (Buchon et al., 2009; Cronin et al., 2009; Jiang et al., 2009). It remains an open question as to whether Upd ligand expression and associated JAK/STAT activity that we report here is a direct manifestation of midgut stress, albeit to a lesser extent in unchallenged animals, or if there is a distinct regulation of Upd ligands under baseline homeostasis. Previous studies indicate that apoptosis and stress signaling are both detectable in the adult midgut at baseline homeostasis (Biteau et al., 2008; Ohlstein and Spradling, 2006). Studies of antimicrobial peptide reporters, which serve as markers of infection, indicate a limited signal in the adult midgut (Tzou et al., 2000). Nonetheless, a degree of infection undetectable by such reporters under conditions of laboratory culture is possible. If activation of Upd ligands is exclusively regulated in response to exogenous factors this would provide an efficient means of coupling the ISC proliferative response to the magnitude, duration and location of the stimulus. However, in this view one might predict that the additive effects of locally generated stress in the midgut under normal circumstances would, over time, lead to a distortion of tissue architecture. On the other hand, if there is also a distinct regulation of Upd ligands under baseline homeostasis, this might contribute to a stereotyped mode of growth control and tissue homeostasis, in conjunction with other signals known to regulate ISC proliferation (Lee et al., 2009; Lin et al., 2008). Such models are not mutually exclusive. Future experiments, which clarify the precise mechanisms of Upd ligand regulation will
provide important insights into the control of the ISC lineage under a broad range of biological conditions.
Figure 3.1: The adult *Drosophila* midgut is maintained by a population of multipotent intestinal stem cells (ISCs). (A) Diagram of the adult midgut in cross section. ISCs (green) occupy an epithelial niche adjacent to the basement membrane and the visceral muscle (red). ISCs give rise to two types of differentiated daughters, enteroendocrine (ee) cells (blue) and enterocytes (ECs; orange). (B) A model of ISC division; the ISC undergoes self-renewal and generates a daughter cell or enteroblast (EB), which can become either an ee or an EC cell. Specification of the EC cell fate requires $N$ signaling. (C, D) All micrographs display superficial views of the midgut, except where indicated. (C) Low magnification view in cross section; *esg > GFP* (green) and phalloidin (red). (D) High magnification view of midgut in cross section showing a newly divided ISC; *vkg-GFP* (green), *esg-lacZ* (red) and phalloidin (blue). CM (circumferential muscle), LM (longitudinal muscle), BM (basement membrane).
Figure 3.2: JAK/STAT signaling is dynamically regulated in midgut ISCs and EBs. (A-F) Characterizing transcriptional reporters of JAK/STAT activity in the midgut (green); nuclei are counterstained with DAPI (blue), except in D. (A-D) 10xSTAT92E-GFP reporter with stable GFP. (A) ISC and EB cells (esg-lacZ; red) express the 10xSTAT92E-GFP reporter. (B) ISCs (Di-lacZ; red) express the 10xSTAT92E-GFP reporter. (C) Differentiated ee cells are marked with anti-Pros (red). (D) Differentiated EC cells are marked with anti-Pdm1 (red). (E, F) 10xSTAT92E-GFP

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reports expression of a destabilized GFP. EB cells \( Su(H)GBE\text{-}lacZ \), red. (E) ISC and EB cells express the destabilized reporter. (F) EBs expressing the destabilized reporter adjacent to ISCs with no detectable expression. High magnification, insert. Scale bar: 50 µm.
Figure 3.3: JAK/STAT signaling is required in the ISC lineage. (A, B) stat92E temperature shift analysis, nuclei (DAPI, grayscale). (A) stat92E<sup>Ts</sup>, unshifted controls. (B) stat92E<sup>Ts</sup>, shifted to non-permissive temperature for 14 days. Global reduction of stat92E leads to the formation of clusters of small cells in the midgut. (C-F) The MARCM system was used to positively identify ISC lineages with GFP 5 days after induction (anti-GFP, green; DAPI, blue). (C) Wild-type ISC lineages. (D) ISC lineages lacking stat92E. Loss of stat92E leads to generation of cells with
abnormal clonal morphology and reduced nuclear size. (E) Quantification of nuclear size. Histogram displays the distribution of nuclear size in wild-type (white) and stat92E lineages (black). stat92E mutant lineages fail to generate cells of the larger nuclear size classes. The average nuclear size of wild-type EB cells (Su(H)GBE-lacZ) is indicated by a triangle; the average nuclear size of wild-type EC cells (anti-Pdm1) is indicated by a circle. (F) ISC lineages lacking hop resemble those lacking stat92E. We note that many of the cells detected in stat92E and hop mutant lineages exhibited the cytoplasmic foot characteristic of EB cells. Scale bar: 50 µm.
Figure 3.4: **stat92E is required for multi-lineage differentiation.** (A–F) The MARCM system was used to positively identify ISC lineages with GFP 5 days following induction. (anti-GFP, green; DAPI, blue). (A) Wild-type ISC lineages contain *esg-lacZ* positive and negative cells (anti-βgal, red). (B) ISC lineages lacking *stat92E* have an increased number of *esg-lacZ* \(^+\) cells and a decreased number of *esg-lacZ* \(^-\) cells (anti-βgal, red). (C, D) *stat92E\(^{85C9}\)* mutant lineages have a significantly reduced number of Pros* \(^+\) cells in both the anterior (A) and posterior (P) midgut. (C)
Representative micrograph. (D) Quantification (n=8; anti-Pros, red). (E, F) stat92E85C9 mutant lineages have a significantly reduced number of Pdm1⁺ cells in both the anterior (A) and posterior (P) midgut. (E) Representative micrograph. (F) Quantification (n=8; anti-Pdm1, red). Error bars denote s.e.m. Scale bar: 50 µm.
Figure 3.5: *stat92E* is epistatic to Dl/N signaling for EC specification. (A–F) The MARCM system was used to positively identify ISC cell lineages with GFP 5 days following induction (anti-GFP, green; DAPI, blue). (A) Wild-type ISC lineages generate EC cells distinguished by large cell morphology and are often Pdm1⁺ (anti-Pdm1, red). (B) ISC lineages expressing *Dl*<sup>wt</sup> generate lineages enriched for EC cells (anti-Pdm1, red). (C) ISC lineages expressing *Dl*<sup>wt</sup> and lacking *stat92E* display the *stat92E*<sup>85C9</sup> phenotype. (D) Quantification of the number of Pdm1⁺ cells in *Dl*<sup>wt</sup> and *Dl*<sup>wt</sup>; *stat92E*<sup>85C9</sup> lineages in both the anterior (A) and posterior (P) midgut. (E) ISC lineages
expressing $N^{\text{intra}}$ generate ECs. (F) ISC lineages expressing $N^{\text{intra}}$ and lacking $\text{stat92E}^{85C9}$ display the $\text{stat92E}^{85C9}$ phenotype. Error bars denote s.e.m. Scale bar: 50 µm.
Figure 3.6: *stat92E* is epistatic to DI/N signaling for ee specification. (A–D) The MARCM system was used to positively identify ISC lineages with GFP 5 days following induction (anti-GFP, green; anti-Pros, red; DAPI, blue). (A) ISC clones lacking *N* generate clusters of Pros⁺ and Pros⁻ cells. (B) ISC clones expressing *N*⁹⁸⁵⁹ generate clusters of Pros⁺ and Pros⁻ cells. Note the presence of some ECs at early time points due to residual N activity. (C) ISC lineages expressing *N*⁹⁸⁵⁹ and lacking *stat92E*⁸⁵⁹⁹ resulted in a loss of the Pros⁺ cells. The occasional ECs observed in the *N*⁹⁸⁵⁹ lineages were not observed in *N*⁹⁸⁵⁹, *stat92E*⁸⁵⁹⁹ lineages. (D) Quantification. The number of Pros⁺ cells in *N*⁹⁸⁵⁹, *stat92E*⁸⁵⁹⁹ lineages is significantly reduced in both the anterior (A) and posterior (P) midgut (*n*=8). Error bars denote s.e.m. Scale bar: 50 µm.
Figure 3.7: JAK/STAT is not required for ISC self-renewal. (A–G) The MARCM system was used to positively identify ISC lineages with GFP between 5 and 20 days after induction (anti-GFP, green; anti-pHH3, red; DAPI, blue). (A, C, E) 5 days after induction, (B, D, F) 20 days after induction. (A, B) Wild-type ISC lineages retain dividing ISCs. (C, D) ISC lineages lacking hop\textsuperscript{C111}. 20 days after induction hop\textsuperscript{C111} clones have detectable pHH3\textsuperscript{+} expression. (B) ISC lineages lacking stat92E. 20 days after induction stat92E\textsuperscript{85C9} clones have detectable pHH3\textsuperscript{+} expression. (G) Wild-type, hop\textsuperscript{C111} and stat92E\textsuperscript{85C9} mutant lineages are detectable in the midgut 20 days following induction. Error bars denote s.e.m. Scale bar: 50 µm.
Figure 3.8: JAK/STAT activation promotes ISC proliferation. (A–D) Expression of upd leads to an increase in ISC proliferation. (A, B) Conditional expression of upd using the esg-Gal4, UAS-GFP, tub-Gal80<sup>Ts</sup> driver (anti-GFP, green; anti-pHH3, grayscale; DAPI, blue). (A) Expression of upd is sufficient to increase the number of pHH3+ cells 48 h after induction. (B) Quantification. Note that simultaneous reduction of N activity using a dominant negative construct leads to a further increase in pHH3+ cell number. (C, D) Expression of upd using the Flip-out (F/O) cassette
(anti-GFP, green; anti-pHH3, grayscale; DAPI, blue). (C) Cells marked in Flip-out experiments were associated with increased numbers of pHH3⁺ cells. (D) Many marked cells rapidly delaminate from the midgut, as seen here in cross section. (E, F) ISC lineages expressing the activated form of hop (hop^{Tum}) are rapidly lost from the midgut (anti-GFP, green; anti-Pros, red; DAPI, blue). (E) hop^{Tum} lineages 5 days after induction. (F) hop^{Tum} lineages 10 days after induction. Error bars denote s.e.m. Scale bar: 50 μm.
Supplemental Figure S3.1: JAK/STAT signaling is dynamically regulated in midgut. (A–D) Characterizing transcriptional reporters of JAK/STAT activity in the midgut. (A, B) A comparison of stable and destabilized JAK/STAT reporters (anti-GFP, grayscale). (A) The stabilized 10xSTAT92E-GFP reporter was consistently found in pairs of small cells and at high levels throughout the length of the midgut. (B) The destabilized reporter, 10xSTAT92E-GFP<sup>D</sup>, demonstrated lower and more variable expression levels than the stabilized reporter. (C, D) ISCs have lower levels of destabilized reporter expression than EBs (Su(H)GBE-lacZ<sup>+</sup> cells). Arrows indicate ISCs with low GFP levels. (C) Merged (anti-GFP, green; anti-βgal, red; DAPI, blue). (D) Single channel showing 10xSTAT92E-GFP<sup>D</sup> (anti-GFP, grayscale). (E–H) Distribution of JAK/STAT ligands in the midgut (anti-GFP, green; DAPI, blue). (E, F) upd1 > GFP. The extent and distribution of reporter gene expression varied in the midgut. (G, H) upd3 > GFP. The extent and distribution of reporter gene expression varied in the midgut. Scale bar: 50 µm.
Supplemental Figure S3.2: Non-autonomous effects of stat92E^{85C9} clones. The MARCM system was used to positively identify wild-type and stat92E^{85C9} mutant lineages. (A) Uninduced. The number of GFP⁻ small cells was scored; no difference between the two genetic backgrounds was detected in the absence of clone induction. (B) Induced. The number of GFP⁻ small cells was scored; an increase was detected in stat92E^{85C9} mosaic animals. (C) Induced. The number of GFP⁺, pHH3⁺ cells was scored; an increase was detected in stat92E^{85C9} mosaic animals. Error bars denote s.e.m.
Supplemental Figure S3.3: ISCs are present in the absence of JAK/STAT signaling. (A, C, E) The MARCM system was used to positively identify ISC lineages with GFP (anti-GFP, green; anti-DI, red; DAPI, blue). (A, B) Wild-type ISC lineages contain DI⁺ cells. Arrow indicates cell with high DI staining. (A) Merged. (B) Anti-DI, grayscale. (C, D) Decreased levels of DI can be detected in stat92E⁸⁵C⁹ mutants. Arrow indicates cell with reduced DI staining. (C) Merged. (D)
Anti-DI, grayscale. (E, F) Decreased levels of DI can be detected in hop$^{C111}$ mutants. Arrow indicates cell with reduced DI staining. (E) Merged. (F) Anti-DI, grayscale. Scale bar: 50 µm.
Supplemental Figure S3.4: Delta expression is sufficient to promote the EC cell fate. (A) DI expressing ISCs can be detected adjacent to EBs transducing the N signal (Su(H)GBE-lacZ+ cells; anti-βgal, green; anti-DI, red; DAPI, blue). (B-F) The MARCM system was used to positively identify ISC lineages with GFP 5 days after induction, except F (anti-GFP-green; DAPI, blue). (B) Wild-type lineages often contain Pros+ cells. (C, D) Expression of DI in ISC lineages resulted in a significant decrease of the Pros+ population (anti-Pros, red). (C) Representative image. (D) Quantification. (E) Expression of DI in ISC lineages, produces cells with large nuclear size that
are Pdm1\(^+\) (anti-Pdm1, red). (F) At later time points (here 10 days after clone induction) expression of \(Dl\) in ISC lineages was often associated with an increased number of cells in comparison to wild-type lineages (anti-Pros, red). Error bars denote s.e.m. Scale bar: 50 \(\mu\)m.
Supplemental Figure S3.5: stat92E\textsuperscript{06346} and stat92E\textsuperscript{85C9} have similar phenotypes in the ISC lineage. (A-D) The MARCM system was used to positively identify ISC lineages with GFP 5 days following induction (GFP, green; DAPI, blue). (A) Wild-type ISC lineages. (B) stat92E\textsuperscript{06346} mutant lineages. (C) D\textsuperscript{tw}, stat92E\textsuperscript{06346} lineages (anti-Pdm1, red). (D) N\textsuperscript{RNAi}, stat92E\textsuperscript{06346} lineages (anti-Pros, red). (E) GFP\textsuperscript{+} cells from stat92E\textsuperscript{06346} mutant lineages are still detectable 20 days following
clone induction, *stat92E*°6346° represented by dotted bars. Error bars denote s.e.m. Scale bar: 50 µm.
Supplemental Figure S3.6: JAK/STAT is not absolutely required for self-renewal. (A-E) The MARCM system was used to positively identify ISC lineages with GFP (anti-GFP, green; DAPI, blue). (A-C) 20-day stat92E clones occasionally display lineages that appear hyperplastic. Images showing superficial and cross sections of the midgut. (A) Superficial (B) Cross-section. Note that clone profile appears smaller in superficial sections (anti-GFP, green; DAPI, blue). (C) High magnification. (D, E) Cells in hop^{C111} mutant lineages could be observed to incorporate BrdU during the 5th week after clone induction (anti-GFP, green; anti-BrdU, red; DAPI, blue). (D) Merged. (E) Arrows indicate BrdU positive cells. Asterisk indicates an unlabeled cell. Scale bar: 50 µm.
Supplemental Figure S3.7: A model for the role of JAK/STAT in the *Drosophila* intestinal stem cell lineage. JAK/STAT functions to coordinate stem cell proliferation and multi-lineage differentiation.
Chapter 4

