5-2016

Integrity of the Barrier Epithelium in Drosophila Gut is Mediated by Factors That Regulate Intestinal Stem Cell Activity

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<https://doi.org/10.33015/dominican.edu/2016.bio.10>

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Kinion, Adam A., "Integrity of the Barrier Epithelium in Drosophila Gut is Mediated by Factors That Regulate Intestinal Stem Cell Activity" (2016). *Graduate Master's Theses, Capstones, and Culminating Projects*. 235.
<https://doi.org/10.33015/dominican.edu/2016.bio.10>

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**Integrity of The Barrier Epithelium in *Drosophila* Gut is Mediated
by Factors That Regulate Intestinal Stem Cell Activity**

By

Adam Ashton Kinion

A culminating thesis submitted to the faculty of Dominican University of California and the
Buck Institute for Research on Aging in partial fulfillment of the requirements for the degree

Master of Science in Biology

San Rafael, California

August 2016

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Buck Institute for Research on Aging – Dominican University of California

Certification of approval

This thesis, written under the direction of the candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree Master of Science in Biology at Dominican University of California. The written content presented in this work represents the work of the candidate alone.

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Abstract

My thesis work on the *Drosophila* intestinal epithelium (IE) involved characterizing the mitochondrial unfolded protein response (UPR_{mt}), validating proliferation specific regulatory genes, and proposing gut barrier function—specific genes as regulators of commensal bacteria density. The results from this project have shown that intestinal stem cells (ISCs) respond systemically in the presence of autonomously induced, visceral muscle (VM)-localized UPR_{mt}. A phenotypic correlation between autonomous electron transport chain (ETC) dysfunction was observed in the visceral muscle and the systemic UPR_{mt} response of ISCs in the posterior midgut. Exploratively, two genome-wide association studies (GWAS) using the *Drosophila* genetic reference panel (DGRP) were performed, and genetic candidates with functionally unknown effects on both ISC proliferation and gut flora homeostasis in an aged invertebrate tissue were found. These candidate genes—Dunce (Dnc), Tfb1 (TF11H Homolog), and Arrowhead (Awh)—were validated to be genetic regulators of proliferative homeostasis with age. In addition, I proposed the genes Dystrophin (Dys) and Rolling Pebbles (Rols) to be mediators of microbial density relative to gut barrier integrity. Collectively, the work presented here has contributed to the fundamental understanding of regenerative biology and gerontology by demonstrating phenotypes of functionally novel, stem cell—specific genetic perturbations interpreted as regulatory factors of both ISC maintenance and activity.

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List of Abbreviations

- INTESTINAL EPITHELIUM – IE
- VISCERAL MUSCLE – VM
- ENTEROCYTE – EE
- INTESTINAL STEM CELL – ISC
- ENTEROENDOCRINE – EE
- ENTEROBLAST – EB
- *DROSOPHILA* GENETIC REFERENCE PANEL – DGRP
- GENOME WIDE ASSOCIATION STUDY – GWAS
- COLONY FORMING UNITS – CFU
- PHOSPHO-HISTONE 3 – PH3
- DYSTROPHIN – DYS
- ROLLING PEBBLES – ROLS
- ARROWHEAD – AWH
- MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE – UPRMT
- ADENOSINE TRI-PHOSPHATE – ATP
- ADENOSINE MONO-PHOSPHATE - AMP
- MOSAIC ANALYSIS OF REPRESSIBLE CELL MARKER – MARCM
- POSTERIOR MIDGUT – PM
- HINDGUT – HG
- ANTERIOR MIDGUT – AM
- *DROSOPHILA* INSULIN LIKE PEPTIDE - DILP
- VEIN - VN
- WINGLESS - WG
- OVER EXPRESSION – OE
- KNOCKDOWN – KD
- KNOCK OUT – KO
- ELECTRON TRANSPORT CHAIN – ETC
- DEOXYRIBONUCLEIC ACID – DNA
- RIBONUCLEIC ACID – RNA
- REAL TIME POLYMERASE CHAIN REACTION – RT-PCR
- ROOM TEMPERATURE – RT

Thanks & Acknowledgements

Dr. Heinrich Jasper, Dr. Maggie Louie, Dr. Emily Willingham

Rachel B. Brem, Walter Shine, Graciela Maria Carranza, Kazutaka Akagi, Kenny Wilson, Mauricio Ortega, Subhash D. Katewa, Jesse Simons, Sven Lang, Pankaj Kapahi, Joanne Van Kampen, Evelyn Crivello, Hai Lu, Imilce Rodriguez-Fernandez, Hansong Deng, Hongjie Li, Devon Haiges, Suzy Jackson, Yanyan Qi, Rebecca Riley, Samantha Haller, Daniel Hu, Mia Konjikusic, Pedro Victor-Souza, Nancy Barnett, Alexis Valdovinos, Ethan Annis, Regina Thomas, Robert Frazer, Paul Kitagawa, Scott Mulligan, Jennifer Beck, Herbert Alexander Kinion Sr, Daniel F. Barrett, Evelyn Britton, Vernon Peppers, Kristylea Ojeda, Randall Hall, Casey Halcro, Pamela Santiago, Hongye Zou, LinLin Guo, Lifen Wang, Alexander Rifkind, Kandyce Hamilton, Tom Starr,

Valerie Wynne Kinion

Background

Overview of Aging and Regeneration

Gerontology, the study of aging, focuses on the naturally progressive deregulation of energy metabolism, genomic stability, and tissue homeostasis in adult organisms (Karpac, 2013). A particularly interesting area of current research is the exploration of the causes of regenerative decline in many adult tissues. Molecular and genetic studies in model organisms have shown that tissue-resident stem cells are maintained and regulated by a variety of conserved longevity pathways (Biteau B. , 2011) (Garcia-Prat, 2016) (Juhyun, 2014).

Drosophila melanogaster has been used a genetic model system to understand the decline of self-renewal and differentiation of stem cells in the intestinal epithelium with age. Critical functions of the intestinal epithelium, including its barrier function, decline with age. This has warranted further investigation of tissue-specific stem cells, and their de-regulation in the aging organism (Wang L. , 2014) (Seaberg, 2003). Regeneration of the *Drosophila* gut epithelium is regulated by endogenously secreted factors, and these factors have been extensively studied as mediators of tissue integrity (Biteau B. , 2011) (Carlson, 2007) (Rera, 2011). Genetic interventions aimed at modulating such paracrine and systemic signals may promote homeostasis and thus deter the onset of age-related diseases (Tuch, 2006) (Oh, 2014) (Langer R, 1993) (Scopelliti, 2014).

Electron Transport Chain (ETC) Dysfunction

Mitochondria responses to changes in electron transport chain (ETC) activity have been shown to promote longevity (Dillin, 2002). Over a species' lifespan, the dysfunctional ETC-driven loss in metabolic homeostasis is in part due to the inability of mitochondria to

manage ATP output (Martinus, 1996) (Broderick, 2014) (Durieux, 2011). Cellular aging studies show that total ATP output of oxidative phosphorylation slowly declines with age (Green RA1, 2011). Studies have also shown that an accumulation of ROS happens with age, a phenomenon known as the “free radical theory” (Paula Ludovico1, 2015). This theory considers the implications of ROS accumulation in the context of proteostasis or the improper folding of proteins in the cytosol and mitochondria. The proper folding of a given protein is mediated by chaperones; however, the ability of chaperones to maintain this responsibility diminishes with age. As a result, cells induce peptide degradation to properly remove the unfolded or misfolded proteins; further, the accumulation of misfolded proteins may then lead to the aggregations shown to drive age related diseases. Currently, little is known about the role of mitochondrial proteostasis in stem cell homeostasis and how these changes in mitochondria affect a tissue’s regenerative capacity. A potential role for mitochondria is managing stem cell death once the stem cell’s genomic profile has accumulated a certain number of mutations (Lu W, 2016).

The Mitochondrial Unfolded Protein Response (UPRmt)

Mitochondrial stress initiates essential cellular activity necessary for managing cellular proteostasis and depends on the up-regulation of chaperones and proteases (Castro P. d., 2012) (Pellegrino MW, 2012). Previous work has shown that the induction of the mitochondrial unfolded Protein response (UPRmt) during development has profound effects on the lifespan of *C. elegans* (Dillin, 2002). More specifically, heat shock protein-60 (hsp60) expression is required for the lifespan extension shown in *C. elegans* (Dillin, 2002) (Houtkooper, 2013). The up-regulation of heat shock proteins by inducing UPRmt is conserved among the fly, worm, and mammalian models, which suggests that more

biologically conserved factors may mediate longevity (Walker, 2013) (Rauthan, 2013). The role of the UPR_{mt} in mediating longevity has been speculated to result from reduced respiration during developmental stages (Dillin, 2002).

Paracrine signals can promote cellular and mitochondrial proteostasis by mediating the removal of dysfunctional proteins (Scopelliti A, 2014). In moderation, mitochondrial stress initiates tissue regeneration, but extreme dysfunction may promote other diseases like cancer (Castro P. d., 2012) (Garcia-Prat, 2016). Tumor cells exhibit a strong reduction in proper protein folding, supporting the speculation that the unfolded protein response may be acting as a tumor suppressor (Pellegrino MW, 2012). Improperly translated peptides serve as mediators of signal transduction and as systemic regulators of endogenous UPR_{mt} (Pellegrino MW, 2012). Systemic regulation stimulates longevity, and several studies suggest that this stimulation is, in part, due to an improvement in targeting gene expression necessary for cell-to-cell communication, which has been shown to mediate cell fate (Andrea H. Brand, 1993) (Gaurav, 2007). Understanding how mitochondrial function influences stem cell maintenance will contribute to further understanding of the natural regenerative capacity of tissues (Figs. 1, 2). The systemic effects of the UPR_{mt} in stem cell maintenance—and therefore in aging—are largely unknown across model systems.

Endogenous Regulation of Intestinal Stem Cell Activity

Stem cells are multipotent and serve as niche-sensitive sources for tissue regeneration in both simple and complex organ systems. Each tissue harbors a distinct niche necessary for promoting the self-renewal of the specific stem cell population (Tuch, 2006). Tissue regeneration requires stem cells that actively self-renew and differentiate in response to changes in the niche environment; therefore, studies targeting the endogenous regulation of

stem cell activity in the context of promoting a tissue's regenerative capacity are necessary. The fly intestinal epithelium has 5 distinct cell types communicating by paracrine signaling to maintain the overall integrity of the epithelia lining. Intestinal stem cells (ISCs) are the only actively dividing cells in the fly gut and become more active as the animal ages (Claesson, 2011). Under various stress conditions, disruption of ISC function is a naturally occurring phenomenon observed in aged flies (Biteau B. , 2010) (Juhyun, 2014). ISCs typically receive signals for activating asymmetric and symmetric divisions. Symmetric divisions maintain the stem cell population, while asymmetric divisions yield differentiated cells that allow for self-renewal of the epithelium. The typical "cell fate" of ISCs in the gut epithelium is to differentiate into enteroblasts (EB) then into enteroendocrine cells (EE) or enterocyte (ECs), which is dependent on paracrine signaling. ISCs remain at the basement membrane adjacent to the visceral muscle, next to the progenitor cells and enterocytes and beneath the enteroendocrine cells of the epithelia. Furthermore, studies have shown that vein (vn), wingless (wg), and dilp3 are the primary secreted factors from the visceral muscle to the ISCs that regulate this differentiation process. Slit is another factor secreted by EEs that influences ISC terminal differentiation (Biteau B. e., 2014) (Fig 1).