Development & Characterization of a Chemically Defined Food for *Drosophila*

The research work in this Chapter is accepted by PLoS One. 2013. Jun Wen-Chih Lee is the first author in this publication.
4.1 Summary

Diet can affect a spectrum of biological processes ranging from behavior to cellular metabolism. Yet, the precise role of an individual dietary constituent can be a difficult variable to isolate experimentally. A chemically defined food (CDF) permits the systematic evaluation of individual macro- and micronutrients. In addition, CDF facilitates the direct comparison of data obtained independently from different laboratories. Here, we report the development and characterization of a CDF for Drosophila. We show that CDF can support the long-term culture of laboratory strains and demonstrate that this formulation has utility in isolating macronutrient from caloric density requirements in studies of development, longevity and reproduction.

4.2 Introduction

Organisms must acquire nutrients from food to meet the energetic and metabolic requirements necessary for life. Deficiency or overabundance of dietary nutrients is a key physiological variable influencing developmental, homeostatic and disease processes (Léopold and Perrimon, 2007; Mattison et al., 2012; Piper and Bartke, 2008; Stafford, 2010; Warden and Fisler, 2008; Willett, 2000). Understanding how nutrient-dependent physiological status can influence cellular processes has been the subject of intensive investigation. For example, in Drosophila, dietary manipulation has been shown to broadly affect global transcriptional programs, as well as specific cellular processes such as the expansion of stem and progenitor cell lineages, maintenance of stem cell niches, development, regeneration, reproduction and longevity (Britton and Edgar, 1998; Chell and Brand, 2010; Cheng et al., 2011; Good and Tatar, 2001; Grandison et al., 2009; Hsu et al., 2008; Li et al., 2010; Mair et al., 2010; Marshall et al., 2012; O'Brien et al., 2011; Skorupa et al., 2008; Tennessen and Thummel, 2011).

While these recent studies in Drosophila underscore the importance of diet-induced changes on cellular function, they have all employed standard complex (undefined) media as a means to manipulate dietary nutrients. Complex media is composed of ingredients of biological origin (e.g. yeast, cornmeal, molasses). Such ingredients are essentially nutrient composites that have different profiles depending on where and when they are sourced. Thus, an important limitation of the complex diet is that its composition is variable and difficult to precisely manipulate (Report of the American Institute of Nutrition ad hoc
Committee on standards for nutritional studies., 1977). While diluting or excluding components of a complex media permits gross nutrient manipulation, it also introduces the confounding variable of altering caloric density (content).

A powerful tool to decipher the effects of diet is the use of chemically defined food (CDF) media, which consists entirely of purified compounds (Winitz et al., 1970a; Winitz et al., 1970b; Wu et al., 2011). Notably, CDFs have only been fully developed in a limited number of experimental model organisms (Lu and Goetsch, 1993; Szewczyk et al., 2003; Szewczyk et al., 2006). Such diets permit the systematic evaluation of individual macro- or micronutrients and facilitate the interpretation and replication of experimental data obtained independently by different investigators (Piper et al., 2005; Tatar, 2011). In addition, use of CDF permits caloric density to be more tightly controlled.

Classic studies in Drosophila have determined the nutritional and metabolic requirements for the developing larvae. Essential components of the media include proteins, carbohydrates, lipids, nucleic acid, vitamins and salts (Hinton et al., 1951; Sang, 1956; Schultz et al., 1946). Together, these studies provided a basis for establishing the first chemically defined media for larval culture (Sang, 1978). In contrast, the dietary requirements for adults have been largely neglected since adults are capable of surviving on an energy source alone (e.g. sucrose) and because it has been assumed that nutritional requirements are similar during all stages of life. In this regard it is worth noting that certain nutritional requirements between larvae and adults can differ by two or three orders of magnitude (King, 1970; Sang and King, 1961).

More recently, CDF recipes have been reported for adult Drosophila (Grandison et al., 2009; Troen et al., 2007). However, previous formulations have been technically flawed (Troen et al., 2007; Troen et al., 2010) or characterized only under a narrow set of conditions (Grandison et al., 2009). Consequently, the overall use and utility of CDF in Drosophila has remained rather limited. Here, we describe an open-source CDF suitable for long-term culture (> 30 generations) of Drosophila laboratory strains. The effects of this CDF were analyzed at different stages of the Drosophila life cycle and compared to standard complex media. Finally, we used the CDF to directly test the requirement of individual dietary macronutrients on Drosophila development, reproduction and longevity.
4.3 Material and methods

4.3.1 Fly strains

*w^1118* flies were used for all feeding assays performed in this study. All experiments were performed at 25 degrees Celsius unless otherwise noted.

4.3.2 Development of chemically defined food (CDF)

CDF was formulated by optimizing the macro- and micronutrient components from several existing studies (Grandison et al., 2009; Lee et al., 2008; Troen et al., 2007; Troen et al., 2010). The concentration of amino acids, ribonucleotides, metals and vitamins was based on the work of Troen et al. (Troen et al., 2007; Troen et al., 2010). We modified the amino acid composition of Troen et al. (Troen et al., 2007) to include amino acids that were previously excluded (see Supplemental File S1). Both the composition and concentration of carbohydrates and lipids was based on the work of Grandison et al. (Grandison et al., 2009). The amino acid to carbohydrate energy ratio was set at 1:4, a proportion shown to optimize overall fitness by Lee et al. (Lee et al., 2008). CDF lipid levels were set at 2%. This value was chosen by surveying a series of standard recipes on the Bloomington Stock Center website (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm) with different lipid compositions and selecting the lipid level associated with best stock propagation (see Supplemental File S1). Thus, the ratio of food energy per mass in CDF for amino acids, carbohydrates and lipids is 1:4:0.1, respectively. To derive the caloric density of CDF, we first estimated the caloric density from a series of standard complex food recipes referenced on the Bloomington stock center website. These fell in a range between 275-991 K-cal/L (see Supplemental File S1). Troen et al. suggested that 300-400 K-cal/L was an optimal caloric density (Troen et al., 2007; Troen et al., 2010). We therefore focused on testing media with caloric density in the range of 100-500 K-cal/L (see Supplemental File S1).

4.3.3 Preparation of chemically defined food

To simplify production of CDF we first created a series of three powered master mixes; essential amino acid mix (TD.10473); non-essential amino acid mix (TD.110036); and basal mix (TD.10475). See Table 4.1A and Supplemental File S4.1 for additional details. These custom reagent mixes can be
obtained from Harlan Laboratories, Inc., IN, US using the TD reference numbers indicated. Two additional stock solutions were prepared (see Supplemental File S1): 1) 5X carbohydrate mix (autoclaved and stored at 4°C) and 2) A freshly prepared 100X slurry of lipid vortexed into water until no solids are visible. Commercial sources for all ingredients above are listed in Supplemental File S1.

To assemble CDF, the appropriate amount of agarose and sugar (5X carbohydrate mix) are combined into a final volume of water (see Supplemental File S1). This mixture is gently brought to a boil using a microwave to minimize evaporation. Once the solution cools to 65°C amino acid mixes (TD.10473 and TD.110036), basal mix (TD.10475), and lipid (100X stock) are added. The final solution is stirred without heating for an additional 5-10 minutes before aliquoting into vials. Plugged, boxed and wrapped vials are stable for 1 month at 4°C.

4.3.4 Feeding assays in adult flies

Newly eclosed adult flies were collected every 12 hours without CO₂ anesthesia. 3 days later, 10 pairs of male and female flies were sorted into a fresh vial and aged for 3 additional days on regular food (RF; Table 4.1B) before initiating the shift to experimental food. We began scoring values for survival, body weight, and egg-lay 12hrs after the initial transfer onto experimental food. The 10 pairs of flies were transferred into fresh food vials of the appropriate type every other day during the course of an experiment.

4.3.5 Measurements of adult body weight and egg-lay

Adult body weight was determined by performing two independent measurements of adult flies in a microcentrifuge tube using a precision balance then recording the average value. Average weight at each time point was normalized to initial average body weight. 12 hour egg-lay was determined every other day by counting the number of eggs present in a vial three times, recording the average value and then normalizing to the average number of living females present in the vial during consecutive time points. The accumulated egg-lay was calculated by summing average egg-lay values to a given time point. Flies used in both the survival and egg-laying studies were never anesthetized using CO₂.
4.3.6 Feeding assays in larvae

Newly eclosed adult flies were collected every 12 hours and grown on RF vials for 6 days before transferring into an egg-collecting bottle with grape juice plate. 24 hour egg-lays were collected on grape juice plates. Egg-lay plates were then inspected at two independent times over a 30 minute period to ensure all hatched larvae were completely removed. Individuals hatching within next 30 minute interval were then collected and 20-25 newly hatched 1st instar larvae were transferred into experimental food vials to measure their development and viability. The time required for larval development was scored every 12 hours by counting the number of pupae present; each pupa was marked on the vial wall and followed to determine the time to eclosion.

4.3.7 Trans-generational feeding assays

10 pairs of adult flies were collected and aged as described above. Flies were transferred into experimental vials at day 6 and into new vials 2 days later. For the second (and subsequent) generations, we collected 10-15 pairs of adult flies that eclosed within 3 days and transferred them into a fresh vial. Measurement of generation time was the same as described above.

4.3.8 Temperature shift experiments

10 pairs of adult flies were collected and aged as described above, then transferred into experimental vials and shifted to 18 or 29 degrees Celsius.

4.3.9 Quantifying effects of dietary macronutrients on egg-lay

10 pairs of w^{1118} flies were collected and aged for 6 days as described above and then transferred into new experimental food every day. Viability of adult flies and egg-lay was scored every 12 hours for 7 days. All CDF deficient media were compensated with remaining macronutrients, while maintaining proportional energetic contributions (see Supplemental File S1). For example, amino acid deprivation CDF is compensated to 400 K-cal/L by adding extra sugar solution and fat mixture at a 4: 0.1 ratio.
4.3.10 Statistics

Statistical analysis was performed using GraphPad Prism software Version 5.0d (GraphPad Software, Inc., CA, USA). Fisher’s exact tests were carried out using the online calculator from GraphPad Prism software homepage. Each statistical method used and corresponding p-values are listed in the Supplemental Tables. In all figure legends, *, **, *** indicate a p value < 0.0500, < 0.0100, < 0.0010, respectively.

4.4 Results and Discussion

In order to develop a chemically defined food (CDF) for Drosophila two general aspects of the media required optimization, dietary composition and caloric density (see Materials and Methods). Our goal was to synthesize a recipe that would functionally substitute for standard laboratory media. However, commonly used food recipes vary widely in their composition (e.g. http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm). As a basis for comparison, we arbitrarily chose one standard Drosophila complex media, which we refer to here as regular food (RF; Table 4.1B; see Supplemental File S4.1). We combined a series of simple feeding assays with an iterative approach to empirically determine the effect of successive CDF formulations on broad indicators of organismal fitness including longevity, body weight and egg-laying ability, developmental time and trans-generation viability (Fig. 4.1). Table 4.1A summarizes the complete list of individual components in the final CDF recipe characterized in this study.

4.4.1 CDF is sufficient to support the culture of adult Drosophila

We first compared the viability of wild type (white^{1118}) flies on both RF and CDF. On RF media, median survival values ranged from 35-41 days under our laboratory culture conditions (Supplemental Table S4.1). Similar values were measured on CDF where median survival ranged from 33-44 days. Gender specific analysis showed that CDF in the range of 100-500 K-cal/L did not significantly affect life span of adult female flies when compared to RF (p > 0.1053; Fig. 4.2A; Supplemental Table S4.1). In contrast, adult males were found to be more sensitive to changes in caloric density, showing shorter life span on CDF^{100K} and CDF^{500K} (p < 0.0001 and 0.0489 respectively) (Fig. 4.2B; Supplemental Table S4.1).
These results suggest that CDF formulated at a caloric density of between 200-400 K-cal/L is optimal to support the co-culture of adult male and female flies.

We next determined the extent to which CDF diets affect adult body weight. On standard RF media, both male and female body weight was observed to gradually increase over time (Supplemental Fig. S4.1A, Supplemental Table S4.2A). We note that young flies exhibited little variation in measured weight, however this variation increased markedly in females with advancing age (Supplemental Fig. S1A; Supplemental Table S4.2A). This variation in weight in aged female flies may be related in part to dietary effects on egg-laying (see below). A similar trend was observed when we monitored changes in body weight in adult flies fed a CDF (Supplemental Fig. S1B, C; Supplemental Table S4.2B, C). When we compared the effect of RF and CDF on the weight of young flies at defined time points, no significant differences were detected, with the exception of 400 K-cal/L and 500 K-cal/L diets on day 7 females (Fig. 4.3A, B; Supplemental Table S4.2B, C). Thus, CDF diets were not associated with significant changes in overall adult body weight compared to standard RF media.

Finally, we wished to determine if CDF diets affect female egg-laying ability. To quantify this effect, we first scored the number of eggs laid per female over the course of adult life (Fig. 4.4A; Supplemental Table S3). This analysis showed that females fed a RF diet lay a maximum of 12±1.8 eggs in 12 hours, whereas females fed a CDF have a maximum egg-lay as high as 24.6±1.9 (Fig. 4.4B; Supplemental Table S3). We next examined whether CDF could influence the female reproductive life span. To quantify this phenotype we calculated the time to reproductive quiescence defined as the number of days a female can lay more than a single egg per day. Females fed a RF diet remain reproductively active period for 21.0±2.4 days (Fig. 4.4C). Females fed a CDF diet showed an increase in reproductive longevity at all caloric densities tested with averages of 35.0±0.8, 42.5±2.1, 40.5±1.0, 36.0±2.1, 30.0±3.5 days on CDF$^{100K}$, CDF$^{200K}$, CDF$^{300K}$, CDF$^{400K}$, CDF$^{500K}$ respectively (Fig. 4.4C; Supplemental Table S3). Finally, to calculate total lifetime egg-lay we summed each independent 12-hour count over the duration of the experiment. Females fed a RF diet lay a lifetime average of 62.7 ±10.1 eggs (Fig. 4.4D). In contrast, females fed a CDF diet showed an increase in reproductive activity at all caloric densities tested with lifetime averages of 182.0±14.1, 220.0±36.9, 230.7±23.1; 169.4±9.8; 118.6±14.3 eggs on CDF$^{100K}$, CDF$^{200K}$, CDF$^{300K}$, CDF$^{400K}$, CDF$^{500K}$ respectively (Fig. 4.4D; Supplemental Table S3). Thus, CDF diets
were associated with an increase in the rate of egg-lay, reproductive longevity and total reproductive capacity of females.

In summary, the effects of a chemically defined food were compared to a standard *Drosophila* media. Gross measures of adult homeostasis were similar on RF and CDF, although in some cases male and female measurements diverged, suggesting distinct dietary requirements. Finally, this analysis directly demonstrates that caloric density affects measures of adult longevity, body weight and egg-lay.

4.4.2 *CDF is sufficient to support the culture of developing Drosophila*

To determine if CDF was sufficient to support early growth of *Drosophila*, we compared the developmental rate and survival of larvae reared on either RF or CDF. Embryos were collected from adults cultured on RF (Fig. 4.1A, C). Following hatching, larvae were either maintained on RF or transferred to a CDF. We first determined if CDF affects the time necessary to complete larval development by scoring the number of larvae that reached pupation and/or eclosion every 12 hours, following a timed egg-lay. These studies showed that without exception CDF diets were associated with a significant developmental delay (Fig. 4.5A). The average time to eclosion was 8.6 days on RF, while time to eclosion on CDF ranged from 13.2-15 days depending on the caloric density of the media (Supplemental Table S4A). Temporal analysis revealed that most, if not all, of this effect occurred during the larval stages of development (Supplemental Fig. S2A).

We then determined if the observed developmental delay was associated with lethality during development. To assess this, we measured the survival rate of embryos from hatching to eclosion on both RF and CDF media. The average survival rate on RF was 89.7±5.2 percent, whereas percent survival on CDF ranged from 70.3±7.3 ~ 92.3±1.5 depending on the caloric density of the media (Fig. 4.5B). Survival on CDF\(^{200-400K}\) trended lower but did not significantly differ from survival on RF (Supplemental Table S4B). Temporal analysis indicated that for those diets associated with significantly lower survival rates (i.e. CDF\(^{100K}\) and CDF\(^{500K}\)) death occurred largely during the pupal period (Supplemental Fig. S2B). Taken together these studies indicate that CDF can also support *Drosophila* development. While CDF is associated with a significant developmental delay, a caloric density 400 K-cal/L was associated with the shortest developmental delay and lowest lethality.
4.4.3 CDF is sufficient to support long-term culture of *Drosophila*

A stringent test of a CDF is the ability to support trans-generational propagation of individual cultures, as incomplete diets ultimately lead to a lack of viability on deficient media. To test the ability of CDF to support long-term culture we monitored both the number of successive generations and generation times of cultures grown on either RF or CDF (Fig. 4.6). Our studies show that CDF was sufficient to support trans-generational growth for 10 successive generations. This was most clearly the case for CDF formulated at higher caloric densities (i.e. 300-500 K-cal/L); CDF at 100 K-cal/L ultimately failed to support growth. Generation times for flies cultured on a particular diet were not observed to change from one generation to the next. As described above most of the developmental delay observed in a given generation is attributable to effects on larval development. Subsequent to these studies, cultures have been continuously propagated for up to 30 generations (Table 2), although generation times were not quantified after the 10\textsuperscript{th} generation. We also noted that CDF is capable of supporting culture growth at common experimental conditions of both 18 and 29 degrees Celsius (Table 2). Taken together, these experiments demonstrate that CDF is sufficient to support long-term culture of *Drosophila* strains under experimentally relevant conditions. Table 2 summarizes our observations concerning the culture of *Drosophila* on RF and CDF of different caloric densities.

4.4.4 CDF can be used to distinguish nutritional requirements from caloric requirements in *Drosophila*

We wished to determine the effect of individual macronutrients (amino acids, carbohydrates and fat) on developmental and homeostatic processes, independent of any potential effects of altered caloric density. Of the caloric densities tested in the experiments described above, CDF formulated at 400 K-cal/L consistently led to measures that were most similar to RF media over a rage of different assays. Thus we selected CDF at 400 K-cal/L for use in these “drop-out” studies. Holding caloric density constant, we examined the effect of deficits in each of the three macronutrients in our assays of adult survival, female egg-lay and larval development. Note that in the “drop-out” studies described here, caloric density that would have been lost from the diet by eliminating amino acids (for example) is compensated by
augmenting both carbohydrates and fat, while holding the overall proportions of remaining macronutrients constant (see Materials and Methods; Supplemental File S1).

We first compared the effects of serially eliminating each macronutrient from CDF on adult survival. In both males and females, dietary amino acids, carbohydrates, and fats were all found to be required for adult survival (Fig. 4.7A, B; Supplemental Table S5A). The median survival of adult females deprived of amino acids (CDF\textsuperscript{400K-AA}), carbohydrates (CDF\textsuperscript{400K-Carb}), or fat (CDF\textsuperscript{400K-Fat}) is 20.5, 4.0, and 26.0 days respectively. In adult males, median survival was, 19.0, 2.5, and 35.0 days on CDF\textsuperscript{400K-AA}, CDF\textsuperscript{400K-Carb}, and CDF\textsuperscript{400K-Fat}. These studies demonstrate that under experimental conditions where caloric density is held constant (i.e. 400 K-cal/L), dietary carbohydrates play the most important role in adult longevity, followed by amino acids and then fat. Although adult male flies are more sensitive to dietary carbohydrate deprivation than females, they are less sensitive to fat deprivation. Thus, nutritional requirements for the survival of adult flies differ between genders.

We next compared the effects of serially eliminating each macronutrient from CDF on female egg-laying ability. In these studies we scored the number of eggs laid per female every 12 hours for 7 consecutive days. We found that female egg-lay was differentially sensitive to macronutrient deprivation (Fig. 4.7C; Supplemental Table S5B). For example, total egg-lay of female flies fed either CDF or CDF lacking fat did not significantly differ (81.8±3.6, 80.7±5.1, respectively). However, females fed CDF lacking either amino acids or carbohydrates produced significantly fewer eggs (18.7±2.5, 28.3±3.0, respectively). Thus, under experimental conditions in which caloric density is held constant (i.e. 400 K-cal/L), both amino acids and carbohydrates are necessary for maintaining female egg-laying ability, while fat is dispensable.

Finally we tested the effects of serially eliminating macronutrients from CDF on developmental progression. In these studies we scored the time required to progress through larval and pupal stages. We found that post-embryonic development was differentially sensitive to the type of macronutrient deprivation (Fig. 4.7D). Not surprisingly, significant lethality and developmental delay was found to be associated with macronutrient deficits. For example, only a small fraction (less than 2%) of larvae grown on CDF lacking carbohydrates grew to adulthood, and were delayed in their development. Even more extreme requirements were observed with deficits in amino acids and fat. Larvae grown on either amino
acid or fat deprived CDF showed developmental arrest and died 7 days after egg-lay. Thus, under experimental conditions in which caloric density is held constant (i.e. 400 K-cal/L) carbohydrates, amino acids and fat are all necessary for larval development.

In summary, we have developed a chemically defined food (CDF) for the analysis of macro- and micronutrients in *Drosophila*. We have characterized the effects of this diet on both developmental and homeostatic processes and show that CDF can functionally substitute for standard media in a number of independent assays. While CDF is sufficient to support the long-term culture of *Drosophila* strains, it is associated with a significant delay in larval development. Replacement of dietary protein with amino acid mixes has previously been shown to prolong larval development and in some insects disrupt osmotic balance during development (House, 1966; Rudkin and Schultz, 1947). Therefore, additional modifications are necessary to optimize CDF for larval growth. Importantly, we demonstrate that CDF allows the effects of macronutrient and caloric density requirements to be distinguished experimentally. The CDF recipe described here should, in principle, permit the systematic experimental manipulation of individual nutrients within the diet (i.e. single essential amino acids). Similarly, this recipe can easily be used to test the effects of augmenting macro- or micronutrient composition or overall caloric density in the range above 500 K-cal/L. In *Drosophila*, methods to manipulate gene function at the single cell level can combine powerfully with the ability to manipulate specific dietary components leading to new insights into the way in which nutrient availability affects developmental, homeostatic and disease processes.
4.5 Figures