Gastrointestinal Commensal Bacteria Homeostasis

Proper breakdown and absorption of the minerals and nutrients is the host may depend on the gut flora (Chaston, 2016). Total cell count, the rate of fission, and the percent composition of a specific species' gut flora changes as the host ages, yet it is unclear whether the human gut flora is a regulator for gastrointestinal diseases (Broderick, 2014). Dysbiosis is a loss in gut flora homeostasis and has been most extensively studied in the fly gut (Chaston, 2016). In humans, dysbiosis develops slowly and influences the onset of inflammatory bowel disease, obesity, and diabetes (Clemente, 2012) (Guo, 2013). Recent human studies

comparing the microbiome of older to young individuals have revealed that the older individuals have higher microbial diversity relative to the younger ones (Claesson, 2011).

Goals of My Thesis Project

The concept that genomic stability loss with age drives disease development is an overarching paradigm in the field of regenerative biology. In my studies, I explored the foundation of this biological paradigm with a highly regenerative and accessible tissue. The goals of this project included: aim 1) to identify a phenotype for the systemic effects of UPRmt on the ISCs and determine if this phenotype is capable of mimicking the aging phenotype of hyperplasia in a young gut; aim 2) to identify genes involved in managing proliferative homeostasis in an aging tissue; and aim 3) to identify genes associated with the loss in commensal bacteria homeostasis by analyzing colony-forming units in relation to both age and genetic variation.

Figure 1 | Stress Signal–Secreted Factors from Other Cell Types to the ISCs Joined with a Cell Lineage
Adapted from (Biteau B. , 2011) (Hochmuth, 2011). Enterocyte (EC), visceral muscle (VM), enteroendocrine (EE), insulin-producing tissues, and enteroblasts (EB) each secrete cell-specific substrates that interact with ISCs, activating self-renewal and differentiating pathways. ECs release cytokines, EEs secrete slit, the VM secretes Vein and wingless and dilp3 peptides, and Drosophila insulin-like peptide also can activate a quiescent ISC. Active ISCs remain active when Delta is high, differentiate into EBs when notch is high, and become EEs when notch levels are low.

Figure 2 | Characterized Mitochondrial Cell Autonomous and Systemic UPRmt Suggests a Paracrine Phenotype. Adapted from (Jensen, 2014). A cartoon UPRmt as an autonomous positive regulator of nuclear chaperone and protease expression, mitophagy, and inhibition of translation. Systemic (non-autonomous) UPRmt elevates chaperone expression and stimulates FoxO pathway. Non-autonomous UPRmt eliciting a systemic response from paracrine signaling between the visceral muscle and the intestinal stem cells has not been characterized.

Materials & Methods

Stock Food Recipe

The recipe for stock fly food is g/1: 5 L Water, Agar = 20, Molasses = 30, Malt = 98, Yeast = 27, Corn Flower = 127, Soy Flower = 15, PA = 9.4 ml, MP EtOH = 3 g/11 ml

Mating and Genotypes

Mitochondrial Unfolded Protein Response

; Da-Gs; DeltaOTC/MKRS X w; gstD1::LacZ/CYO; Dr/TM3
w / yw; tubGal80ts / CyO; HovGal4, UAS-CD8GFP / TM6.b X;; dOTC/Tm6b
w / yw; tubGal80ts / CyO; HovGal4, UAS-CD8GFP / TM6.b X W1118
w / yw; tubGal80ts / CyO; HovGal4, UAS-CD8GFP / TM6.b X ; ND75; RNAi
w; esglacZ{sw}, tubG80ts / CyO; HovG4/TM3 X; ND75 RNAi;
w; esglacZ{sw}, tubG80ts / CyO; HovG4/TM3 X W1118

Dunce

w; tub::G80ts / CYO; Hov::Gal4, UAS::CD8-GFP/TM6.b X y1 v1;; P {TRiP.GL01226}attP2/TM3.Sb1
w; ,tub::G80ts / CYO; Hov::Gal4, UAS::CD8-GFP/TM6.b X W1118
;5961/CYO; X y1 v1 ;; P{TRiP.GL01226}attP2/TM3.Sb1
; Su (H)::GBE, UAS::CD8-GFP/CyO; Tub::G80ts/TM3 X y1 v1;; P{TRiP.GL01226}attP2/TM3.Sb1
; Su (H)::GBE,UAS::CD8-GFP/CyO; Tub::G80ts/TM3 X W1118
; Esg::Gal4/CyO; Su (H)::G80, tub::G80ts/tm3.sb X y1 v1;; P {TRiP.GL01226} attP2/TM3.Sb1
; Esg::Gal4/CyO; Su (H)::G80, tub::G80ts/tm3.sb X W1118
;5961/CYO; X y1 v1 ;; P{TRiP.GL01226}attP2/TM3.Sb1
MARCM19a (hsFLP-FRT19, Tub::Gal80, UAS::CD8-GFP) X y1 w1118 dnc221b
P{neoFRT}19A/FM6:
MARCM19a (hsFLP-FRT19, Tub::Gal80; UAS::CD8-GFP) X neo-FRT19a

Arrowhead

; *Esg::Gal4/CyO; Su (H)::G80, tub::G80ts/tm3.sb X W1118*

; *Esg::Gal4/CyO; Su (H)::G80, tub::G80ts/tm3.sb X y1 v1; P {TRiP.JF02403} attP2*

; *5961gs/CyO; X y1 v1; P {TRiP.JF02403} attP2*

(*MARCM2A*) *hsFLP, UAS::CD8-GFP, Act::Gal4/FM6;;FRT2A,Tub::Gal80/Tm3 X y1w*;;*
P{FRT(wbs)}2AAwb16/TM6b,Tb1

(*MARCM2A*) *hsFLP, UAS::CD8-GFP, Act::Gal4/FM6;;FRT2A, Tub::Gal80/Tm3 X yw;;FRT2A,*
TubG80/TM3

; *NP1::G4/Tub::Gal80ts; X y1 v1; P {TRiP.JF02403}attP2 FLIP 1*

; *NP1::G4/Tub::Gal80ts; X W1118 FLIP 1*

w / yw; tubGal80ts / CyO; HowGal4, UAS-CD8GFP / TM6, tb X y1 v1; P {TRiP.JF02403} attP2 FLIP
1

w / yw; tubGal80ts / CyO; HowGal4, UAS-CD8GFP / TM6, tb X W1118 FLIP 1

Tfb1

; *Da-GS; X y1 w67c23; P{EPgy2} Tfb1EY02347/CyO*

; *Da-GS; X y1sc*v; P {TRiP.HMC04714} attP40;*

*w; 5961-GS/CyO; X y1sc*v; P {TRiP.HMC04714} attP40;*

w; 5961-GS/CyO; X y1 w67c23; P{EPgy2} Tfb1EY02347/CyO

; *Su (H)::GBE, UAS::CD8-GFP/CyO; Tub::G80ts/TM3 X y1sc*v; P{TRiP.HMC04714} attP40;*

; *Su (H)::GBE, UAS::CD8-GFP/CyO; Tub::G80ts/TM3 X W1118*

; *Su (H)::GBE, UAS::CD8-GFP/CyO; Tub::G80ts/TM3; X y1 w67c23; P{EPgy2}Tfb1EY02347/CyO*

; *NP1::Gal4/Tub::G80ts; X y1sc*v; P{TRiP.HMC04714} attP40; FLIP 1*

; *NP1::Gal4/Tub::G80ts; X W1118 FLIP 1*

*w; tub::Gal80-ts / CyO; How::Gal4, UAS::CD8-GFP/TM3 X y1sc*v; P {TRiP.HMC04714} attP40;*

w; ,tub::Gal80-ts / CyO ; How::Gal4, UAS::CD8-GFP/TM3 X W1118

Dystrophin

; *5966-gs; X w1; P{EP}DysEP3397/TM6B, Tb1*

Rols

;5966-gs; Xy1 v1; P {TRiP.HMJ22326} attP40

; DA-Gs; Xy1 v1; P {TRiP.HMJ22326} attP40

Nutrient-rich Ru486 Food Recipe for Drug Inducible Gal4

Ad libitum feeding was provided as a food composition of 85% water, 8.5% cornmeal, 5% sugar, 5% yeast extract, and 0.46% agar (Camarella, Investigating the Interactions between Circadian Rhythms and Dietary Restrictions, 2014).

Fly Husbandry and Genotypes

All crosses were maintained on stock food recipe media. Female progeny used for experiments were taken at 3–4 days post eclosion unless indicated otherwise. Posterior midguts or whole guts were analyzed in assays for statistical significance

Transgenic Activation by a Temperature-inducible Expression System

Crosses with TubGal80ts insertion was generated at 18 °C . Drug-inducible drivers were generated at 25 °C . Expression of transgenic lines was used with the Gal4/Gal80/Gal80ts system. The animals used in the studies were reared and aged at 18 °C . Once animals reach 3–5 days of adulthood, half of the population was then moved to 29 °C, allowing the Gal4 system to activate to express the transgenes via UAS (Arshad Ayyaz, 2015).

Clonal Analysis

The MARCM (mosaic analysis of repressible cell marker) system was used to characterize lineage-dependent phenotypes. The crosses were reared and maintained at 25°C for three to five days of adulthood to reach maturity. For post-selection of genotypes, the flies were heat

shocked for 45 minutes at 37°C if MARCM2a and twice if MARCM19a, and then returned to 25°C (Lee T, 2001).

Lifespans

Flies for the lifespan assay were maintained at 25 °C in an incubator controlling circadian rhythms by 12/12 light–dark cycles. The progeny used for assays were extracted from flies maintained with the standard fly husbandry protocol. Day 0 of the lifespan is at approximately 14 days after the parent generation is crossed and has completed mating.

Quantifications and Statistics: (Adapted from (Scopelliti A, 2014)

Total Clonal Analysis

Clonal analysis was first done by counting the number of colonies in the entire gut for each group. A clone was defined as three or more GFP+ cells adjacent to one another. Analyzing the size of the clones was done by counting each individual GFP+ cell in a clone located in the gut previously used quantify the amount of clones per gut. These principles are used in the both the experiment and the control.

Clone Composition Analysis

Staining for delta and Prospero was achieved with DAPI (4',6-diamidino-2-phenylindole), a fluorescent molecule with a high affinity for adenine–thymine rich regions. Clone composition was analyzed between snapshots of two samples using ImageJ or Zen software. Thirty clones were counted for at least five different guts in one sample. The overall goal was to count all of them without bias.

Clone Size Analysis

The size of clones was evaluated by imaging areas of the guts extracted from the experiments, and then by counting the number of GFP+ cells in a single area. The amount of clones counted in the experiment group is statistically compared to the control group.

Quantification of EE+, DI+, PH3+, β -gal+ Cells Relative to DAPI

Delta positive cells were normalized to DAPI-positive cells in a given three areas of the posterior midgut and averaged with 7–15 other animals from the same experiment.

Quantification of Transcription Levels

Relative mRNA levels were normalized to actin and RP49 by dividing the desired transcript levels by the mRNA levels of controls. These controls were chosen because they are ubiquitously expressed.

Statistical Analysis

The data sets were analyzed for statistical significance using Microsoft Excel version 16.0.6769.2040. The graphs corresponding to the data sets were also generated using Excel, and two-tailed t-tests were used to calculate the p values.

mRNA Analysis: Adapted from Jasper Lab Protocol

To analyze the fly gut transcriptome, gut tissue was collected in 1 ml trizol (5–10 thoraces / 3–6 whole flies) on ice and homogenized with a mechanical mortar and pestle. Samples were extracted by adding 1/5 volume (200 μ l) of chloroform and centrifuged for 15 min at 12,000 $\times g$ at 4°C. The aqueous phase (top layer) was removed and placed into a new tube. A total of 500 μ l of isopropanol (1/2 of the initial trizol volume) was added, mixed, and incubated for 15

min at room temperature. Samples were centrifuged for 10 min at $12,000 \times g$ at 4°C. RNA pellets were washed in 70% ethanol, dried, and suspended in ~30–50 µl of RNAase free water. RNA was stored at -20°C for up 2 months or -80°C for 4 months. mRNA was transcribed into cDNA prior to PCR analysis with gene specific primers.

ECC15 Infection Assay for Proliferation Induction

To complete an infection, assay media broth to suspend bacteria was made with 10 g of tryptone, 5 g yeast extract, and 10 g NaCl all in 950 ml of D1 H₂O. In order to adjust pH to ~7.0 NaOH was mixed into the broth prior to autoclaving media. ECC15 bacteria is added to media, and inoculated overnight. Using a photometer to measure bacterial content, the concentration of the infection solution is brought to OD 100. The ECC15 broth with bacteria was stocked in 200 µl aliquots of the OD100 solution, and adapted from (Buchon, 2009).

X-gal Staining (Verduzco 2013) (x-gal)

Ten guts per well were dissected and suspended in PBS. After PBS was removed, tissues were fixed with 25 µl of 8% Gluteraldehyde and 375 µl of PBS at room temperature for 10 minutes. The fixative was removed and the sample washed twice with 500 µl PBS. Tissues were stained with the staining solution (10 µl K3, 10 µl K4, 20 µl X-gal, 360 µl PBS) for at least 3 hours at 37°C and mounted (Moviol) with elongating of the stained guts.

SMURF

Stock food was heated in a microwave or surface burner until liquid (measure max temperature if possible). Blue dye (2.5% w/v (mass of solute (g)/ volume of solution (ml))) was added to the heated stock food and allowed to mix (with stirring) for 15 minutes. Ten

milliliters of heated/mixed food were added to fly vials and allowed to cool overnight with a mesh net. Flies were placed on SMURF food at the beginning of the perturbation and analyzed daily and imaged accordingly to identify measurable time points for loss of gut integrity phenotype. Percent composition of SMURF as a function of time was calculated (Rera, 2011).

Histology and Staining - Tissue and Cell Imaging Adapted from (Goyal, 2007)

To complete immunohistochemistry, 24-well plates were used to dissect fly guts in PBS. Following fixation, tissues were washed with buffer A (90% FCS/10% DMSO) for 10 minutes at room temperature before application of primary antibody diluted in 200 μ l buffer (adapted from (Biteau B. e., 2014). The images of whole flies were taken with a Zeiss dissecting fluorescent microscope, and all other images were taken with a Zeiss LSM 700 confocal microscope and processed using Adobe Photoshop, Illustrator, and ImageJ (Ayyaz 2015) (Pellegrino MW, 2012). The following primary antibodies and dilutions were used: rabbit anti-phospho-Histone H3 Ser 10 (Upstate, 1:1000), mouse anti-Prospero (Developmental Studies Hybridoma Bank (DSHB), 1:250), mouse monoclonal anti-Cut (DSHB, 1:100), mouse anti-Armadillo (DSHB, 1:1000), and rat anti-Delta (gift from M. Rand, University of Vermont, USA, 1:1000). Secondary antibodies were obtained from Jackson Immuno-research. DAPI and phalloidin (Invitrogen) were used to stain total DNA and actin filaments, respectively, in the visceral muscle.

Phenotyping for Proliferation Screening in Aged Flies Assay Using the *Drosophila* Genetic Reference Panel

Flies from the DGRP are controlled by comparing data values to the Bloomington fly lines W1118 and ORER directly from the stock to account for batch variance. Female adults,

aged for 30 days at 25°C, were sorted and 10 guts dissected per sample and stained for PH3+ cells. Cells per gut were quantified and analyzed by the bioinformatics department at the Buck Institute.

Phenotyping Colony-forming Unit Screening in the Aged Flies Assay Using the *Drosophila* Genetic Reference Panel (DGRP)

Female adults, aged for 30 days at 25°C, were sorted and dissected as described above. Guts were mashed and homogenized using a sterile pestle. A total of 1000 µl of sterile PBS was added to the tube, and serial dilutions were performed to desired concentrations. The target concentrations were plated by pipetting 150 µl of sample incubated at 29°C for 36–48 hours, and the number of colonies was counted. Two controls, W1118 and ORER, directly from the stock were used to account for batch variance.

Chapter 1 | The Mitochondrial Unfolded Protein Response Systemically Regulates *Drosophila* Intestinal Stem Cell Self-renewal in the Posterior Midgut

Overview

The importance of systemic stem cell regulation and the role of UPR_{mt} induction in tissue regeneration were explored. Based on previous studies, we postulated that ISC activity would mimic a phenotype similar to that of an aging hyperplasia that is often observed in the posterior midgut of flies at age 40 days or more. To test this hypothesis, UPR_{mt} was activated using the UAS-Gal4 system in *D. melanogaster* specifically in visceral muscle of the intestinal epithelium, by over expressing a mammalian, missense-mutated, matrix-confined,

and misfolded ornithine transcarboxylase (OTC) enzyme (Hodges, 1989). ISC-specific responses in the presence of electron transport chain (ETC) dysfunction of the visceral muscle were also evaluated. Furthermore, we investigated ISC response during conditions of reducing ETC activity in the visceral muscle using UAS driven RNA-mediated interference (RNAi). Results from our missense insertion enzyme dOTC demonstrated a UPRmt phenotype similar to reducing respiration through the impairment of ETC function in the visceral muscle.

Chapter 1: UPRmt Results

1.1 Over-expression of dOTC in Visceral Muscle Systemically Affects Intestinal Stem Cells

To understand how the ISCs respond to a systemic induction of UPRmt in the visceral muscle, young progeny of *Drosophila* females with a UAS (upstream activating sequence)-Gal4 were crossed with a male carrying UAS-driven dOTC to induce the UPRmt in a temperature-dependent manner. W1118 was used as an experimental control (Table 1). The parents of the progeny were healthy maintained male and female stocks, which were back-crossed to match the W1118 genomic profile to reduce variation (Fig S.1A-A'). Prior to the experiments, I validated the baseline expression of Delta (ISC marker), Prospero (EE marker), and DAPI (Hoechst) staining in the posterior midgut (Table 1) (Fig S.1E). The GAL4-UAS system is a method used to study gene expression and function in *Drosophila* and allows monitoring of the Gal4 protein activity and location of induction, shown to be inhibited at 18°C (Fig S.1B). GFP was used to visualize the location of UAS-Gal4 activity when activated at 29°C (Fig S.1B'). Controls were compared by DI/DAPI ratio between female progeny W1118 WT flies at 18°C and their genetic counterparts at 29°C (Fig. 3A-A'). Flies with the transgene dOTC placed at 18°C had a DI/DAPI ratio similar to that of the

control at 29°C (Fig 3B-B'). In the presence of activated Gal4, the W1118 control continued to show normal distribution with GFP, which was indicative of activated Gal4 (Fig 3C-C'). The treated group carrying dOTC showed normal GFP, indicative of Gal4 expression, and also a significant increase in the ratio of DI/DAPI post UPRmt induction (Fig. 3D-D'). The images were also quantitatively analyzed for statistical significance, and an increase in the ratio of ISCs to total nuclei (DI/DAPI) in the posterior midgut is graphically represented in (Fig. 3E). This experiment was also repeated with Prospero, which stains and allows for quantification of EE cells in comparison to total nuclei. The data are not statistically significant, which suggests an inability of ISCs to terminally differentiate into EEs (Fig S.1 C'-C''').

In a repeated experiment, we again induced UPRmt in the visceral muscle of the fly intestinal epithelia for 5 days to repeat the phenotype, but this time we extended the induction for 3 days before proceeding with assays. At 18°C, which suppresses Gal4 expression, results showed an unchanged ISC density in the posterior midgut of W1118 control flies (Fig. 3G-G'). In flies carrying the dOTC insertion (UPRmt induction), the ratio was not different in comparison to WT controls (Fig. 3H-H'). At 29°C with Gal4 activation, flies with W1118 control showed a normal distribution of GFP expression (Fig. 3I-I'), whereas inducing UPRmt in the visceral muscle increased the DI/DAPI ratio significantly (Fig 3J-J'). In short, activating UPRmt in the visceral muscle of adult female flies caused a response that resulted in increased stem cell density that was likely dependent on paracrine signaling within the posterior midgut (Fig. 3K).

The increase in ISC density in the posterior midgut was cross analyzed by quantifying post-translational modifications to histone H3 in ISCs using phospho-histone H3 (PH3) staining. The data indicated a statistically significant increase in PH3+ cells in gut

tissue expressing dOTC relative to the control W1118 at 29°C (Fig. 3L-L'). The bar graph in Fig. 3M indicates the amount of baseline PH3 expression in cells per gut and indicates a statistically significant increase in mitotic figures in the flies overexpressing dOTC (Fig. 3M). A qualitative experiment of the same population of the PH3-positive data demonstrated an upregulation of Gstd1 (Glutathione S Transferase D1) expression upon ubiquitous activation of UPRmt shown by X-gal staining fly midgut, suggesting that UPRmt may have a reactive oxidative species (ROS)–dependent regulatory effect on ISC proliferation observable by measuring changes in Nrf2 expression (Hochmuth, 2011) (Fig. 3N-N').