A. Feeding Assay

B. Assay effects on adults

C. Assay effects on larvae

Figure 4.1. Experimental design. (A) General scheme for the feeding assays performed. Flies were grown and aged on regular food (RF) before shifting to chemically defined food (CDF). (B) Assays performed on adult flies. Body weight, survival, and egg-lay were measured after adult flies were shifted to chemically defined food. (C) Assays performed on developing flies. Larval development and survival were measured after newly hatched 1st instar larvae were shifted onto chemically defined food.
Figure 4.2. The effect of CDF on adult survival. (A) Survival of adult female flies cultured on chemically defined food as a function of caloric density. Comparison of all survival curves by long-rank (Mantel-Cox) test shows no statistical difference (n = 40; p ≥ 0.1053 for all). (B) Survival of adult male flies cultured on chemically defined food as a function of caloric density. Paired comparison of survival curves between RF to other CDFs shows that the life span of males is only significantly reduced on 100 K-cal/L CDF (n = 40; p < 0.0001) and slightly reduced on 500 K-cal/L CDF (n = 40, p = 0.0489). See Supplemental Table S4.1 for additional details.
Figure 4.3. The effect of CDF on adult weight. (A) Average body weight of adult female flies cultured on chemically defined food as a function of caloric density. At day 7, only flies on 400 and 500 K-cal/L CDFs have less body weight than on RF (Mann Whitney test; n = 4, p = 0.0286 for both; see Supplemental Table S4.2A for details). At day 13 and 21, the body weights of flies cultured on CDFs are not statistically different from flies cultured on RF. (B) Average body weight of adult male flies on chemically defined food as a function of caloric density. The body weights of flies cultured on all CDFs are not significantly different from flies cultured on RF at day 7, 13, and 21 (Mann Whitney test; n = 4, p ≥ 0.1143 for all; see Supplemental Table S4.2C for additional details).
Figure 4.4. The effect of CDF on adult female egg-laying. Chemically defined food extends the egg-laying ability of adult female flies. (A) Average egg-lay in 12 hours of adult female flies on chemically defined food as a function of caloric density. Females fed on CDF show an increase in both maximal egg-lay and reproductive lifespan (n = 4). B) Maximal 12-hour egg-lay on chemically defined food. CDF$^{100K-400K}$ enhances the maximal egg-laying ability compared to RF (Mann Whitney test; n = 4, p = 0.2454 for CDF$^{500K}$ and 0.0286 for others). (C) Number of days a female is capable of producing more than one egg per day. CDF$^{100K-400K}$ extends the reproductive lifespan compared to RF (Mann Whitney test; n = 4, p = 0.1441 for CDF$^{500K}$ and ≤ 0.0294 for others). (D) Total lifetime egg-lay per female on chemically defined food. Females lay more eggs on CDF$^{100K-400K}$ than on RF (Mann Whitney test; n = 4, p = 0.0571 for CDF$^{500K}$ and 0.0286 for others). See Supplemental Table S3 for additional details.
Figure 4.5. The effect of CDF on larval development and survival. (A) Days required for first instar larvae to eclose on chemically defined food. Larvae grown on CDFs show statistically significant developmental delay (Mann Whitney test; $n \geq 65$, $p < 0.0001$ for all; see Supplemental Table S4A for details). (B) Eclosion rates for first instar larvae cultured on chemically defined food. Larvae on CDF$^{200K-400K}$ show no statistical difference in survival compared to RF (one-tailed Fisher's exact test; $n \geq 65$, $p \geq 0.0544$ for all; see Supplemental Table S4B for details), but lower survival is observed on CDF$^{100K}$ and CDF$^{500K}$ ($p = 0.0025$ and 0.0202 respectively).
Figure 4.6. CDF is sufficient to support long-term culture of *Drosophila* strains. Generation number as a function of caloric density. CDFs over 200 K-cal/L successfully support trans-generational propagation of *Drosophila* strains.
Figure 4.7. The effects of macronutrient deficiency on adult survival, female egg-lay and larval development. (A) Survival of adult female flies on chemically defined food (CDF) formulated at a caloric density of 400 K-cal/L and lacking either amino acids (AA), carbohydrates (Carb) or fats. Comparison of survival curves among all groups by long-rank (Mantel-Cox) test shows that life span is significantly reduced under each of the deprivation conditions (n = 40, p ≤ 0.0002 for all; see Supplemental Table S5A for details). (B) Survival of adult male flies on chemically defined food formulated at a caloric density of 400 K-cal/L and lacking either amino acids, carbohydrates or fats. Comparison of all survival curves in male flies shows the life span of males is significantly reduced on tested deprivation conditions (n = 40, p ≤ 0.0241 for all; see Supplemental Table S5A for details). (C) Total egg-lay per viable female on chemically defined food lacking either amino acids, carbohydrates or fats. Females lay fewer eggs on CDF lacking either amino acids or carbohydrates (Mann Whitney test; n = 4, p = 0.0286 for both), but not fat (p = 0.7715; see Supplemental Table S5B for additional details). (D) Larval development on chemically defined food lacking either amino acids, carbohydrates or fats. Larvae fed on CDF
lacking amino acids show growth arrest at 1st instar stage. Larvae fed on CDF lacking carbohydrates show high lethality in 2nd instar stage, but escapers can progress to adulthood (see text). Larvae fed on fat deprived CDF show growth arrest at 2nd instar stage. CDF: CDF$^{400K}$; - AA: amino acid deprived CDF$^{400K}$; - Carb: carbohydrate deprived CDF$^{400K}$; - Fat: fat deprived CDF$^{400K}$; Dashed line indicates lethality.
### Table 4.1A. Recipe for 400 K-cal/Liter chemically defined food (CDF$^{400K}$).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gram / Liter</th>
<th>Ingredients</th>
<th>gram / Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td><strong>Vitamins, Minerals, and Nucleic Acids</strong></td>
<td></td>
</tr>
<tr>
<td>L-arginine HCl</td>
<td>1.67</td>
<td>Vitamin B12 (0.1% in mannitol)</td>
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<tr>
<td>L-histidine HCl-H$_2$O</td>
<td>0.47</td>
<td>Biotin</td>
<td>0.0002</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.81</td>
<td>p-Aminobenzoic Acid</td>
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<td>L-leucine</td>
<td>1.32</td>
<td>Inositol</td>
<td>0.04200</td>
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<tr>
<td>L-lysine HCl</td>
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<td>Niacin</td>
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<td>L-methionine</td>
<td>0.58</td>
<td>Calcium Pantothenate</td>
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<tr>
<td>L-phenylalanine</td>
<td>0.94</td>
<td>Folic Acid</td>
<td>0.00599</td>
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<tr>
<td>L-threonine</td>
<td>0.90</td>
<td>Pyridoxine HCl</td>
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<td>L-tryptophan</td>
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<td>Riboflavin</td>
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<td>L-valine</td>
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<td>Thiamin HCl</td>
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<tr>
<td>L-alanine</td>
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<td>Choline Bitartrate</td>
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<td>L-asparagine</td>
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<td>Vitamin A Palmitate (500,000 IU/g)</td>
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<tr>
<td>L-aspartic acid</td>
<td>0.53</td>
<td>Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)</td>
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<td>L-cystine</td>
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<td>Vitamin D3, cholecalciferol (500,000 IU/g)</td>
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<tr>
<td>L-glutamic acid</td>
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<td>Vitamin K, MSB complex</td>
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</tr>
<tr>
<td>L-glutamine</td>
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<td></td>
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<tr>
<td>Glycine</td>
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<td>Zinc Carbonate</td>
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<td>L-proline</td>
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<td>L-serine</td>
<td>0.98</td>
<td>Chromium Potassium Sulfate, dodecahydrate</td>
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<td>L-tyrosine</td>
<td>0.81</td>
<td>Potassium Phosphate, dibasic</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Potassium Phosphate, monobasic</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Calcium Chloride</td>
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<td>Carbohydrates</td>
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<td>Ferrous Sulfate, heptahydrate</td>
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<td>Sucrose</td>
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<td>Magnesium Sulfate, heptahydrate</td>
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<td>Glucose</td>
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<td>Manganese Sulfate, monohydrate</td>
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<tr>
<td>Lactose</td>
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<td>Sodium Chloride</td>
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<tr>
<td>Trehalose</td>
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<td>RNA</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>DNA</td>
<td>0.49996</td>
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<td><strong>Lipids</strong></td>
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<td>Agarose</td>
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<td>Cholesterol</td>
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<tr>
<td>Lecithin</td>
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### Table 4.1B. Recipe for regular food (RF).

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<th>Ingredients</th>
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<tbody>
<tr>
<td>Yeast</td>
<td>35.00</td>
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<tr>
<td>Yellow cornmeal</td>
<td>80.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>50.00</td>
</tr>
<tr>
<td>10% p-Hydroxy-benzoic acid methyl ester in 95% ethanol (ml)</td>
<td>27.00</td>
</tr>
<tr>
<td>Agar</td>
<td>9.00</td>
</tr>
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Table 4.2. Summary: chemically defined food versus regular food.

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<tr>
<th>Parameters</th>
<th>CDF&lt;sup&gt;100K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;200K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;300K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;400K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;500K&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Body weight</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lifetime egg-lay</td>
<td>2.9 fold increase</td>
<td>3.5 fold increase</td>
<td>3.7 fold increase</td>
<td>2.7 fold increase</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td>6 days shorter</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>2 days shorter</td>
</tr>
<tr>
<td>Body weight</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Larvae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Survival</td>
<td>19.3% decrease</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>16.3% decrease</td>
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<tr>
<td>Growth</td>
<td>6.05 days delayed</td>
<td>4.25 days delayed</td>
<td>4.25 days delayed</td>
<td>4.14 days delayed</td>
<td>4.31 days delayed</td>
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<tr>
<td>Pupation period</td>
<td>0.43 days delayed</td>
<td>0.38 days delayed</td>
<td>0.47 days delayed</td>
<td>0.42 days delayed</td>
<td>0.52 days delayed</td>
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<tr>
<td><strong>Transgeneration</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Generation time</td>
<td>5.6 days delayed</td>
<td>5.2 days delayed</td>
<td>3.6 days delayed</td>
<td>2.6 days delayed</td>
<td>2.8 days delayed</td>
</tr>
<tr>
<td>Growth @ 29°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth @ 18°C</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Generation #</td>
<td>6</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
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</table>

n.s.: not statistically significant; +: vigorous culture growth; +/-: poor culture growth; -: fail to support culture; a vials often have fungi / bacterial growth.
Supplemental Figure S4.1. The effect of CDF on adult weight (continued). (A) Average body weight of adult flies cultured on regular food (RF) as a function of age. Females gain 5.7±2.2
(Mean±SE), 14.9±3.9, 21.2±7.8% of body weight at day 21, 27, 35 respectively (Mann Whitney test; n = 4 except at day 35; p ≥ 0.1288 for all; see Supplemental Table S4.2A for additional details). Males lose 7.0±2.8 and 1.3±2.0% of body weight by day 21 (n=4, p = 0.0289) and day 27 respectively, then gain 2.5±5.9% of body weight by day 35 (n = 4, p = 0.4754). (B) Average body weight of adult female flies cultured on chemically defined food (CDF) as a function of caloric density. In the first week on CDF, females first lose about 10% of their initial body weight which is recovered by day 5. Females gain 21.2±7.8, 10.1±5.7, 4.7±2.6, 13.3±2.0, 8.1±4.2 and 12.4±5.1 % of body weight after 35 days on RF, CDF_{100K}, CDF_{200K}, CDF_{300K}, CDF_{400K} and CDF_{500K} respectively (Mann Whitney test; n = 4, p ≥ 0.1143 for all; see Supplemental Table S4.2B for details). Females on CDF show a similar trend of increasing body weight as they age on RF (Friedman test; n = 4, p ≤ 0.0006 for all; see Supplemental Table S4.2D for details). (C) Average body weight of adult male flies cultured on chemically defined food as a function of caloric density. Males gain 2.5±5.9, 7.8±3.4, 10.2±1.2, 14.0±2.0, 11.2±3.2 and 12.7±5.6 % of body weight changes after 35 days on RF, CDF_{100K}, CDF_{200K}, CDF_{300K}, CDF_{400K} and CDF_{500K} respectively (Mann Whitney test; n = 4, p ≥ 0.200 for all; see Supplemental Table S4.2C for details). Male flies on CDFs show an increasing trend in body weight compared to males aged on RF (Friedman test; n = 4, p ≤ 0.0116 for all; see Supplemental Table S4.2D for details).
Supplemental Figure S4.2. The effect of CDF on larval development and survival. (A) Days required for larvae to complete different stages of development when cultured on chemically defined food. All larvae grown on CDFs show a statistically significant developmental delay (Mann Whitney test; n ≥ 65, p < 0.0001 for all; see Supplemental Table S4A for details). (B) Survival rates for larvae cultured on chemically defined food by stage. Larvae cultured on CDF^{200K-400K} show no statistical difference in survival compared to RF (one-tailed Fisher’s exact test; n > 65, p ≥ 0.0544 for all; see Supplemental Table S4B for additional details); significant differences in survival are observed on CDF^{100K} and CDF^{500K} (p = 0.0025 and 0.0202 respectively).
Supplemental Table S4.1. Longevity of adult flies on CDF (n = 40, 4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female median survival</td>
<td>Days</td>
<td>35</td>
<td>33</td>
<td>36</td>
<td>35</td>
<td>37</td>
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<tr>
<td>Female median survival p value for Mantel-Cox test to RF</td>
<td>0.6796</td>
<td>0.2577</td>
<td>0.1053</td>
<td>0.2425</td>
<td>0.7777</td>
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<tr>
<td>Male median survival</td>
<td>Days</td>
<td>41</td>
<td>35</td>
<td>40</td>
<td>44</td>
<td>43</td>
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<tr>
<td>Male median survival p value for Mantel-Cox test to RF</td>
<td>&lt;0.0001</td>
<td>0.1789</td>
<td>0.1600</td>
<td>0.6631</td>
<td><strong>0.0489</strong></td>
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Supplemental Table S4.2A. Body weight of adult flies on RF or CDF (normalized to day 0; 4 replicates, 10 pairs of flies for each replicate; ‡3 replicates). Statistically significant p values are labeled with bold text.

<table>
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<th>Day 0</th>
<th>Day 7</th>
<th>Day 13</th>
<th>Day 21</th>
<th>Day 27</th>
<th>Day 35</th>
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<tr>
<td>Female</td>
<td>Mean</td>
<td>100</td>
<td>105.4</td>
<td>104.2</td>
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<td>Female</td>
<td>Std. Error</td>
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<td>2.1</td>
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<td>p value for Mann Whitney test to Day 0</td>
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<tr>
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<td>Mean</td>
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<tr>
<td>Male</td>
<td>Std. Error</td>
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<td>2.0</td>
<td>2.8</td>
<td>2.0</td>
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<tr>
<td>Male</td>
<td>p value for Mann Whitney test to Day 0</td>
<td>0.5308</td>
<td>0.2408</td>
<td>0.0289</td>
<td>0.1057</td>
<td>0.4754</td>
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Supplemental Table S4.2B. Body weight of adult female flies on CDF (normalized to day 0; 4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text.