Figure 3 | Over-expression of dOTC in Visceral Muscle (VM) Changes Delta+/DAPI Ratio in Posterior Midgut

(A-A') IHC staining for Delta in ISCs in white, GFP for VM in green, DAPI blue stains for nuclei shown in W1118 control flies at 18°C; 5 days' post UPR induction and 8 days of age, imaging of the posterior midgut 40× magnification.

(B-B') dOTC genetic control in flies at 18°C.

(C-C') W1118 experimental control flies at 29°C. UAS-Gal4 activated shown in GFP.

(D-D') dOTC overexpression in flies at 29°C at 5 days' post induction and 8 days of age showing more DI staining.

(E) Bar Graph showing Y-axis avg. ratio of DI/DAPI. X-axis shows control vs UPRmt induction through 5 days' post induction. Error bars are standard deviation. T-test p-values: [*dOTC18/W18* $p = 0.93$] [*w18/w29* $p = 0.12$] [*dOTC29/w29* $p = 0.004$]

(F) Y-axis is the ratio of Pros/DAPI cells Ttest p-values: [*W18/W29* $p = 0.11$] [*dOTC18/dOTC29* $p = 0.27$] [*W29/dOTC29* $p = 0.19$] IHC staining with DI+GFP+DAPI. 18°C indicates suppression of UAS-Gal4 by temperature sensitive Gal80.

(G-G') IHC staining with Delta, GFP, and DAPI in W1118 control flies at 18°C at 8 days' post induction and 11 days of age.

(H-H') dOTC overexpression in flies at 29°C at 8 days' post induction and 11 days of age.

(I-I') W1118 control flies at 18°C at 8 days' post induction and 11 days of age.

(J-J') In dOTC OE flies at 29°C at 8 days' post induction and 11 days of age.

(K) Y-axis is the ratio of total DI per total DAPI PM. Controls are statistically similar while UPRmt is statistically different. Ttest p-values: [*dOTC29/W29* $p = 0.001$].

(L-L') OE of dOTC in the VM (green, GFP) induces proliferative PH3, CY3 PM. Control on left, dOTC on right. Quantification in PM post-dOTC activation w/ G80ts.

(M) Y-axis is PH3+ cells per gut. T-test p-values: [*dOTC18/dOTC29* $p = 0.001$] [*dOTC29/W111829* $p = 0.00002$]

(N-N') OE of dOTC increases Gstd1 expression in the midgut Gstd1::LacZ X-gal. The images are of the PM. Saturation normalized to the background region of the middle midgut.

Images taken, edited, and quantified with Zen and ImageJ of Posterior.

Chapter 1: ETC Dysfunction Results

1.2 Knockdown of Electron Transport Complex 1, Ubiquinone Reductase (ND75) Expression in the Visceral Muscle Affects ISC Renewal and Gut Barrier Integrity

To investigate how ISCs systemically respond to ETC dysfunction in the visceral muscle, we used a UAS driven RNA-interference (RNAi) construct for ETC complex 1 ubiquinone reductase (ND75). NAD⁺ (nicotinamide adenine dinucleotide), a co-enzyme, influences mitochondrial stress responses and regulates energy metabolism (Pellegrino MW, 2012). Using the same genotype as the dOTC experiment, we showed that W1118 control flies have a normal DI/DAPI ratio in the posterior midgut with no GFP in the posterior midgut (Fig. 4A-A'). When crossed with ND75 RNAi and held at 18°C, the density of ISCs was similar to that of W1118 control flies (Fig. 4B-B'). The W1118 group at 29°C with GFP expression also exhibited a normal DI/DAPI ratio (Fig. 4C-C'). When ND75 was actively knocked down with the RNAi in the visceral muscle, a statistically significant increase in DI/DAPI and GFP expression was observed (Fig. 4D-D'), suggesting that autonomous ETC dysfunction in the VM systemically promotes ISC self-renewal.

Using the muscle-specific fluorescent stain phalloidin, changes in morphology, rigidity, and shrinkage were observed in these ND75 knocked down flies (Fig. S.4D). As depicted in Fig. 4E, knockdown of ND75 was similar to the overexpression of dOTC in the visceral muscle, with both showing a statistically significant increase in the DI/DAPI ratio in the posterior midgut and a similar but stronger response when dOTC was expressed (Fig. 4E). Using the SMURF assay, which involves feeding flies with a food containing a blue dye mixed into the media, we tested the effects of systemic induction on gut barrier function. The control W1118 flies did not have a blue abdomen because the dye is primarily confined

to the midgut and proboscis (Fig. 4F-F'). The distribution of the true ISCs in the intestinal epithelium of a young, healthy animal did not show crowding or non-linear divisions, as demonstrated using the ISC-specific LacZ reporter and DI staining relative to total nuclei (DAPI) (Fig. 4G-G'). In contrast, results of ND75 knock-down experiments were consistent in DI staining of animals at 29°C (induced UPR_{mt}) (Fig. 4H). Reducing the activity of the ETC in visceral muscle resulted in a statistically significant increase in β -gal and DI-positive cells in the posterior midgut relative to DAPI (Fig. 4H'). The ratio of both the β -gal and DI-positive cells was much higher in flies expressing ND75 RNAi, suggesting that true ISCs are directly responsible for responding to stress signals from the visceral muscle during changes in metabolic activity (Fig. 4I). We demonstrate a clear loss of barrier function at 29°C in flies expressing ND75 RNAi, with these flies exhibiting a 60% increase in gut permeability at 18 days and 30% increase in gut permeability at 27 days of age (Fig. 4J).

Figure 4 | Knockdown (KD) of Electron Transport Chain Complex 1 Protein, ND75 in the Visceral Muscle Elicits a ISC Response in the Posterior Midgut

(A-A') IHC staining with DI+GFP+DAPI in W1118 control flies at 18°C at 5 days' post induction and 8 days of age.
 (B-B') ND75 KD flies at 18°C at 5 days' post induction and 8 days of age.
 (C-C') W1118 control flies at 29°C at 5 days' post induction and 8 days of age.
 (D-D') ND75 KD flies at 29°C at 5 days' post induction and 8 days of age.
 (E-E') Graphical depiction of KD shows no statistical difference in controls and a significant statistical difference between ND75 KD and control; T-test p-value: $[ND29/W29\ p = 0.005]$.
 (F-F') The first group was assayed 14 days with SMURF food in the dye. The control is W1118, and ND75 KD to elicit the response. (Fig. 9A) shows the control.
 (G-G') IHC staining with DI+ Bgal+ DAPI in W1118 control flies at 29°C at 5 days' post induction and 8 days of age.
 (H-H') ND75 KD flies at 29°C at 5 days' post induction and 8 days of age.
 (I) KD of ND75 in visceral muscle increases the density of specific ISCs in the posterior midgut; graphical depiction of KD shows no statistical difference in controls and a significant statistical difference between ND75 KD and control; T-test p-value: $[W1118\ 29/ND75\ 29\ p = 0.01]$
 (J) KD of ND75 in visceral muscle causes loss of barrier epithelia function SMURF flies placed on SMURF food. Taken at day 14, day 18, and day 27. W1118 control in black.

Chapter 1: UPRmt & ETC Dysfunction Discussion

1.3 UPRmt and Systemic Intestinal Stem Cell Renewal

Overexpressing dOTC in the visceral muscle increases the ratio of DI/DAPI in the posterior midgut, and the data support claims that mitochondrial proteostasis potentially changes endogenous signaling factors from the visceral muscle to the ISCs. Increasing the induction time of UPRmt suggests that the density of Delta positive (DI) cells relative to DAPI stained cells continues to increase. In addition, overexpressing dOTC in the visceral muscle increases mitotic figures, indicative of self-renewal ligands being secreted in the whole gut. We specifically observed a large increase in the posterior midgut, despite baseline mitotic figures being relatively high in comparison to other studies (Biteau B. , 2010) (Hochmuth, 2011) (Wang L. , 2014). Plausible reasons for this outcome are that the parents were not healthy and young, the genetic construct disrupted endogenous proliferative homeostasis, or the quantifier also counted false positives. In logical contrast, the experimental group demonstrated a statistically significant increase relative to the control.

While assaying for ISCs, we also stained for EE cells by looking at Prospero expression in the same experiment to determine if the systemic phenotype was driving ISC terminal differentiation. We showed that overexpressing dOTC in the visceral muscles led to no statistically significant change in the ratio of EEs to total nuclei in the posterior midgut, suggesting that the phenotype in the ISCs may be in favor of driving symmetric rather than asymmetric divisions. In previous studies, progenitor cells were more likely to become ECs rather than EEs, and the interpretation of the absence of change in the amount of EE cells is that endogenous Enteroblast differentiation is unaltered, or has a lineage favoring ECs (Andrea H. Brand, 1993). Future analysis to further support this observation should involve measuring transcript levels of Slit expression in the gut using quantitative qRT-PCR; in the current work, attempts to complete a conclusive transcriptome analysis was unsuccessful (data not shown), possibly in part because of an inability to extract only visceral muscle RNA in sufficient quantities, hampering this measurement. A potential approach would be to compare fluorescent localization, intensity, and distribution by using immunohistochemistry with primary antibodies individually specific for *vn*, *wg*, *upds*, and *dilps*. Under conditions of ubiquitous dOTC overexpression, we observed changes in the staining of LacZ due to an increase in transcription of *GstD1*. What is increasing the *GstD1* expression is unknown, but given that the induction of UPRmt is ubiquitous, this result may still indicate higher Nrf2 activity, possibly because of changes in cell-specific oxidative challenges (Wang L. , 2014). This finding warrants a comparative analysis of Nrf2 expression in the visceral muscle and in the ISCs.

One aim was to develop a drug-inducible fly line that allows the control of UPRmt to demonstrate a change in longevity when UPRmt is induced during development and adulthood. The purpose of our experiments was to establish a model for UPRmt that is

genetically accessible enough for studying the systemic effects on stem cell self-renewal. When we knocked down the expression of ND75 in the visceral muscle, we observed changes in DI/DAPI and β -gal/DAPI ratios in posterior midgut, which mimics dOTC expression. This result may be used as readout for systemic effects of ETC dysfunction in visceral muscle to ISCs, but more important, it signifies that ETC dysfunction may be a primary phenotype of autonomous UPRmt in muscle tissue. A previous study involved knocking down ND75 expression in ISCs to autonomously induce proliferation by adding stress to the ETC (Hochmuth, 2011). In relation to this, we demonstrated that disrupting the ETC in a cell type adjacent to the ISCs could elicit a response similar to an autonomous ISC response shown in another study (Hochmuth, 2011). As we began postulating a testable pathway linking autonomous and systemic effects of UPRmt on stem cell self-renewal, we then speculated that the observed hyperplasia develops quickly into a dysplasia resulting in gut permeability. From the results presented here, we also speculate that UPRmt is causing ROS-generated ETC dysfunction.