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<th></th>
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</thead>
<tbody>
<tr>
<td>Female (day 7)</td>
<td>Mean</td>
<td>105.4</td>
<td>96.2</td>
<td>97.0</td>
<td>98.4</td>
<td>95.2</td>
</tr>
<tr>
<td>Female (day 7)</td>
<td>Std. Error</td>
<td>2.1</td>
<td>4.7</td>
<td>2.3</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Female (day 7)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.3429</td>
<td>0.0571</td>
<td>0.0571</td>
<td>0.0286</td>
<td>0.0286</td>
</tr>
<tr>
<td>Female (day13)</td>
<td>Mean</td>
<td>104.2</td>
<td>101.5</td>
<td>97.0</td>
<td>102.1</td>
<td>95.3</td>
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<tr>
<td>Female (day13)</td>
<td>Std. Error</td>
<td>1.3</td>
<td>4.9</td>
<td>2.6</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Female (day13)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.8857</td>
<td>0.0814</td>
<td>0.6857</td>
<td>0.0571</td>
<td>0.0571</td>
</tr>
<tr>
<td>Female (day21)</td>
<td>Mean</td>
<td>105.7</td>
<td>106.0</td>
<td>103.1</td>
<td>102.0</td>
<td>99.0</td>
</tr>
<tr>
<td>Female (day21)</td>
<td>Std. Error</td>
<td>2.2</td>
<td>4.7</td>
<td>4.9</td>
<td>3.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Female (day21)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.8857</td>
<td>0.4857</td>
<td>0.3836</td>
<td>0.4857</td>
<td>1.0000</td>
</tr>
<tr>
<td>Female (day27)</td>
<td>Mean</td>
<td>114.9</td>
<td>108.0</td>
<td>102.8</td>
<td>102.3</td>
<td>105.3</td>
</tr>
<tr>
<td>Female (day27)</td>
<td>Std. Error</td>
<td>3.9</td>
<td>2.0</td>
<td>3.6</td>
<td>2.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Female (day27)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.3429</td>
<td>0.0571</td>
<td>0.0571</td>
<td>0.1143</td>
<td>0.4857</td>
</tr>
<tr>
<td>Female (day35)</td>
<td>Mean</td>
<td>121.2‡</td>
<td>110.1</td>
<td>104.7</td>
<td>113.3</td>
<td>108.1</td>
</tr>
<tr>
<td>Female (day35)</td>
<td>Std. Error</td>
<td>7.8‡</td>
<td>5.7</td>
<td>2.6</td>
<td>2.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Female (day35)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.2286</td>
<td>0.1143</td>
<td>0.4000</td>
<td>0.2286</td>
<td>0.4000</td>
</tr>
</tbody>
</table>
Supplemental Table S4.2C. Body weight of adult male flies on CDF (normalized to day 0; 4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>CDF&lt;sup&gt;100K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;200K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;300K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;400K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;500K&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (day 7)</td>
<td>Mean</td>
<td>98.6</td>
<td>99.0</td>
<td>98.1</td>
<td>101.2</td>
<td>100.6</td>
</tr>
<tr>
<td>Male (day 7)</td>
<td>Std. Error</td>
<td>2.0</td>
<td>3.2</td>
<td>2.0</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Male (day 7)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.8857</td>
<td>1.0000</td>
<td>0.3429</td>
<td>0.4857</td>
<td>0.4857</td>
</tr>
<tr>
<td>Male (day 13)</td>
<td>Mean</td>
<td>96.9</td>
<td>101.0</td>
<td>99.9</td>
<td>99.6</td>
<td>100.6</td>
</tr>
<tr>
<td>Male (day 13)</td>
<td>Std. Error</td>
<td>2.0</td>
<td>2.6</td>
<td>3.0</td>
<td>2.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Male (day 13)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.2000</td>
<td>0.6857</td>
<td>0.2454</td>
<td>0.2000</td>
<td>0.2000</td>
</tr>
<tr>
<td>Male (day 21)</td>
<td>Mean</td>
<td>93.0</td>
<td>99.5</td>
<td>99.5</td>
<td>100.5</td>
<td>100.5</td>
</tr>
<tr>
<td>Male (day 21)</td>
<td>Std. Error</td>
<td>2.8</td>
<td>2.2</td>
<td>3.4</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Male (day 21)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.2000</td>
<td>0.1465</td>
<td>0.1143</td>
<td>0.1143</td>
<td>0.1143</td>
</tr>
<tr>
<td>Male (day 27)</td>
<td>Mean</td>
<td>98.7</td>
<td>103.5</td>
<td>103.7</td>
<td>104.9</td>
<td>105.1</td>
</tr>
<tr>
<td>Male (day 27)</td>
<td>Std. Error</td>
<td>2.0</td>
<td>1.2</td>
<td>2.9</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Male (day 27)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.1143</td>
<td>0.3094</td>
<td>0.1143</td>
<td>0.1143</td>
<td>0.4857</td>
</tr>
<tr>
<td>Male (day 35)</td>
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<td>107.8</td>
<td>110.2</td>
<td>114.0</td>
<td>111.2</td>
</tr>
<tr>
<td>Male (day 35)</td>
<td>Std. Error</td>
<td>5.9</td>
<td>3.4</td>
<td>1.2</td>
<td>2.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Male (day 35)</td>
<td>p value for Mann Whitney test to RF</td>
<td>0.6857</td>
<td>0.3429</td>
<td>0.2000</td>
<td>0.3429</td>
<td>0.3429</td>
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</tbody>
</table>

Supplemental Table S4.2D. Trends in body weight changes of adult flies on different food (4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text. Day 33 is the last data point eligible for Friedman test in female flies.

<table>
<thead>
<tr>
<th></th>
<th>CDF&lt;sup&gt;100K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;200K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;300K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;400K&lt;/sup&gt;</th>
<th>CDF&lt;sup&gt;500K&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (day 1-21)</td>
<td>p value for Friedman test to RF</td>
<td>0.0006</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Female (day 1-33)</td>
<td>p value for Friedman test to RF</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Male (day 1-21)</td>
<td>p value for Friedman test to RF</td>
<td>0.0116</td>
<td>0.0006</td>
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</tr>
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<td>Male (day 1-45)</td>
<td>p value for Friedman test to RF</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>
Supplemental Table S4.3. Effect of CDF on egg-lay (4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>CDF^{100K}</th>
<th>CDF^{200K}</th>
<th>CDF^{300K}</th>
<th>CDF^{400K}</th>
<th>CDF^{500K}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal 12 hr egg-lay</td>
<td>Mean</td>
<td>12.7</td>
<td>24.6</td>
<td>22.8</td>
<td>23.6</td>
<td>18.5</td>
</tr>
<tr>
<td>Maximal 12 hr egg-lay Std. Error</td>
<td>1.8</td>
<td>1.9</td>
<td>2.4</td>
<td>1.2</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Maximal 12 hr egg-lay p value for Mann Whitney test to RF</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.2454</td>
<td></td>
</tr>
<tr>
<td>Days of maximal egg-lay</td>
<td>Mean</td>
<td>6.5</td>
<td>10.5</td>
<td>11.0</td>
<td>12.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Days of maximal egg-lay Std. Error</td>
<td>1.9</td>
<td>1.5</td>
<td>1.4</td>
<td>1.7</td>
<td>2.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Days of maximal egg-lay p value for Mann Whitney test to RF</td>
<td>0.2308</td>
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<td>0.1416</td>
<td>0.1416</td>
<td>0.3688</td>
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<tr>
<td>Time to Reproductive Quiescence (days)</td>
<td>Mean</td>
<td>21.0</td>
<td>35.0</td>
<td>42.5</td>
<td>40.5</td>
<td>36.0</td>
</tr>
<tr>
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<td>2.4</td>
<td>0.8</td>
<td>2.1</td>
<td>1.0</td>
<td>2.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Time to Reproductive Quiescence (Days) p value for Mann Whitney test to RF</td>
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<td>0.0284</td>
<td>0.0284</td>
<td>0.0294</td>
<td>0.1441</td>
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<tr>
<td>Lifetime egg-lay</td>
<td>Mean</td>
<td>62.7</td>
<td>182.0</td>
<td>220.0</td>
<td>230.7</td>
<td>169.4</td>
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<tr>
<td>Lifetime egg-lay Std. Error</td>
<td>10.1</td>
<td>14.1</td>
<td>36.9</td>
<td>23.1</td>
<td>9.8</td>
<td>14.3</td>
</tr>
<tr>
<td>Lifetime egg-lay p value for Mann Whitney test to RF</td>
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<td>0.0286</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.0571</td>
<td></td>
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</table>
### Supplemental Table S4.4A. Larval development on CDF (n ≥ 65, 3 replicates, 20-25 larvae for each replicate). Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th>Days (Hatch to Eclosion)</th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days (Hatch to Eclosion)</td>
<td>Mean</td>
<td>8.61</td>
<td>15.00</td>
<td>13.29</td>
<td>13.19</td>
<td>13.23</td>
</tr>
<tr>
<td>Days (Hatch to Eclosion)</td>
<td>Std. Error</td>
<td>0.06</td>
<td>0.15</td>
<td>0.09</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td>Days (Hatch to Eclosion)</td>
<td>p value for Mann Whitney test to RF</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Days (Hatch to Pupal)</th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days (Hatch to Pupal)</td>
<td>Mean</td>
<td>4.71</td>
<td>10.76</td>
<td>8.97</td>
<td>8.97</td>
<td>8.85</td>
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<tr>
<td>Days (Hatch to Pupal)</td>
<td>Std. Error</td>
<td>0.06</td>
<td>0.15</td>
<td>0.09</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td>Days (Hatch to Pupal)</td>
<td>p value for Mann Whitney test to RF</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
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</table>

<table>
<thead>
<tr>
<th>Days (Pupal to Eclosion)</th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days (Pupal to Eclosion)</td>
<td>Mean</td>
<td>3.92</td>
<td>4.35</td>
<td>4.30</td>
<td>4.39</td>
<td>4.34</td>
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<tr>
<td>Days (Pupal to Eclosion)</td>
<td>Std. Error</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
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<tr>
<td>Days (Pupal to Eclosion)</td>
<td>p value for Mann Whitney test to RF</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

### Supplemental Table S4.4B. Larval survival on CDF (n ≥ 65, 3 replicates, 20-25 larvae for each replicate). One-tailed Fisher’s exact tests were performed with the total counts, not the mean values. Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th>Eclosed % (Hatch to Eclosion)</th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eclosed % (Hatch to Eclosion)</td>
<td>Mean</td>
<td>89.7</td>
<td>70.3</td>
<td>81.0</td>
<td>77.7</td>
<td>92.3</td>
</tr>
<tr>
<td>Eclosed % (Hatch to Eclosion)</td>
<td>Std. Error</td>
<td>5.2</td>
<td>7.3</td>
<td>6.7</td>
<td>3.8</td>
<td>1.5</td>
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<tr>
<td>Eclosed % (Hatch to Eclosion)</td>
<td>p value for Fisher’s exact test to RF</td>
<td>0.0025</td>
<td>0.1118</td>
<td>0.0544</td>
<td>0.3816</td>
<td>0.0202</td>
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</table>

<table>
<thead>
<tr>
<th>Survival % (Hatch to Pupal)</th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival % (Hatch to Pupal)</td>
<td>Mean</td>
<td>94.7</td>
<td>84.0</td>
<td>90.3</td>
<td>91.7</td>
<td>92.3</td>
</tr>
<tr>
<td>Survival % (Hatch to Pupal)</td>
<td>Std. Error</td>
<td>5.3</td>
<td>6.0</td>
<td>7.3</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Survival % (Hatch to Pupal)</td>
<td>p value for Fisher’s exact test to RF</td>
<td>0.0485</td>
<td>0.2652</td>
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<td>0.5000</td>
<td>0.3719</td>
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</table>

<table>
<thead>
<tr>
<th>Survival % (Pupal to Eclosion)</th>
<th>RF</th>
<th>CDF100K</th>
<th>CDF200K</th>
<th>CDF300K</th>
<th>CDF400K</th>
<th>CDF500K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival % (Pupal to Eclosion)</td>
<td>Mean</td>
<td>95.0</td>
<td>83.3</td>
<td>89.7</td>
<td>85.2</td>
<td>100.0</td>
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<tr>
<td>Survival % (Pupal to Eclosion)</td>
<td>Std. Error</td>
<td>5.0</td>
<td>3.2</td>
<td>0.2</td>
<td>4.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Survival % (Pupal to Eclosion)</td>
<td>p value for Fisher’s exact test to RF</td>
<td>0.0217</td>
<td>0.2207</td>
<td>0.0592</td>
<td>0.1250</td>
<td>0.0185</td>
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</table>
Supplemental Table S4.5A. Effect of macro-nutrient deprivation on adult longevity (n = 40, 4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th>Female median survival</th>
<th>Days</th>
<th>CDF(^{400K})</th>
<th>CDF(^{400K-\text{AA}})</th>
<th>CDF(^{400K-\text{Carb}})</th>
<th>CDF(^{400K-\text{Fat}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>35.0</td>
<td>20.5</td>
<td>4.0</td>
<td>26.0</td>
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<tr>
<td>Female median survival</td>
<td>p value for Mantel-Cox test to CDF(^{400K})</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0002</td>
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<tr>
<td>Female median survival</td>
<td>p value for Mantel-Cox test to CDF(^{400K-\text{AA}})</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Female median survival</td>
<td>p value for Mantel-Cox test to CDF(^{400K-\text{Carb}})</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Male median survival</td>
<td>Days</td>
<td>41.0</td>
<td>19.0</td>
<td>2.5</td>
<td>35.0</td>
</tr>
<tr>
<td>Male median survival</td>
<td>p value for Mantel-Cox test to CDF(^{400K})</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0241</td>
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</tr>
<tr>
<td>Male median survival</td>
<td>p value for Mantel-Cox test to CDF(^{400K-\text{AA}})</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Male median survival</td>
<td>p value for Mantel-Cox test to CDF(^{400K-\text{Carb}})</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Supplemental Table S4.5B. Effect of macro-nutrient deprivation on egg-lay (4 replicates, 10 pairs of flies for each replicate). Statistically significant p values are labeled with bold text.

<table>
<thead>
<tr>
<th>Total egg-lay (7 days)</th>
<th>Mean</th>
<th>CDF(^{400K})</th>
<th>CDF(^{400K-\text{AA}})</th>
<th>CDF(^{400K-\text{Carb}})</th>
<th>CDF(^{400K-\text{Fat}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>81.8</td>
<td>18.7</td>
<td>28.3</td>
<td>80.7</td>
<td></td>
</tr>
<tr>
<td>Total egg-lay (7 days)</td>
<td>Std. Error</td>
<td>3.6</td>
<td>2.5</td>
<td>3.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Total egg-lay (7 days)</td>
<td>p value for Mann Whitney Test to CDF(^{400K})</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.7715</td>
<td></td>
</tr>
<tr>
<td>Total egg-lay (7 days)</td>
<td>p value for Mann Whitney Test to CDF(^{400K-\text{AA}})</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.0286</td>
<td></td>
</tr>
<tr>
<td>Total egg-lay (7 days)</td>
<td>p value for Mann Whitney Test to CDF(^{400K-\text{Carb}})</td>
<td>0.0286</td>
<td>0.0286</td>
<td>0.0286</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5

Summary and future directions
5.1 Summary

In this thesis I investigated the roles of WNT/APC and JAK/STAT in regulating stem cell properties of *Drosophila* ISCs, and established a chemically defined food recipe adequate for nutritional studies in adult *Drosophila*. In this Chapter, I will summarize the major findings, discuss the topics for future studies and propose experiments accordingly to elucidate the mechanisms regulating the properties of ISCs under the influences of genetic mutations, signaling pathways and nutrients.

5.2 Insulin promotes the expansion of *APC*<sub>lof</sub> lineages

Experiments discussed in Chapter 2 demonstrated that APC is required in ISCs to suppress proliferation, and APC loss-of-function (*APC*<sub>lof</sub>; both APC1 and APC2 loss of function) ISC clones undergo hyper-proliferation and grow into adenoma-like multi-layered polyps. This finding suggested that the *APC*<sub>lof</sub> ISCs can function as the cells-of-origin of intestinal tumors, consistent with the mouse model (Barker et al., 2009).

Pioneered clonal study initiated by Vogelstein’s group not only demonstrated that APC is the gate-keeper gene for colon and rectal cancer (CRC) initiation but also identified multiple mutations associated with CRC progression (Fearon et al., 1987; Kinzler and Vogelstein, 1996). These observations suggested that certain mutations can promote the expansion and the transformation of intestinal lineages after the loss of APC. Although many studies have identified additional mutations capable of promoting the expansion of *APC*<sub>lof</sub> lineages, the involvement of the ISC during this process remains at large. Thus I designed a clone-autonomous screen aimed to identify signaling pathways involved in regulating the expansion of *APC*<sub>lof</sub> ISC lineages in *Drosophila*. By modifying the MARCM system (Fig. 1.3D), I can ectopically express UAS constructs to alter the major signaling pathway activities in a clone-autonomous manner. The pathways selected for the screen were EGF, Hedgehog, Insulin, JAK/STAT, JNK, mTOR, Notch, p53, and TGFβ (See Material and methods section 5.4.1).

To assess whether altering the activity of each signaling pathway can sustainably promote or suppress *APC*<sub>lof</sub> clone expansion, midguts were dissected at 1 week, 2 weeks, and 4 weeks after
clone induction for analysis. Measured by clone size, only activation of the Insulin pathway, activation of the EGF pathway, and inactivation of the Notch pathway can continuously promote the expansion \(APC^{lof}\) lineages over a 4 week period (Fig. 5.1, 5.2, and 5.3). Also, inactivation of the Insulin pathway, inactivation of the EGF pathway, and activation of the Notch pathway all suppress the expansion of \(APC^{lof}\) lineages (Fig. 5.4, 5.5, and 5.6). However, inactivation of the Notch pathway in \(APC^{lof}\) background leads to significant clone loss at 4 weeks (Fig. 5.3) suggesting that Notch activity is not fully dispensable for ISC self-renewal. Compared to activation of the EGF pathway, activation of the Insulin pathway is more potent to promote the expansion of \(APC^{lof}\) lineage (Fig. 5.1, and 5.2). Of note, the Insulin-like growth factor 2 (IGF2) - IGF1R - IRS2 - PI3K signaling axis has recently been shown to be associated with CRC and is suggested to be a therapeutic target for CRC through a large scale sequencing analyses (Cancer Genome Atlas Network, 2012).

For the future directions of this project, I propose to focus on the cellular and molecular mechanisms to dissect both the growth advantages given by Insulin activation and the essential signaling components utilized by the Insulin pathway to promote the expansion of tumorigenic \(APC^{lof}\) lineages. The knowledge learned from the following studies will provide new insights about how tumorigenic ISC clones acquire growth advantages over normal cells and can be used as a model for the intestinal tumor progression that is initiated in ISCs.

5.2.1 Cellular mechanisms by which Insulin signaling promotes the expansion of \(APC^{lof}\) lineages

To study the cellular mechanism by which Insulin activation promotes the expansion of \(APC^{lof}\) lineages, I proposed to focus on both clone-autonomous and clone-non-autonomous behaviors of \(InR^{act}, APC^{lof}\) ISCs (clonal activation of the Insulin pathway in \(APC^{lof}\) ISCs). For clone-autonomous behavior, I hypothesizde that \(InR^{act}, APC^{lof}\) lineages acquire growth advantages through four possible mechanisms: 1) increase in lineage output, 2) increase in clone initiation, 3) reduction in clone maintenance effect, and 4) increase in symmetric division.
To measure lineage output, the clone size and the mitotic index can be counted at selected experimental time points. The measurements can be conducted using the MARCM system. However, as mentioned in Chapter 2, the fusion of the hyperplastic APC<sup>lof</sup> clones limits analyses to the first 5-10 days after clone induction. If activation of the Insulin pathway further promotes the expansion of APC<sup>lof</sup> lineages, the fusion of InR<sup>act</sup>, APC<sup>lof</sup> clones would be expected within 5-10 days after clone induction thus further limiting the clonal analysis to earlier time points. Although reduction of clonal marking frequency can be achieved by reducing the heat-shock stress of the classic MARCM system, it also reduces the ratio of animals getting clone induction and alters the constancy of clone numbers induced per animal in our preliminary experiments. These variables dramatically affect the results of clonal analysis using the MARCM system. In order to reduce the clone marking frequency without altering the heat shock stress, I genetically modified the classic MARCM system into a double MARCM system (Fig. 5.7). The clonal marking rate is dramatically reduced in the double MARCM system (~30 clones per gut for the double MARCM system versus ~200 clones per gut for the classic MARCM system). I further tested whether the double MACRM system is capable of revealing each individual hyperplastic InR<sup>act</sup>; APC<sup>lof</sup> clone in late experimental stages for counting the clone size. My preliminary data shows that the individual InR<sup>act</sup>, APC<sup>lof</sup> clones can be identified even at 20 days after clone induction (Fig. 5.8) (See Material and methods section 5.4.2).

The number of clones at early versus late stages during an experimental period can be used to assess the influence of Insulin activation on APC<sup>lof</sup> ISCs in their abilities to initiate a clone and to be maintained in the intestinal epithelium. However, two different types of clones can be induced in the MARCM system, the transient clones and the stable clones (Ohlstein and Spradling, 2006). After a labeling event occurs in a mitotic ISC, either the self-renewing ISC or newly produced EB daughter cell will inherit the lineage tracing marker (usually GFP). A GFP marked EB will then differentiate into either an EC or an EE, and will be lost from the midgut epithelium due to the routine turn-over of the epithelium. Since the EB clone does not contain an ISC, and thus cannot undergo self-renewal and proliferation, it is also visualized as a single cell clone. Due to their short living nature, EB clones are referred to as transient clones. In contrast, a
GFP marked ISC can undergo self-renewal and produce additional daughter cells, which yields a multi-cellular clone. Due to their sustainable nature, the ISC clones are termed stable clones. Since the normal turn-over rate of midgut epithelium is 7-10 days (Buchon et al., 2010), the MARCM based clone number analysis in early experimental stages is subjected to the influence of transient clones. In addition to the single cell transient clones, multi-cellular transient clones can arise from the clonal loss of ISCs in late experimental stages. Because this type of clone will eventually be lost from the midgut epithelium, it is also considered a transient clone. To accurately assess ISC properties using the MARCM system, the type of clones have to be taken into consideration. For clone size and clone initiation analyses, the measurements should be done on stable clones. For the maintenance effect, the numbers of both stable clones and transient clones have to be taken into consideration.

To determine an event of symmetric division, a system must mark both daughter cells of a mitotic ISC. The use of the twin-spot MARCM system to determine the division pattern of a normal ISC has been reported during the growth stage of newly eclosed adult flies (O'Brien et al., 2011; Yu et al., 2009), however the recombination event in the twin-spot system does not generate any homozygous loss-of-function cell as in the classic MARCM system. Thus, I propose to modify this system in two ways. First, I will combine the classic MARCM system with the twin-spot MARCM system (O'Brien et al., 2011; Yu et al., 2009) to follow both daughter cell lineages from an ISC in a desired background (See Material and methods section 5.4.3a). The major concern for this modified twin-spot MARCM system is whether the twin-spot system can be brought into the classic MARCM background as it requires intensive genetics. An alternative approach is to generate the $APC1^{lod}$ twin-spot MARCM system (See Material and methods section 5.4.3b). Since the twin-spot MARCM system does not generate homozygous loss-of-function cells, the experiment to determine whether a cell in a gene loss-of-function condition increases the frequency of symmetric divisions using the twin-spot MARCM system would have to be performed in a homozygous background. The Apc1 and Apc2 double loss-of-function mutant is homozygous lethal, and thus the twin-spot MARCM system can only be used in either $APC1^{lod}$ or $APC2^{lod}$ background. My preliminary data and other studies (Cordero et al., 2012) both
suggest that $APC1^{lof}$ is more potent than $APC2^{lof}$ to induce ISC proliferation, thus I propose to perform this experiment in an $APC1^{lof}$ background. However, the ubiquitous Gal4 used in the twin-spot MARCM is located on the 3rd chromosome as Apc1, and therefore this method would require making a recombined chromosome carrying both the ubiquitous Gal4 and $APC1^{lof}$.

To determine how Insulin activation may promote the expansion of tumorigenic $APC^{lof}$ lineages in a clone-non-autonomous manner, I propose to focus on cell competition for multiple reasons. First, the $APC^{lof}$ and Insulin activation ($InR^{act}; APC^{lof}$) clones show accelerated clone growth in comparison to $APC^{lof}$ clones. Second, the $InR^{act}; APC^{lof}$ clones take over the surrounding non-labeled area, suggesting that they grow at the expense of surrounding cells (Fig. 5.1). This phenomena is consistent with the description of cell-competition (Moreno, 2008).

Because growth at the expense of the surrounding cells is the essential definition for cell-competition, both increasing the cell death or decreasing the lineage output from the surrounding cells are possible mechanisms used by over-growing cells to gain growth advantages. For cell death, I propose to use anti-cleaved Caspase-3 (Asp175) antibody (#9661, Cell Signaling) to determine the number of cell death events in the surrounding cells. To calculate the lineage output of the surrounding cells, I propose to use esg-LacZ; MARCM we generated in Chapter 2 (See Material and methods section 5.4.4). The use of esg-LacZ; MARCM permits the counting of progenitor cells and differentiated daughters outside the marked clones, which can be used to assess the number of progenitor cells and their lineage outputs over time. These two measurements can be used to determine whether and how the surrounding lineage outputs are affected by marked over-growing lineages. In addition to cell death, cell shedding is also a common mechanism during intestinal epithelium renewal (Bullen et al., 2006; Watson et al., 2009). However, a quantitative method to measure cell shedding is not currently available. Experimentally, if the lineage output is reduced from surrounding tissue but cell death is not increased, increased cell shedding of the surrounding cells can be an alternative mechanism used by $InR^{act}; APC^{lof}$ cells to outcompete other cells in the Drosophila midgut.
5.2.2 Molecular mechanisms by which Insulin signaling promotes the expansion of $A_{PC}^{lof}$ lineages

To dissect out the molecular component required by the Insulin pathway to promote the expansion of $A_{PC}^{lof}$ lineages, I propose to use the MARCM system to identify downstream components of the Insulin signaling pathway that are sufficient to promote the expansion of $A_{PC}^{lof}$ lineages. Following identification of the Insulin signaling component sufficient for the expansion of $A_{PC}^{lof}$ lineages alone, I propose to confirm its requirement for the expansion of $lnR_{act}; A_{PC}^{lof}$ lineages (See Material and methods section 5.4.5).