Chapter 2 | *Drosophila* Genetic Reference Panel Identifies Genes Associated with Two Aging Phenotypes

Chapter 2: Overview

A genome-wide analysis study (GWAS) is an experimental approach designed to reveal novel genetic variants associated with a phenotype within a given population, and this relationship is comparatively analyzed with bioinformatics tools (Pearson, 2008) (Nachman, 2001). In nature, genetic variations fundamentally differentiate the survival and organismal fitness of individuals within a species (Manolio, 2010). The association of a phenotype with genetic variance in a population is statistically verified by the frequency of single nucleotide polymorphisms, and the *Drosophila* genetic reference panel (DGRP) stocked at Bloomington is currently an accepted resource for performing a GWAS in the field of fly genetics (Mackay, 2012). Genetic variations are represented by the frequency of single nucleotide polymorphisms (SNPs) occurring in measurable segments of DNA. Genomic variations within a species may contribute to unique physiological responses to changes in diet, exposure to toxins, and pathogen resistance; methodically understanding these genetic variations can unveil plausible pathways controlling an organism's health and longevity (Nachman, 2001). In order to establish a phenotypic correlation with naturally segregating genetic variations, we used the DGRP to perform a GWAS of 192 inbred strains (Mackay, 2012). The goal of this study was identify novel functions for proliferative and commensal bacteria homeostasis for known genes in the context of aging.

Chapter 2: GWAS Results

2.1 Establishment of New Phenotypic Correlations with Naturally Segregating Genetic Variations

I hypothesized that by using a proliferation marker as a readout to gather data for aged flies (30 days), we would identify genetic associations between the observed phenotypes and SNPs within the DGRP collection. These data were expected to yield new gene candidates that may play an important role in influencing age-related ISC over-proliferation. To accomplish this, the flies were stained and counted for phospho-histone 3 positive (PH3+) marker in the entire gut, and PH3+ cells were averaged and documented with their respective DGRP number (Fig. 5A). This study was a 52-line, blind sampling of the population, and the p values of gene candidates were calculated to determine the promise of pursuing a candidate based on the numerical distance from the threshold of significance.

In this study, using the GWAS method, we identified two interesting genes involved in the promotion or inhibition of proliferative homeostasis in an aging animal (Fig. 5B). Each individual data point (numerical value corresponding to one DGRP line) has a SNP, and we represent the data value relative to the SNP. Tfb1 (RNA polymerase II transcription factor TFIIH, nucleotide excision repair subunit) has point mutations exchanging adenine to cytosine and vice versa. Tfb1 is necessary for nucleotide excision repair (DiLello, 2008) (Fig. 5C). Dunce (Dnc), is a candidate with a change from adenine to thymine and is a phosphodiesterase involved in controlling cytosolic cAMP levels (Dyke, 2002) (Fig. 5D). The SNP in Dunce is located in the intron, which suggests that the mutation is not found in the peptide sequence but rather in the regulatory region of the gene, which may alter the transcription levels of the mRNA. In the case of Tfb1, the SNP is upstream of the Tfb1

coding region, suggesting that the mutation leading to a phenotype may not only regulate transcription of Tfb1 but also may regulate genes between the mutation and the Tfb1 coding region.

Evidence from recent studies suggests that commensal bacteria homeostasis declines with age in both vertebrates and invertebrates. The loss of homeostasis of the gut flora affects longevity, and both the density of microbes and composition of the population affect metabolic homeostasis (Broderick, 2014) (Claesson, 2011) (Guo L1, 2014). The importance of the GWAS study, in the context of my project, was to identify genes that may serve as inhibitors of the age-related loss in commensal bacteria homeostasis.

In this study, quantifying colony-forming units (CFUs) was used to produce usable data that could be analyzed through bioinformatics. To identify genes, a simple assay was developed for identifying CFUs in the *Drosophila* midgut which best represents the population (Fig. 5E). I hypothesized that by quantifying the average CFUs from a sample of each DGRP line, I could identify candidate genes involved in maintaining homeostasis of age-related gut flora population (Fig 5F). Using this method, I identified Dystrophin as a candidate, with a cytosine to thymine exchange; it is involved in myofibril formation (Fig. 5G). A second candidate, with an adenine to thymine change, was Rolling Pebbles (Rols) was also identified. Rols is involved in adherin junction maintenance, which is important for proper tissue formation (Fig. 5H). More specifically, the SNPs in both Dystrophin and Rols are positioned in the introns, suggesting a transcriptional regulation, and expression levels are likely not affected.

B

Marker	Gene	Position	P value	Annotation
Stem Cell Count				
X_3207337_SNP	dnc	INTRON	8.08E-07	FBgn0000479
2R_10250446_SNP	Tfb1	UPSTREAM	1.19E-06	FBgn0033929

F

Marker	Gene	Position	P value	Annotation
CFUs				
3R_15360886_SNP	Dys	INTRON	3.12E-06	FBgn0260003
3L_12041883_SNP	Rols	INTRON	4.38E-06	FBgn0041096
2R_12860363_SNP			4.72E-06	

G

H

Figure 5 | GWAS for Proliferative and Commensal Homeostasis Associated with Aging

(A) Graphical representation of the specific *Drosophila* genetic reference panel (DGRP) fly lines assayed on the x-axis. Average PH3+ cells per gut. In each DGRP line, there could be up to as many as 100,000 position differences. The software takes the top 25 SNPs associated with the phenotype and averages the differences between them to calculate the variance.

(B) In this table, the p value represents the chance that this candidate will land under the area of the null. The annotation is from Fly Base, indicating the number corresponding to that gene listed. The position will either be exon, intron, or upstream. For Duncle, the SNP is located in the intron, meaning that the SNP is spliced out, but that does not rule out a change in function.

(C) Dot plot for Tfb1 (RNA polymerase II transcription factor TFIIH), a nucleotide excision repair subunit. The site of the SNP is shown at the top right of the box. Tfb1 is located at 2R_10250446_SNP; each dot is a data point taken in a sample from the population.

(D) Dot plot for Dnc, located at X_3207337_SNP. Each dot is a data point taken in a sample from the population.

(E) DGRP lines that were assayed and the average CFU per gut.

(F) The position is located either in an exon, intron, or upstream regulatory region. For Dys and Rols, the positions are located at the intron. Dys is located at 3R_15360886_SNP.

(G) Dot plot for Dystrophin. The site of the SNP is shown at the top right of the box. Each dot is a data point taken in a sample from the population.

(H) Dot plot for Rolling Pebbles. Rols is located at 3L_12041883_SNP. Each dot is a data point taken in a sample from the population.

Chapter 2: GWAS Discussion

2.2 New Regulator of Proliferative and Commensal Homeostasis with Age

The goal of the study was to identify genes with regulatory functions in the intestinal epithelium. We identified three candidates for proliferative homeostasis and two for commensal bacteria homeostasis. A false detection rate (FDR) of 65% is relatively high compared to other working studies, indicating that 65% of all candidates will be false. Increasing the amount of *Drosophila* genetic reference panel (DGRP) lines used may alleviate the high FDR. Of all the candidates that we discovered, Dnc and Tfb1 were chosen as the best candidates based on their p values, which were above the threshold of allele frequency associated with the proliferation data. A potential importance of Dnc is that cytosolic cAMP levels are in part responsible for activation of the PKA pathway, characterized as a pathway inducing proliferation (Iijima-Ando, 2009). This role may have clinical implications for interventions and treatments, which may target the regulation of cytosolic cAMP.

Tfb1 is a checkpoint component of the mitotic cell cycle demonstrated in a past study, which investigated a loss-of-function phenotype supported by previous data suggesting an arrest cell cycle in G2 phase. The dot plot data for Dnc and Tfb1 suggest that a selective process for which the SNP at the gene loci is responsible for the phenotype provides pharmacological targets for deterring a loss in proliferative homeostasis in an aging tissue. Our GWAS study indicated an age-related change in gut flora population size as a supporter of genetic expression. This study produced a FDR of 10%, indicating that 1 out of every 10 hits would be a false positive. We showed that the difference in gut bacteria population size between the individual lines statistically suggest genes that promote or inhibit the growth or reduction of the commensal bacteria population in the fly gut, but in humans, the genetic

regulators of this phenomenon is still very much a mystery. Maximizing the number of lines assayed will yield the most precise genetic hits, but it is a very labor-intensive way to discover new genes.

To pursue further more microbial screening for genes, we used 30-day aged flies using the DGRP lines. The hits that were received from this study are above the p value threshold for statistical significance, suggesting that these genes are likely required in the regulation of commensal bacteria in the gut of *Drosophila*; further, dot plots could potentially be used to select for which missense or point mutation of a gene locus gives the desired phenotype to combat the loss in proliferative homeostasis with age. Our logic is that losing smooth muscle function and cell-to-cell adhesion in the gut would increase permeability and reduce integrity; further, the candidate Rols could conceivably modulate commensal bacteria homeostasis by controlling cell-to-cell adhesion and reducing or promoting permeability with age. A way to follow up on any commensal bacteria hits would be to express RNAi in the whole gut of the genetic candidates at 30 days of age and place the flies on SMURF food to determine gut permeability. In the future, we may consider measuring the changes in gut microbial composition in flies produced from an experiment in which we perturb the expression of Dystrophin and Rols separately (Broderick, 2014).

Chapter 3 | Regulators of ISC Maintenance, Activity, Cell Lineage, and Commensal Bacteria Homeostasis

Chapter 3: Overview

Using the DGRP for GWAS, we have identified genes that have not been previously characterized as constituents of ISC activity in the *Drosophila* intestinal epithelium model.

The intestinal epithelium of *Drosophila* has been used to study the regenerative capacity of intestinal stem cells (ISCs). The importance of properly maintained endogenous ISC regulation is the maintenance of gut epithelial integrity. Without ISCs, there would be no cells responsible for maintaining gut barrier function through regeneration. The ability to maintain barrier function is lost with age (Guo L1, 2014) (Kapuria, 2012); aging results in a loss of genomic stability, which negatively affects tissue maintenance factors by reducing ISC regenerative capacity. From our GWAS data, we find that known genes may have unknown functions in ISC regulatory pathways required in tissue maintenance.

Overview: Arrowhead (Awh)

A recent proliferation study demonstrated an increase in LIM homeobox domain 4 (LHX4) mRNA as a function of age in mammals (Wang Z. , 2013). The age-related increase of LHX4 expression in malignant colorectal tissues suggests a relationship between cancer development and age through the mediation of LHX transcription factors for LIM proteins. The HOX family gene Awh in *Drosophila* is orthologous to the LHX family genes found in mammals (Sun, 2006). Similar to LIM proteins, Awh plays a role in the differentiation and proliferation. In addition to Awh, escargot (esg), which is expressed in *Drosophila* intestinal epithelium ISCs and EBs, is also required for cell cycle regulation, and expression of the Awh gene family suppresses Wnt. This study further shows that downstream effectors of the Wnt pathway— β -catenin and T-Cell Factor (TCF)—are required for both benign and malignant colorectal growth (Sato, 2013). Data from our GWAS identified Awh as a potential candidate involved in the regeneration capacity of gut ISCs and warrant further investigation.

Overview: Dunce (Dnc)

The functional peptide that the Dunce (Dnc) gene codes is a phosphodiesterase (PDEs) (Qiu, 1993). PDEs are enzymes that break phosphodiester bonds of cyclic adenosine monophosphate (cAMP), a secondary messenger of the protein kinase A (PKA) pathway. The PKA pathway begins with the binding of a ligand to a G-protein–coupled receptor (GPCR), which triggers a cascade leading to an increase in cAMP levels and activation of PKA. Cytosolic concentration of the substrate cAMP is controlled upstream of the PKA pathway, and this pathway influences proliferation and hyperplasia (Wang C. , 2010). The expression of PDEs can be used to regulate downstream effectors involved in the PKA by synergistic activation (Gervasi, 2010). This synergistic activity has been further studied as a component of neurodegenerative diseases (Walker, 2013) (Iijima-Ando, 2009).

Dunce plays a role in behavioral phenotypes associated with learning and memory; additionally, Dunc studies have demonstrated varying expression levels throughout the lifespan of an animal (Qiu, 1993). Dnc is expressed in *Drosophila* into adulthood. Previous studies have shown Dnc mutants have moderately higher levels of Ca^{2+} release, and Dnc serves as a regulatory factor of adenylyl cyclase, which catalyzes the conversion of ATP to cAMP (Gervasi, 2010). Some studies suggest that a deregulation of the cAMP pathways may be both directly and indirectly responsible for most cancer growths, development of neurodegenerative diseases, and the age-related loss in water balance (Dyke, 2002) (Gervasi, 2010) (Pietrobon, 2011) (Wang C. , 2010).

Figure 6 | Illustration of the GPCR pathway upstream of cAMP conversion and the activation of the PKA pathway via cytosolic cAMP. Adapted from (Gervasi, 2010). The phosphodiesterase (PDE) shown in the cartoon is the enzyme that is coded by the gene *Dunce*. High levels of cAMP activate the PKA pathway, resulting in various downstream affects. PDE binds and converts cAMP to AMP.

Overview: Tfb1 (RNA polymerase II transcription factor of the TFIIH-Nucleotide excision repair subunit

Tfb1 in yeast is a subunit of a complex named TF11H (Fregoso, 2007) and unwinds DNA for site-specific excision in the nucleotide excision repair (NER) pathway. TF11H activity and expression in aged flies are associated with proliferative homeostasis. The developmental significance of TF11H has been reported as an important factor of UV-induced DNA damage (Castro J. , 2002). In our GWAS study, Tfb1 was identified as a candidate gene, and we hypothesized that Tfb1 may trigger endogenous DNA repair mechanisms in an attempt to manage proliferative homeostasis decline during aging. The enzymatic activity of TFIIH has three main functions that are essential for transcription initiation and elongation; furthermore, DNA-supported ATPase, ATP-dependent helicase,

and CTD kinase are mediated by Tfb1 (Tirode F, 1999). Tfb1's role in DNA damage repair makes it important to further investigate in terms of its role aging.

Overview: Dystrophin (Dys) and Rolling Pebbles (Rols)

Gut barrier function is a component of digestive disease formation in humans, flies, and other aging organisms. Embryonic progenitor cells eventually become muscle cells as a part of myogenesis, and this fusion is essential for maintaining skeletal muscle structure and smooth muscle integrity, mediated by Dystrophin. A second age-related deficiency in barrier function is associated with cell-to-cell adhesion via formation of adherens junctions (Houtkooper, 2013). Rols has been functionally characterized as one of the primary peptide groups exhibiting deregulated cell adhesion as a component of age, and this functional deregulation leads to dysbiosis and epithelial dysplasia, both shown in the *Drosophila* gut barrier (Buchon, 2009) (Guo, 2013).

The advancement of diseases may arise from the requirement for the maintenance of appropriate synaptic retrograde communication and the stabilization of muscle cell architecture or physiology; more specifically, it may play a role in anchoring the cytoskeleton to the plasma membrane. Dystrophin is involved in the biological process described by muscle cell-to-cell interaction, homeostasis, and muscle organ development. Dystrophin is required in actin binding and cytoskeleton formation and is one of the largest genetic constituents responsible for muscle structure (Mariska C. , 2006) (Scherbata, 2007). It is also responsible for actin filament formation during muscle development and muscle repair across different species. Results of several studies have indicated that dystrophin is significant in neuromuscular synaptic transmission by establishment of cell polarity. More specifically, the presence of dystrophin isoforms post-synaptically evokes the release of

various neurotransmitters, whereas its absence leads to no obvious changes in myofibril morphology (Mariska C. , 2006).

Rols is involved in myoblast fusion and has genetic and physical interactions with genes that are involved in proximity-dependent cell-to-cell interaction (Avirneni-Vadlamudi, 2012) (Menon, 2005). The presence of Rols supports dependent localization of Mhcl (myosin heavy chain-like), which is necessary for myoblast fusion (Bettina, 2013). Properly characterizing the functional importance of Rols in the maintenance of gut barrier function is important.

Chapter 3: Awh Results

3.1 Arrowhead Is Required for ISC Lineage Maintenance

The mosaic analysis with a repressible cell marker (MARCM) system was used to provide insights to the proliferative effects of a mutated Awh gene. The MARCM system in flies allows the observer to trace the mutation of a given gene through cell lineage in a given organismal model by providing the genetic control construct, an FLP-FRT (flippase recombinase–FLP recombinase target) fly line. In the *Drosophila* epithelia, according to previous reports, Awh may have autonomous effects on ISC differentiation (Lee T, 2001). In our study, we determine if the Awh gene is responsible for mediating cell/tissue integrity by conducting a comparative analysis after perturbing Awh expression (Table 1 and Fig. S.2 A-A'). Clones of Awh mutants were generated to determine how Awh expression affects clone size and frequency at 7 and 15 days' post heat shock (Fig. 6A-A'). The graph in Fig. 6B represents Awh mutants generating a much larger average clone size than the control, with on average fewer clones per gut when compared to the control (Fig. 6C). Similar results were seen with the same genotype when assayed 15 days' post heat shock (Fig 6D-D'); however,

as expected, the Awh mutant produced a larger clone size in after 15 days of heat shock compared with the 7-day time point (Fig. 6E), with Awh mutants continue to generate fewer clones for gut in comparison to the control (Fig. 6F).

The data were also binned to compare the R squared values for both the 7- and 15-day time points (Fig. 6G and 6H). The experiment was repeated, demonstrating a similar trend (Fig S.2 B-B' and Fig. S.2 C-E). The R squared value is a statistical measure of the closeness of the data to the fitted line and is the percentage of the variation to the total. The control groups had a high R squared value, while the Awh mutant groups exhibited a lack of consistency suggesting a disruption of endogenous gut maintenance factors.

In Chapter 2, the GWAS of the DGRP identified the gene Arrowhead (Awh) as a potential candidate involved in proliferative homeostasis. Here, we validate our GWAS finding by observing differences in proliferation through Awh knockdown (KD) in the ISCs and EBs. We also evaluated the proliferation of the Awh mutants during exogenous stress such as the addition of the ECC15 microbes. Our results show that Awh KD in EBs and ISCs increased endogenous mitotic figures (Fig. 6 I-I'). The cells in *Drosophila* gut responded to the exogenous stress, and ECC15 microbes were added to these flies (Fig. 6J-J'). The amount of GFP+ cells that are also PH3+ positive was less in the ECC15 infection groups relative to the sucrose control groups. This result suggests that cells not expressing Awh are not proliferating in response to stress. The proliferation frequency when Awh expression was reduced in young intestinal stem cells. Young adult flies expressing Awh RNAi under ubiquitous Gal4 control failed to survive an ECC15 assay; additionally, ECs and EBs expressing Gal4 driven Awh RNAi using NPI-1 (nucleoprotein interactor 1) insertion also failed to survive. These findings suggest a potential novel role of Awh in mediating epithelia stress.

Figure 7 | Arrowhead (Awh) is a Regulator of ISC Proliferation

(A-A') Control Flippase - Recombinase Target construct used for clonal analysis showing GFP+ Cells in a clone with DI staining ISC-specific antibodies and DAPI. Awh mutant generated clones qualitatively showing larger clone size.

(B) Graphical depiction of 7 days' post heat shock. Y-axis average GFP+ cells in a single clone. Lineage analysis showing statistically significantly larger clones in Awh mutants compared to the control. *T-test p-value* = 0.001

(C) Graphical depiction of 7 days' post heat shock. Y-axis depicts the average clones per gut. Lineage analysis showing a statistically significant lesser amount of clones in Awh mutants compared to the control. *T-test p-value* = 0.01

(D-D') Control FRT2A MARCM clonal analysis showing GFP+ cells in a clone with DI staining ISC-specific antibodies and DAPI. Awh mutant generated clones qualitatively showing larger clone size.

(E) Lineage analysis of Awh Mutant 15 days' post heat shock. Recombination control FRT2A MARCM clonal analysis showing GFP+ cells in a clone with DI staining ISC specific antibodies and DAPI. Awh mutant generated clones qualitatively showing larger clone size.

(F) Graphical depiction of 15 days' post heat shock. Y-axis depicts average GFP+ cells in a single clone. Lineage analysis showing statistically significantly larger clones in Awh mutants compared to the control. *T-test p-value* = 0.01

(G) Data binning groups of samples based on count of GFP+ cells. Curve generated to produce R² value. Graphical depiction of 7 days' post heat shock. Y-axis depicts average clones per gut. Lineage analysis showing statistically significant lesser amount of clones in Awh mutants compared to the control. *T-test p-value* = 0.0002

(H) Data binning groups of samples based on count of GFP+ cells. Curve generated to produce R² value.

(I-I') Knock down (KD) of Awh expression in ISCs and EBs. The driver 5961-gene switch crossed with Awh KD line 31772. CY3 represents antibody for PH3+ cells; GFP+ corresponds to cells expressing the Gal4 system. DAPI blue represents total nuclei.

(J-J') Mitotic figures post KD of Awh expression in ISCs and EBs showing paracrine effect. The driver 5961-gene switch crossed with Awh KD line 31772. CY3 represents antibody for PH3+ cells; GFP+ corresponds to cells expressing the Gal4 system. DAPI blue represents total nuclei.