To identify the possible downstream pathways utilized by $lnR_{act}; A_{PC}^{lof}$ lineages to promote cell competition, I will focus on the Myc, Hippo, and Notum pathways for the following reasons. Myc is a known direct target negatively regulated by APC and is involved in regulating cell competition (He et al., 1998; Levayer and Moreno, 2013). The transcriptional coactivator Yokie, negatively regulated by the Hippo pathway, has recently been shown to promote the transcription of Myc (Stocker, 2011). Myc then negatively regulates the concentration of Yokie to restrain the growth induced by Yokie. Recent studies also identified the Hippo pathway as a common regulator of cell competition (Levayer and Moreno, 2013). The secretion of a Myc-independent negative feedback inhibitor, Notum, has been demonstrated to be required for WNT signaling dependent cell competition (Vincent et al., 2011). Notum secreted by WNT activating cells is known to suppress the response to WNT in surrounding cells, which in turns maintains the growth advantage to WNT activating cells. Although Notum is required for maintaining the growth advantage of $Axin^{lof}$ clones in Drosophila imaginal discs, its functional roles during cell competition induced by $lnR_{act}; A_{PC}^{lof}$ lineages in midgut remains at large. For these reasons, I propose to test whether Myc, Hippo, and Notum are required for cell competition caused by $lnR_{act}; A_{PC}^{lof}$ ISC lineages. (See Material and methods section 5.4.6).

In addition to cell competition, an increase in cytokine production can be another non-clone-autonomous growth advantage acquired by $lnR_{act}; A_{PC}^{lof}$ ISC lineages. The non-clone-autonomous induction of the unpaired (UPD) ligand for the JAK/STAT pathway, a mediator of cytokine signaling, has been reported in $APC1^{lof}$ midguts (Cordero et al., 2012). Consistent with
this observation, in my clonal modifier screen for \(APC^{lof}\) lineages, I found that Stat92E is required for the growth of \(APC^{lof}\) lineages but is dispensable for normal ISC lineages (Fig. 5.9). These data suggest a model where \(APC^{lof}\) lineages induce cytokine production in a non-clone-autonomous manner, which in turns upregulates the JAK/STAT signaling in all adjacent cells but brings more beneficial effects for the growth of \(APC^{lof}\) lineages. Activation of the Insulin pathway in \(APC^{lof}\) lineages may further increase the non-autonomous expression of Upd to provide additional growth benefits to \(APC^{lof}\) lineages.

To determine whether cytokine levels are upregulated by Insulin activation in the \(APC^{lof}\) background, I propose to use Quantitative PCR and LacZ reporters (Cordero et al., 2012) to measure the relative gene expression level of Unpaired (Upd) ligands in the midguts of wild-type, \(APC^{lof}\), \(InR^{act}\) and \(InR^{act}\), \(APC^{lof}\) MARCM flies (See Material and methods section 5.4.7a). To test whether upregulated cytokine is sufficient to promote the expansion of the tumorigenic ISC lineage, I propose to use double MACRM to ectopically express Upd for clonal analysis as proposed in the previous section (See Material and methods section 5.4.7b). To further test whether upregulated cytokine is necessary to promote the expansion of tumorigenic ISC lineages, I propose to use the classic MACRM system to knock-down Upd (See Material and methods section 5.4.7c). If Upd is sufficient or necessary to promote the expansion of tumorigenic ISC lineages, I propose to examine whether the JAK/STAT signaling is sufficient or necessary for the expansion of \(InR^{act};\ \ APC^{lof}\) lineage (See Material and methods section 5.4.7).

5.3 Nutritional requirements for normal and tumorigenic ISC lineages

In order to support life and maintain body functions, organisms must acquire nutrients from food to sustain their energetic and metabolic needs. Understanding how dietary nutrients influence a variety of biological processes has been the subject of active investigation. However, it is difficult to dissect out the precious role of a specific nutrient from a regular complex diet as reasoned in Chapter 4. To establish the requirement of individual nutrients for a biological process of an organism, the most direct route is using a chemically defined food to quantify the biological process at different nutrient levels from deficiency to overabundance (Douglas and
Simpson, 2013). However, to my knowledge, most developed CDFs are either stressful to model organisms or have allowed only certain parameters to be varied (Grandison et al., 2009; Lu and Goetsch, 1993; Pleasants and Johnson, 1986; Steinberg et al., 2003; Szewczyk et al., 2003). Conducting nutritional experiments with stressed animals on non-optimized CDF may complicate the results. Therefore a CDF capable of supporting cultured animals with similar overall performance as those grown on regular complex medium is essential. Experiments discussed in Chapter 4 described the development of a chemically defined food capable of culturing *Drosophila* over 30 generations, where flies fed on CDF at 400 K-cal/L have similar overall performance as flies grown on regular medium. Although larvae fed on this CDF showed some growth delay as most CDFs for *Drosophila*, most, if not all, measurements of adult performance are not affected. Thus, this CDF is adequate to use for nutritional studies in adult *Drosophila*.

ISCs reside in the unique interface between the internal and external environment - the gut epithelium, where nutrients are absorbed to supply the energy needed for the whole body. An important stimuli for the growth of the intestinal epithelium is known to be dietary nutrition (enteral nutrition) (Bragg et al., 1991). Total parenteral nutrition has been correlated with the atrophy and architectural remodeling of the gut mucosa (Groos et al., 2003). Both findings suggest that enteral nutrition regulates the homeostasis of the intestinal epithelium. Because ISCs function as fundamental machinery for the intestinal epithelium renewal, ISCs may alter their behavior in response to nutrients. However, the response of ISCs to nutrients is poorly described. Therefore, I propose to test the hypothesis that ISCs can respond to dietary changes at the level of specific nutrients or caloric densities to maintain gut homeostasis using CDF.

To generate new daughter cells, proliferating cells uptake more glucose and glutamine for the energy and metabolic needs (Muñoz-Pinedo et al., 2012). Synthetic pathways like the pentose phosphate pathway and glutaminolysis are utilized in proliferating cells to metabolize glucose and glutamine, which contribute to the synthesis of building blocks for new cells (Dang, 2012). Like most proliferating cells, cancer cells alter many signaling pathways to increase nutrient uptake to meet their metabolic requirements (Levine and Puzio-Kuter, 2010). Because we also developed a model for studying tumorigenic ISC lineages (Chapter 2 and 5.2), I propose to test the hypothesis
that tumorigenic ISCs can respond to dietary changes at the level of specific nutrients or caloric densities.

To determine the influence of dietary caloric densities in both normal and tumorigenic ISC lineages, I propose to use the double MARCM system to perform clonal analyses on flies fed with CDF at different caloric densities as mentioned in Chapter 5.2. Similar approaches can also be used to determine the influence of a specific dietary nutrient using CDFs at identical caloric densities with different amount of an individual nutrient as mentioned in Chapter 4. To facilitate the identification of important dietary nutrients required for the homeostasis of normal ISC lineages and the expansion of tumorigenic ISC lineages, I propose to use macronutrient deprived CDFs developed in Chapter 4 to determine which macronutrient plays the most important role in regulating behavior of each ISC lineage. One potential caveat for this approach is that flies cannot survive on carbohydrate deprived food for more than 4 days as shown in Chapter 4, thus this analysis cannot be conducted under the carbohydrate deprivation condition. Adding minimal nutrient for survival, 5% sucrose for example, to the experimental CDFs can be an alternative means to assess the effect of dietary carbohydrate on ISC behavior.

The knowledge learned from the above studies will provide new insights about which and how dietary nutrients influence the homeostasis and the expansion of normal and tumorigenic ISC lineages. Most importantly, the experiments described here can be used as a model to study the dependence or sensitivity of both normal and tumorigenic ISC lineages to specific nutrients or chemicals. Targeting the altered cellular metabolism emerges as a common strategy to improve cancer therapeutics (Cheong et al., 2012; Zhao et al., 2013). Identification of the difference in dependence or sensitivity of tumorigenic ISC lineages to specific nutrients or chemicals might provide new opportunities for the development of new cancer therapeutics.

5.4 Material and methods

5.4.1 The classic MARCM for clonal screen (pathway activation then inactivation in alphabetical order by pathway names):

    \[ y, w, hsFLP, UAS-GFP / +; UAS-EGFR / +; FRT^{82B} + / tub-Gal4, FRT^{82B} tub-Gal80. \]
y, w, hsFLP, UAS-GFP / +; UAS-EGFR / +; FRT<sup>82B</sup> APC<sup>Δ10</sup> APC<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-EGFR<sup>RNAi</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-EGFR<sup>RNAi</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Ci<sup>U</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Ci<sup>U</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Ci<sup>cell</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Ci<sup>cell</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-InRA<sup>1325D</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-InRK<sup>K1409A</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-InRK<sup>K1409A</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Socs<sup>RNAi</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Socs<sup>RNAi</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Stat<sup>RNAi</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Stat<sup>RNAi</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Puc<sup>RNAi</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Puc<sup>RNAi</sup> / +; FRT<sup>82B</sup> APC2<sup>Q10</sup> APC1<sup>Q8</sup> / tub-Gal4,
FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Bsk<sup>RNAi</sup> / +; FRT<sup>82B</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-BskRNAi / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Rheb / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Rheb / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-RaptorRNAi / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-RaptorRNAi / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-NotchRNAi / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-NotchRNAi / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-P53 / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-P53 / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-P53 RNAi / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-P53 RNAi / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UASp-Tkv / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UASp-Tkv / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-TkvRNAi / +; FRT62B + / tub-Gal4, FRT62B tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-TkvRNAi / +; FRT62B APC2g10 APC1Q8 / tub-Gal4,
FRT62B tub-Gal80.

5.4.2 The double MARCM:
y, w FRT<sup>19A</sup> / w, tub-Gal80, hsFLP, FRT<sup>19A</sup>; UAS-mCD8::GFP / +; FRT<sup>82B</sup> + / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

5.4.3a The modified twin-spot MARCM:

y, w FRT<sup>19A</sup> / w, tub-Gal80, hsFLP, FRT<sup>19A</sup>; UAS-mCD8::GFP / +; FRT<sup>82B</sup> APC2<sup>g10</sup> APC1<sup>Q8</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w FRT<sup>19A</sup> / w, tub-Gal80, hsFLP, FRT<sup>19A</sup>; UAS-mCD8::GFP / +; FRT<sup>82B</sup> APC2<sup>g10</sup> APC1<sup>Q8</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w FRT<sup>19A</sup> / w, tub-Gal80, hsFLP, FRT<sup>19A</sup>; UAS-mCD8::GFP / UAS-InR<sup>A1325D</sup>, FRT<sup>82B</sup> + /

tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w FRT<sup>19A</sup> / w, tub-Gal80, hsFLP, FRT<sup>19A</sup>; UAS-mCD8::GFP / UAS-InR<sup>A1325D</sup>, FRT<sup>82B</sup> APC2<sup>g10</sup> APC1<sup>Q8</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

5.4.3b The APC<sup>1Q8</sup>o twin-spot MARCM:

y, w, hsFLP / +; UAS-CD8::GFP UAS-miCD2 FRT<sup>40A</sup> / UAS-CDF::RFP UAS-miGFP FRT<sup>40A</sup>, FRT<sup>82B</sup> + / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP / +; UAS-CD8::GFP UAS-miCD2 FRT<sup>40A</sup> / UAS-CDF::RFP UAS-miGFP FRT<sup>40A</sup>, FRT<sup>82B</sup> APC2<sup>g10</sup> APC1<sup>Q8</sup> / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP / UAS-InR<sup>act</sup>; UAS-CD8::GFP UAS-miCD2 FRT<sup>40A</sup> / UAS-CDF::RFP UAS-miGFP FRT<sup>40A</sup>, FRT<sup>82B</sup> + / tub-Gal4, FRT<sup>82B</sup> tub-Gal80.

y, w, hsFLP / UAS-InR<sup>act</sup>; UAS-CD8::GFP UAS-miCD2 FRT<sup>40A</sup> / UAS-CDF::RFP UAS-miGFP FRT<sup>40A</sup>, FRT<sup>82B</sup> APC2<sup>g10</sup> APC1<sup>Q8</sup> /
tub-Gal4, FRT<sup>82B</sup> tub-Gal80.
y, w, hsFLP / UAS-InRact; UAS-CD8::GFP UAS-miCD2 FRT40A /
UAS-CDF::RFP UAS-miGFP FRT40A; tub-Gal4, APC1Q8 / APC1Q8.

5.4.4 esg-LacZ; MARCM:
y, w, hsFLP, UAS-GFP / +; esg-LacZ / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InR\textsuperscript{A1325D} / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InR\textsuperscript{A1325D} / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.