(K) Graphical representation of experiments showing PH3+ cells per gut in the 5% sucrose control. Graphical representation of experiments showing PH3+ cells per gut in the ECC15 proliferative stress induction group. *T-test p-value*: [ECC15-/5%- 0.01] [ECC15+/5%+ 0.0003].

Chapter 3: Awh Discussion

3.2 Arrowhead's Involvement in *Drosophila* Intestinal Epithelia Regenerative Capacity

Awh mutant increases GFP+ clone relative to the FRT2A construct, and an increase in the size of the clones suggests that Awh is required in proliferative homeostasis by possibly pushing ISCs towards terminal differentiation rather than symmetric division. Awh KD in ISCs and EBs increases mitotic figures during ECC15 exposure, suggesting that Awh is necessary for endogenous proliferation stress responses; furthermore, Awh may be an inhibitory component mediating the Armadillo/Wg pathway. Binning the data allowed for the creation of a standard curve of the experimental group relative to the control group to compare clone size based on interactions between Awh and factors of the Wnt pathway by evaluating differences in the area under the curves in Awh clone, KD, or OE constructs. A

shift in the experimental curve to mimic the control curve would indicate a genetic interaction. The pitfall with this kind of data is that the assay is not cell specific; furthermore, KD in ISC and EB elicits a loss of proliferative homeostasis but does not directly indicate cell specificity. The ECC15 experiment shows a paracrine effect observed by noticing the cells that GFP+ are not the cells that are also proliferating. Given that Awh has previously been shown to localize to the nucleus, we postulate that measuring Awh expression relative to Armadillo activity may validate an interaction.

Chapter 3: Dnc Results

3.3 Phosphodiesterase Expression Influences ISC Differentiation and Aging

To investigate the role of Dnc in mediating stem cell function, we designed a lineage-tracing experiment with the MARCM19a construct (Table 3) (Fig. 7A-A'). Results show more clones present per gut in the Dnc mutant relative to the control (Fig. 7B). Additionally, the Dnc mutant exhibited higher levels of DI-positive cells per clone (Fig. 7C). The clone analysis data suggested that Dnc mutant clones may be regulating ISC self-renewal by influencing symmetric divisions, which is reinforced by the statistically insignificant changes in Prospero-positive cells relative to total DAPI cells in each clone (Fig. 7D). Changing overall gut composition conceivably may disrupt excretion and clone composition, as shown in Fig. 7E. Our results indicate that knockdown of phosphodiesterase expression (Dnc) specifically in progenitor cells increases the ratio of PH3+/DAPI (Fig. 7F-F'). The graphical data represent the quantification of the posterior midgut, validating our GWAS results (Fig. 7G). Increasing cytosolic cAMP (adenosine monophosphate) elevates activity of the PKA (protein kinase A) pathway, which also influences regeneration by activating proliferation. In contrast, cAMP has not been validated as a potential target for age-related diseases despite affecting mitotic activity, synaptic stability, neural plasticity, and the PKA pathway (Renger, 2000). Our results showed that Dnc mutation can generate relatively large clones; however, which environmental conditions influence this phenotype has not been explored (Fig. S.3B).

Figure 8 | Clonal Analysis of Dnc Mutant in FRT19a Line

(A-A') FRT19a and Dnc mutant for clonal analysis.

(B) Graph of average clones per gut. *T-test p-value* [0.01].

(C) Dnc mutant clone composition has more ISCs in per clone. *T-test p-value* [DI+/GFP+ *p* = 0.04]. Dnc mutants do not affect EEs *T-test p-values*: [Pros+/GFP+ 0.12][Unknown/GFP+ 0.01] Demonstrating Self-Renewal or EC favored terminal differentiation

(D) The average number of DI+ in any given clone of random selection shows Dnc mutant generating more ISCs *T-test p-value* = 0.01

(E) Graph representing no statistically significant differences between the amounts of Pros+ cells in Dnc mutant clones.

Enteroblast specific expression of an RNAi responsible for reducing activity of Dnc. DAPI+ in Blue, PH3+ CY3.

(E) Graphical representation of PH3+ cells normalized to DAPI+ cells in the posterior midgut, *T-test p-value*: [W18/ W29 *p* = 0.8] [Dnc18/Dnc29 *p* = 0.002] [Dnc29/W29 *p* = 0.03].

(F-F') Ratio of PH3+ cells to DAPI cells in the posterior midgut. Knock down of PDE expression with Dnc RNAi specifically in EBs increased the ratio of proliferating cells.

(G) Graph representing a statistically significant increase in PH3+/DAPI during knockdown of Dnc in the EBs.

Chapter 3: Dnc Discussion

3.4 Managing Cytosolic cyclic AMP Levels May Improve Mitotic Stability

Initially, the finding that Dnc is involved in proliferative homeostasis was not striking; however, Dnc has been extensively studied and potentially links cytosolic adenine mono-phosphate (AMP) and its dependent kinase-induced autophagy (Castro J. , 2002). The PKA pathway is activated or inhibited downstream of an increase or decrease in cAMP respectively, as a secondary messenger in signaling pathways associated with gut flora pathogenesis (Buchon, 2009). AMP and cAMP conversion is a regulator of multiple mechanisms upstream of mitochondrial activity, and phosphodiesterase activity may indirectly regulate mitochondrial activity (Walker, 2013). Stress response pathways, which respond to the ATP/AMP ratio in the cytosol, lend further support that PDEs may act indirectly in stress response by activating immune proteins (Weinberg, 2015).

Chapter 3: Tfb1 Results

3.5 Nucleotide Excision Repair, Proliferation, and Aging Tissue

Inhibiting nucleotide excision repair results in mutations that accumulate as a result of non-excised damaged DNA. To study how these changes affect the regenerative capacity of the ISCs, Tfb1 was genetically knocked down (KD) in actively dividing cells. Results show that flies with ubiquitous KD of Tfb1 expression exhibited an increase in endogenous proliferation rates when compared to W1118 control (Fig. 9A-A'). Ubiquitous KD of Tfb1

decrease lifespan by 21%, but this effect was only seen in ~15% of the population, and ubiquitous overexpression of Tfb1 was associated with no change in lifespan (Fig. S.4 C-D).

Furthermore, the increased endogenous proliferation rates observed flies with ubiquitous KD of Tfb1 expression in comparison to the W1118 control was specifically observed in ISCs (Fig. 9B-B'), while the overexpression of Tfb1 specifically in ISCs reduced lifespan of these cells by ~18%. A graphical representation of the increase in mitotic figures indicates a significant increase in baseline proliferation rates in response to DNA damage accumulation. In addition, we expressed Tfb1 RNAi specifically in ISCs and then specifically in EBs after ~4 hours of stress using ECC15 exposure, and graphs in Fig S.4A-B represent a statistically significant increase in mitotic figures of flies expressing Tfb1 RNAi. Moreover, flies expressing Tfb1 RNAi in EBs and ECs together could not survive during ECC15 exposure, and a proliferative phenotype in the ISCs was observed when DNA repair was genetically disrupted in the visceral muscle (Fig 9E-E', F-F'). These changes were statistically significant and are graphically represented in Fig. 9G. In summary, the observed proliferative phenotype in the ISCs when DNA repair was genetically disrupted in the visceral muscle suggests that accumulated DNA damage in the visceral muscle affects proper secretion of functionally necessary paracrine signaling ligands.

Figure 9 | KD of Tfb1 in EBs, ISCs, EBs, ISCs, and Ubiquitously Affects Mitotic Figures

(A-A') Image of DAPI+ cells in blue for nuclei, and PH3+ cells green (FITC)

(B-B') Image of DAPI+ cells in blue representing nuclei, and PH3+ cells green (FITC).

(C) Graph showing control without Ru486, then the experiment group.

(D) Mitotic figures post ubiquitous knockdown of Tfb1 14 Days Induction. DAPI+ cells in blue representing nuclei, and PH3+ cells green (FITC). Graph showing control without Ru486, then the experiment group.

(E-E') Tfb1 in visceral muscle affects mitotic figures in the posterior midgut. Mitotic figures post knockdown of Tfb1 in visceral muscle post 4.5 h stress @ 14 days. PH3+ image of DAPI+ cells with blue representing total nuclei, PH3+ cells in CY3, and GFP for induction of Gal4.

(F-F') Knock down of Tfb1 in visceral muscle affects, mitotic figures in the posterior midgut. Mitotic figures post knockdown of Tfb1 in visceral muscle post 4.5 h stress @ 14 days. PH3+ image of DAPI+ cells with blue representing total nuclei, PH3+ cells in CY3, and GFP for induction of Gal4.

(G) Graph showing control and experiment groups. *T-test p-values: [57409ECC15/Wecc15 p = 0.05] [0574095%/W5% p = 0.005] [57409ecc15/5% p = 0.002]*. Mitotic figures taken after knockdown of Tfb1 in visceral muscle with 4.5 h stress from ECC15 exposure @ 14 days. PH3+ Image of DAPI+ cells with blue representing total nuclei, PH3+ cells in CY3, and GFP for induction of Gal4.

Chapter 3: Tfb1 Discussion

3.6 Endogenous Repair of Damaged DNA with Age

Autonomous NER phenotype knockdown of Tfb1 in EBs increased mitotic figures during ECC15 exposure, suggesting that with knockdown of Tfb1 expression in progenitor cells, a greater proliferation rate change was observed during stress than in the absence of stress. When cells accumulate DNA damage, NER is initiated to remove and fix the damage. Tfb1 works with other factors in the NER to responsibly unravel DNA so that DNA repair proteins can bind to the target DNA area for excision. When DNA damage occurs in progenitor cells, they cannot unwind the DNA for repair, which may arrest the cell cycle at G2 or may not be recognized by the NER proteins. Without Tfb1, DNA with genetic changes is replicated, which can lead to changes in endogenous growth rates similar to those observed with aging.

In a similar experiment to knockdown of Tfb1 in EBs, we also showed that the knockdown of Tfb1 in ISCs increases mitotic figures during ECC15 assay for proliferative stress. These findings suggest an importance for NER in the regulation of tissue specific regeneration shaped by ISC activity during stress responses. The results prompted us to further investigate the knockdown of Tfb1 in both ISCs and EBs at the same time to reinforce the phenotype that demonstrated an increase in proliferation rate in the guts of young healthy adult flies when not under a proliferative stress condition. Our results suggest that a systemic NER phenotype is present when Tfb1 is ubiquitously knocked down. The increase in proliferation does not directly indicate which physiological response is mediating the changes in proliferation; however, this experiment plausibly creates a correlation between the regenerative capacity of the fly intestine.

I predict that we may have a genetically accessible inducer of indirect DNA damage that we can use to modulate the rate of proliferation autonomously or to arrest the cell cycle. Lastly, ubiquitous knockdown and overexpression have no obvious effects on lifespan (data not shown). Aside from a systemic phenotype, we also demonstrated a paracrine phenotype shown in the Tfb1 knockdown in the visceral muscle. This finding suggests unknown, naturally occurring DNA damage that essentially goes unrepaired. I speculate that the DNA damage is generating a change in the expression of ISC self-renewal secreted factors, further suggesting a paracrine phenotype.

Chapter 3: Dys and Rols Results

3.7 Potential Commensal Bacteria Homeostasis Genes Rolling Pebbles Affects Gut Barrier Integrity

Cell-to-cell adhesion in *Drosophila* is regulated by proper cytoskeleton arrangement because of myoblast founders interacting with fusion-competent myoblasts (Pütz, 2005) (Menon, 2005). Studies have shown that Rols has a responsibility in Malpighian tubule formation, and a mutation in this gene causes a strong loss in myoblast fusion (Rau A1, 2001) (Pütz, 2005). Recent studies aimed at combatting the advancement of Rhabdomyosarcoma (RMS) have given a translational significance to characterizing the functional responsibility of genes like Rols in *Drosophila* barrier function. RMS is a malignancy of primarily striated tissue and affects the endogenous cell cycle mechanisms leading to impairment of proper differentiation of smooth, connective, and skeletal muscle cells; however, the pathogenic effects are unknown (Avirneni-Vadlamudi, 2012).

Reducing Rols expression in the intestinal epithelium may cause an increase in permeability, allowing microbes in the abdominal space surrounding the GI tract to enter and exit more freely. To test this hypothesis, the expression of Rols was reduced in ECs and

EBs, which resulted in a statistically significant increase in microbial density read out by measuring colony-forming units (Fig. 10A). To place Rols in the context of aging, I also completed a lifespan analysis by ubiquitously knocking down Rols expression, which resulted in a 22% increase in total lifespan; however, this finding was limited only to ~10% of the population (Fig. 10B). The total days of the fly life are relatively short, possibly in part because of the parental generation being older than a parental generation under natural conditions. In addition to the Rols, I also conducted a lifespan analysis of flies knocked down in Dystrophin in ECs and EBs. The results demonstrated a reduction in total lifespan; however, this result was limited to ~15% of the population.

Figure 10 | Confirming Rolling Pebbles as a Genetic Candidate for Commensal Bacteria Homeostasis
 (A) Graphical representation of knocking down Rolling Pebbles expression specifically in ECs and EBs. KD after 5 days shows a significant increase in gut flora density; *Ttest p-value* = 0.01.

(B) Lifespan curve for ubiquitous KD of Rolling Pebbles.

Chapter 3: Rolling Pebbles Discussion

3.7 Intestinal Epithelium Permeability and Gut Flora Homeostasis

The knockdown of Rols expression in ECs relative to the control increased CFUs. Previous studies have shown that when cells are dysfunctional for expressing the Rols gene, there is also a suppression of PAX-FOXO1 lethality in a *Drosophila* model, giving relevance to fusion molecules in the context of aging and cancer development (Avirneni-Vadlamudi, 2012) (Linardic, 2008). Reducing the expression of PAX-FOXO1 in myoblasts results in the up-regulation of Tanc1 (mammalian ortholog of Rols) expression, linking Rols to FOXO in mammalian systems with RMS cancer phenotypes (Avirneni-Vadlamudi, 2012) and suggesting that Rols may be a good target for studying the relationship between cell–cell junctions and lifespan extension in *Drosophila*, based upon findings that fusion molecules may be a regulatory component of stem cell activity (Linardic, 2008). My prediction is that by knocking down the expression of Rols in ECs, we may disrupt the adherens junction, leading to increased permeability of the gut barrier. Rols plays a role in supporting muscle integrity by regulating Mhcl localization at contact sites of fusing myoblasts. Furthermore, when the expression of Rols7 is reduced at the contact site, less myoblast fusion occurs (Bettina, 2013). Taken together, the preliminary results presented here strongly suggest that Rols should be a target for further analysis to understand how intestinal epithelium permeability and gut flora homeostasis may be impacted with age.

Concluding Statement

Our results demonstrate an important role for mitochondrial proteostasis, proliferative homeostasis, and commensal homeostasis in the management of aging phenotypes. The findings we have shown here represent the beginning stages for studying

new mechanisms contributing to ISC activity as a function of aging. The contribution of our proliferation studies to gerontology is to provide additional support for the concept that proliferative homeostasis debilitates with age and that currently unknown factors in this decline remain to be discovered. The commensal bacteria contribution to gerontology is the identification of regulatory genes required in gut flora management with age. In our studies, we presented new roles for known genes and functions, which may translate to improving the genetic expression profile of aging organisms. Of the hallmarks of aging, we showed that genomic instability, loss of proteostasis, mitochondrial dysfunction, and altered intracellular communication affect stem cell self-renewal and differentiation. .

Ethical Review and Approval

No mammals were harmed in these studies. All materials used from other labs were approved prior to requesting and using the item(s). All scientific findings are contributions to Professor Heinrich Jasper, PhD, the Jasper Lab, the Buck Institute for Research on Aging.

Supplemental Figures & Tables

Table 1 – Experiments from UPRmt Only Qualitative

Number	Experiment	Date	Name	Assay	P/F?	Inference/Observation	Future
1	Validation of Esg::LacZ	01082015	X-gal	X-gal	P	We can measure ISC's	Use both
2	Validation of UAS::GFP in VM	03032015	G137vsG144	Mito::GFP	P	Gal4 System is specific	All Stocks are Clean
3	Validation IHC Protocol	04012015	Mars	Delta, Prospero, Dapi, Phalloidin	P	Protocol is reproducible	Follow
4	IHC & Bgal for Esg::LacZ	03062015	Neptune	DAPI, FITC	P	Esg::LacZ using CFX	Image ISC's
5	Normalizing Delta to DAPI	08042015	Thunder F1	Delta, DAPI	P	Precision	Use Ratio
6	Inducing Known Reporters	03232015	Reporters	UAS::GFP/ UAS::LacZ	P	Oxidative Challenge	GSTD1+ UPRmt reporter
7	UPRmt in Brain via ELAV-gs	04132015	ElavLS	Lifespan	F	Flies on Ru486 + laid minimal eggs, no diff in curve	Check Genetics

Figure S.1 – Experiments from UPRmt, Only Qualitative

(A-A') G144 w; esg::LacZ, tub::G80ts/Cyo ;How::G4/TM3 G137 y(w); Tub::G80ts/Cyo;
How::Gal4,esg::LacZ/TM6b
(B-B') G144 at 18°C G144 at 29°C.
(C) Image showing the contorted morphology of a region of the PM from Cell Autonomous UPRmt Induction with phalloidin staining. Number 3 (S.1) G144 X ND75.
(D-D'') Pros+/DAPI+ showing no effect w/yw; tub::Gal80ts/+; How::Gal4, UAS-CD8::GFP/UAS-dOTC Control - W1118 - 18°Celsius UPRmt – dOTC 18°C Control - W1118 - 29°C UPRmt - dOTC 29°C.
(E) ND75 at 18 Composite with Armadillo (GFP), DI (CY3), Pros (EGFP), DAPI (Blue). This shows no change in DI+/DAPI or Pros+/DAPI in GFP. This image represents the null young guts against which the UPRmt experiments were analyzed.
(F-F') Bgal of 18 and 29°C ND75 KD.

Table 2 | Non-Statistical Experiments for Arrowhead Project

Number	Experiment	Date	Name	Assay	P/F?	Inference	Future
1	Validate Genetics for MARCM	04132015	FRT2A	GFP	P	Valid Genetics, Phenotypes	IHC
2	Awh KD in ISCs	08032015	F1	PH3, Age	F	FP	Multi-viewers
3	Awh KD in EBs	08032015	F1	PH3, Age	F	FP	Multi-viewers
4	Awh KD in ISCs	07142015	N/A	Ecc15, PH3	F	Ecc15 incorrect, Mock NS	Detail
5	60+ Clone Size	09082015	Model	DAPI, DI, PROS, GFP, Age	P	Cancer Model	Trend Developed
6	UB KD of Awh	10062015	DA31772	Survival @ 24h Post Ecc15	P	Awh maybe Qui<->Act.	Time Curve 0h-24h of Ecc15

Figure S.2 | Table of Non-Statistical Experiments for Arrowhead Project

(A-A') Validate genetics for MARCM2A X Awh Mutant Experiment (Number 1 of Fig S.5) FRT2A Awh 36501.

(B-B') Lineage analysis of Awh mutant 15 days' post heat shock. Recombination: (Number 5 of S.5) hsFLP, UAS::GFP, Ac::tGal4/FM6;;FRT2A, Tub::G80/P{FRT(whs)}2A, Awh16 – FRT2A 36501- Awh Mutant.

(C) Statistically significant increase in avg. GFP+/Clone.

(D) Statistically significant reduction in avg. Clone/Gut.

(E) Data binning to generate curve with R^2 values for comparison.

(F-F') Large clone generated with Awh mutant FRT clonal analysis MARCM2A producing clones of 60+ GFP+ cells at 15 days' post heat shock. hsFLP, UAS::GFP, Ac::tGal4/FM6;;FRT2A, Tub::G80/P{FRT(whs)}2A, Awh16 control – FRT2A 36501 – Awh mutant.

Table 3 - Table of Non-Statistical Experiments for Dnc (Dunce) Project

Number	Experiment	Date	Name	Assay	P/F?	Inference	Future
1	Validate FRT19a Genetics	05122015	F1FRT19a	DAPI, DI, PROS, GFP	P	Phenotype	Repeat
2	KD of Dnc 53344 in ECs	06012015	NPI	Ecc15, no Mock, PH3+	F	Control, Ecc15 NS	Pass
3	Validate Protocol to Form Clones	12122015	2FRT19a	DAPI, DI, PROS,	F	No Recombination Event	Success W/ Cordero Protocol HS 2X for 1h, assay

Figure S.3 - Non-Statistical Experiments for Dnc (Dunce) Project

(A-A') Validation of MARCM19a X FRT19a protocol and genetics for Dnc clonal analysis. Number 3 (Fig S.3) hsFLP, FRT19Gal80/Dnc221b P{neoFRT}19A, Gal4, Tub::8al, GFP/+ clonal analysis of Dnc mutant in FRT19a Line FRT19a 7396 Dnc1.

(B) Large clone generated by Dnc mutant containing multiple ISCs.

Table 4| Table of Non-Statistical Experiments for Nucleotide Excision Repair

Number	Experiment	Date	Name	Assay	P/F?	Inference	Future
1	KD of Tfb1 in ECs	10112016	NPI	Ecc15 -> 9h, Survival	P	Tfb1 Expression required for Stress Response	Assay Time Dependent
2	UB OE of Tfb1 2X	09282016	DA	Ecc15 -> 9h, Survival	P	Tfb1 Activity necessary for Stress Response	Rescue

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