5.4.5a MARCM for Insulin signaling component sufficiency screen:
y, w, hsFLP, UAS-GFP / +; UAS-PI3K / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-PI3K / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Ras\textsuperscript{act} / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Ras\textsuperscript{act} / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-PTEN\textsuperscript{RNAi} / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-PTEN\textsuperscript{RNAi} / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Akt\textsuperscript{act} / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Akt\textsuperscript{act} / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Rheb / +; FRT\textsuperscript{82B} + / tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Rheb / +; FRT\textsuperscript{82B} APC2\textsuperscript{910} APC1Q8 / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-S6K^{STDTE} / +; FRT^{82B} + / tub-Gal4, FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-S6K^{STDTE} / +; FRT^{82B} APC2^{g10} APC1^{Q8} / tub-Gal4,
FRT^{82B} tub-Gal80.

5.4.5b MARCM for Insulin signaling component necessity screen: (PI3K as an example)
y, w, hsFLP, UAS-GFP / +; UAS-PI3K^{RNAi} / +; FRT^{82B} + / tub-Gal4, FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-PI3K^{RNAi} / +; FRT^{82B} APC2^{g10} APC1^{Q8} / tub-Gal4,
FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-PI3K^{RNAi} / UAS-InR^{A1325D}; FRT^{82B} + / tub-Gal4,
FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-PI3K^{RNAi} / UAS-InR^{A1325D}; FRT^{82B} APC2^{g10} APC1^{Q8} / 
tub-Gal4, FRT^{82B} tub-Gal80.

5.4.6 MARCM for mechanism of cell competition:
y, w, hsFLP, UAS-GFP / +; esg-LacZ / UAS-Myc^{RNAi}; FRT^{82B} + / tub-Gal4, FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ / UAS-Myc^{RNAi}; FRT^{82B} APC2^{g10} APC1^{Q8} / tub-Gal4,
FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InR^{A1325D} / UAS-Myc^{RNAi}; FRT^{82B} + / tub-Gal4,
FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InR^{A1325D} / UAS-Myc^{RNAi}; FRT^{82B} APC2^{g10} APC1^{Q8} / 
tub-Gal4, FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InR^{A1325D} / UAS-Myc^{RNAi}; FRT^{82B} + / tub-Gal4,
FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InR^{A1325D} / UAS-Myc^{RNAi}; FRT^{82B} APC2^{g10} APC1^{Q8} / 
tub-Gal4, FRT^{82B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; esg-LacZ / UAS-MycRNAi; FRT82B + / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; esg-LacZ / UAS-MycRNAi; FRT82B APC2\textsuperscript{g10} APC1\textsuperscript{Q8} / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InRA1325D / UAS-MycRNAi; FRT82B + / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; esg-LacZ, UAS-InRA1325D / UAS-MycRNAi; FRT82B APC2\textsuperscript{g10} APC1\textsuperscript{Q8} / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; esg-LacZ / UAS-NotumRNAi; FRT82B + / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; esg-LacZ / UAS-NotumRNAi; FRT82B APC2\textsuperscript{g10} APC1\textsuperscript{Q8} / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; Upd-LacZ / UAS-MycRNAi; FRT82B + / tub-Gal4, FRT82B tub-Gal80.

5.4.7a Upd-LacZ; MARCM:

y, w, hsFLP, UAS-GFP / +; Upd-LacZ / +; FRT82B + / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; Upd-LacZ / +; FRT82B APC2\textsuperscript{g10} APC1\textsuperscript{Q8} / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; Upd-LacZ / UAS-InRA1325D; FRT82B + / tub-Gal4, FRT82B tub-Gal80.

y, w, hsFLP, UAS-GFP / +; Upd-LacZ/ UAS-InRA1325D; FRT82B APC2\textsuperscript{g10} APC1\textsuperscript{Q8} / tub-Gal4, FRT82B tub-Gal80.

5.4.7b Double MARCM for cytokine sufficiency analysis:

y, w FRT19A / w, tub-Gal80, hsFLP, FRT19A; UAS-mCD8::GFP / UAS-Upd; FRT82B + / tub-Gal4, FRT82B tub-Gal80.
y, w FRT\textsuperscript{19A} / w, tub-Gal80, hsFLP, FRT\textsuperscript{19A}; UAS-mCD8::GFP / UAS-Upd; FRT\textsuperscript{62B} APC2\textsuperscript{10} APC1\textsuperscript{Q8} / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w FRT\textsuperscript{19A} / w, tub-Gal80, hsFLP, FRT\textsuperscript{19A}; UAS-mCD8::GFP, UAS-\textit{InR}\textsuperscript{K1409A} / UAS-Upd; FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

5.4.7c Classic MARCM for cytokine necessity analysis:

y, w hsFLP, UAS-GFP / +; UAS-Upd\textsuperscript{RNAi} / +; FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w hsFLP, UAS-GFP / +; UAS-Upd\textsuperscript{RNAi} / +; FRT\textsuperscript{62B} APC2\textsuperscript{10} APC1\textsuperscript{Q8} / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w hsFLP, UAS-GFP / +; UAS-Upd\textsuperscript{RNAi} / UAS-\textit{InR}\textsuperscript{A1325D}, FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w hsFLP, UAS-GFP / +; UAS-Upd\textsuperscript{RNAi} / UAS-\textit{InR}\textsuperscript{A1325D}, FRT\textsuperscript{62B} APC2\textsuperscript{10} APC1\textsuperscript{Q8} / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w hsFLP, UAS-GFP / +; UAS-Upd\textsuperscript{RNAi} / UAS-\textit{Socs} RNAi; FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

5.4.7d Double MARCM for JAK/STAT sufficiency analysis:

y, w FRT\textsuperscript{19A} / w, tub-Gal80, hsFLP, FRT\textsuperscript{19A}; UAS-mCD8::GFP / UAS-\textit{Socs} RNAi; FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w FRT\textsuperscript{19A} / w, tub-Gal80, hsFLP, FRT\textsuperscript{19A}; UAS-mCD8::GFP / UAS-\textit{Socs} RNAi, FRT\textsuperscript{62B} APC2\textsuperscript{10} APC1\textsuperscript{Q8} / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w FRT\textsuperscript{19A} / w, tub-Gal80, hsFLP, FRT\textsuperscript{19A}; UAS-mCD8::GFP, UAS-\textit{InR}\textsuperscript{K1409A} / UAS-\textit{Socs} RNAi; FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

y, w FRT\textsuperscript{19A} / w, tub-Gal80, hsFLP, FRT\textsuperscript{19A}; UAS-mCD8::GFP, UAS-\textit{InR}\textsuperscript{K1409A} / UAS-\textit{Socs} RNAi, FRT\textsuperscript{62B} APC2\textsuperscript{10} APC1\textsuperscript{Q8} / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.

5.4.7e Classic MARCM for JAK/STST necessity analysis:

y, w hsFLP, UAS-GFP / +; UAS-\textit{Stat} RNAi / +; FRT\textsuperscript{62B} + / tub-Gal4, FRT\textsuperscript{62B} tub-Gal80.
y, w, hsFLP, UAS-GFP / +; UAS-Stat\textsuperscript{RNAi} / +; FRT\textsuperscript{82B} APC\textsuperscript{2010} APC\textsuperscript{1Q8} / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Stat\textsuperscript{RNAi} / UAS-In\textsuperscript{A1325D}; FRT\textsuperscript{82B} + / tub-Gal4,
FRT\textsuperscript{82B} tub-Gal80.

y, w, hsFLP, UAS-GFP / +; UAS-Stat\textsuperscript{RNAi} / UAS-In\textsuperscript{A1325D}; FRT\textsuperscript{82B} APC\textsuperscript{2010} APC\textsuperscript{1Q8} /
tub-Gal4, FRT\textsuperscript{82B} tub-Gal80.
Figure 5.1: Clonal activation of the Insulin signaling pathway promotes the expansion of
\textit{APC}\textsuperscript{lof} lineages. (A). Migduts with Insulin activation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Migduts with Insulin activation and \textit{APC}\textsuperscript{lof} clones at 1 week
(B1), 2 week (B2), and 4 week (B3) after clone induction. $\text{ins}^{\text{act}}$: Insulin receptor constitutively activative form expression construct.
Figure 5.2: Clonal activation of the EGF signaling pathway promotes the expansion of \( \textit{APC}^{\text{lof}} \) lineages. (A). Midguts with EGF activation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Midguts with EGF activation and \( \textit{APC}^{\text{lof}} \) clones at 1 week (B1), 2 week (B2), and 4 week (B3) after clone induction. \( \text{egfr}^{\text{wt}} \): EGF receptor wild type isoform 1 expression construct.
Figure 5.3: Clonal inactivation of the Notch signaling pathway promotes the expansion of 

**APC^{lof} lineages.** (A). Midguts with Notch inactivation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Midguts with Notch inactivation and **APC^{lof}** clones at 1 week (B1), 2 week (B2), and 4 week (B3) after clone induction. **notch^{RNAi}:** Notch receptor RNA interference construct.
Figure 5.4: Clonal inactivation of the Insulin signaling pathway suppresses the expansion of APC<sup>lof</sup> lineages. (A). Midguts with Insulin inactivation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Midguts with Insulin inactivation and APC<sup>lof</sup> clones at 1 week (B1), 2 week (B2), and 4 week (B3) after clone induction. inr<sup>dn</sup>: Insulin receptor dominant negative form expression construct.
Figure 5.5: Clonal inactivation of the EGF signaling pathway suppresses the expansion of APC<sup>lof</sup> lineages. (A). Midguts with EGF inactivation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Midguts with EGF inactivation and APC<sup>lof</sup> clones at 1 week (B1), 2 week (B2), and 4 week (B3) after clone induction. egfr<sup>RNAi</sup>: EGF receptor RNA interference construct.
Figure 5.6: Clonal activation of the Notch signaling pathway suppresses the expansion of

*APC*<sup>lof</sup> lineages. (A). Midguts with Notch activation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Midguts with Notch activation and *APC*<sup>lof</sup> clones at 1 week (B1), 2 week (B2), and 4 week (B3) after clone induction. *notch*<sup>intrac</sup>: Notch receptor intracellular domain expression construct.
Figure 5.7: Comparison of the classic MARCM and the double MARCM systems. (A) In the classic MARCM system, the clonal labeling event requires an induced recombination between a pair of homozygous chromosomal arms (dark blue and light blue). (B) In the double MARCM system, both induced recombination events in each pair of homozygous chromosomal arms (dark blue and light blue; dark purple and light purple) are required to generate a labeling cell. Although a labeled cell in the double MARCM system is also homozygous for gene-of-interest (GOI), not all homozygous GOI cells are labeled. Only homozygous GOI cells are shown in (B) for simplicity.
Figure 5.8: Comparison of induced clones using the classic MARCM and the double MARCM systems. (A-D) Midguts with classic MARCM clones at 2 week after clone induction. (E-H) Midguts with double MARCM clones at 20 days after clone induction. (A, E) Wild-type clones. (B, F) APC loss-of-function clones. (C, G) Insulin activation clones. (D, H) Insulin activation and APC loss-of-function clones.
Figure 5.9: Clonal inactivation of Stat92E suppresses the expansion of $APC^{l o f}$ lineages. (A).
Midguts with STAT92E inactivation clones at 1 week (A1), 2 week (A2), and 4 week (A3) after clone induction. (B). Midguts with STAT92E inactivation and $APC^{l o f}$ clones at 1 week (B1), 2 week (B2), and 4 week (B3) after clone induction. $stat^{RNAi}$: STAT92E RNA interference construct.
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2006 – Present Ph.D. in Developmental, Regenerative and Stem Cell, Biology, expected Aug 2013
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1998 – 2000 Masters in Fisheries Science
Laboratory of Dr. Huai-Jen Tsai, National Taiwan University, Taipei, Taiwan
Thesis: Molecular Structure, Dynamic Expression and Promoter Analysis of Zebrafish (Danio rerio) Muscle Regulatory Factor, Myf-5

1994 – 1998 B.S. in Biology
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RESEARCH EXPERIENCE:

2006 – Present Graduate Student, Laboratory of Dr. Craig Micchelli
Department of Developmental Biology, Washington University in St. Louis, United States
• Established the roles of Apc and Stat92E in regulating intestinal stem cell properties.
• Developed the chemically defined food for nutritional studies in Drosophila.
• Current experiments are investigating the roles major signaling pathways for the expansion of the tumorigenic intestinal stem cells and dissecting the nutritional requirement for the tumorigenic and normal intestinal stem cells.

2005 – 2006 Research Assistant, Laboratory of Dr. Cheng-Chen Huang
Institute of Cellular and Organismic Biology, Academia Sinica, Taiwan
• Developed a drug screen method for inhibiting vasculogenesis in Zebrafish.
• Assisted in fishroom setup.

2005 – 2006 Research Assistant, Laboratory of Dr. Chun-Che Chang
Department of Entomology, National Taiwan University, Taiwan
• Investigated the role of Vasa and Nanos in germline development in pea Aphids.
• Assisted in the development of the in situ hybridization and immunofluorescence staining protocol for pea Aphids.

2000 – 2005 Research Assistant for Military Service, Laboratory of Dr. Bon-Chu Chung
Institute of Molecular Biology, Academia Sinica, Taiwan
• Fishroom manager.
• Established and investigated the roles of orphan nuclear receptor 5A family members for the slow muscle, gonad, and brain development in Zebrafish.
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1998 – 2000 Graduate Student, Laboratory of Dr. Huai-Jen Tsai
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• Cloned and established the roles of muscle regulatory factor genes in Zebrafish.
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HONORS AND AWARDS

2000  Dean’s Prize of the College of Science, National Taiwan University

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