5-1-2014

Indy Encodes a Regulator of Intermediary Metabolism that Modulates Longevity in Drosophila melanogaster

Ryan P. Rogers
rrogers@student.uchc.edu

Indy Encodes a Physiological Regulator of Intermediary Metabolism that Modulates Longevity in *Drosophila melanogaster*

Ryan Patricia Rogers, Ph.D.

University of Connecticut, 2014

Abstract

The *Drosophila I'm Not Dead Yet (Indy)* gene encodes a plasma membrane transporter of Krebs cycle intermediates, with robust expression in tissues associated with metabolism. Reduced INDY alters metabolism and extends longevity in a manner similar to caloric restriction (CR) with few biological tradeoffs; however, little is known about the tissue specific physiological effects of INDY reduction or how such changes contribute to fly health and longevity. The experiments described in this dissertation are focused on the effects of INDY reduction in the *Drosophila* midgut due to the importance of intestinal tissue homeostasis in healthy aging and longevity. The expression of *Indy* mRNA in the midgut was found to change in response to aging and nutrition. Genetic reduction of *Indy* expression leads to increased midgut expression of the mitochondrial regulator *spargel/dPGC*-1, which is accompanied by increased mitochondrial biogenesis and reduced reactive oxygen species (ROS). These physiological changes in the *Indy* mutant midgut preserve intestinal stem cell (ISC) homeostasis and are associated with healthy aging. The data suggest INDY may be a physiological regulator that modulates intermediary metabolism in response to changes in nutrient availability and organismal needs. Genetic studies confirm that *dPGC-1* mediates regulatory effects of INDY, as illustrated by lack of longevity extension and ISC homeostasis in flies with mutations in both *Indy* and *dPGC1*. Together, the data support a model of INDY as a physiological regulator that is responsive to aging and nutritional conditions, which acts by modulating *dPGC-1*.

Indy Encodes a Physiological Regulator of Intermediary Metabolism that Modulates Longevity in *Drosophila melanogaster*

Ryan Patricia Rogers

B.S., Wagner College 2009

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of Doctor of Philosophy

at the

University of Connecticut

2014

APPROVAL PAGE

Doctor of Philosophy Dissertation

Indy Encodes a Physiological Regulator of Intermediary Metabolism that Modulates Longevity in *Drosophila melanogaster*

Presented by
Ryan Patricia Rogers, B.S.

Major Advisor_____
Dr Blanka Rogina

Associate Advisor_____
Dr James (Yuanhao Li

Associate Advisor_____
Dr William Mohler

Associate Advisor_____
Dr Gordon Carmichael

University of Connecticut
2014

For all strong females, especially my mother, who led by example, taught me to be strong and inspired me in my never-ending pursuit of success.

Acknowledgments

This chapter of my life has been unpredictable and filled with challenges. There are many individuals who provided valuable insight into surviving graduate level science and took an active interest in my personal success. Dr. Blanka Rogina has been an inspiring mentor and I am grateful that she allowed me to work under her guidance. She is passionate about her work and instills immense pride in all that she does. I would like to thank Jared Woods, for his camaraderie during our shared time in the Rogina lab; he provided balance and humor when it was greatly needed. I would also like to acknowledge the past and present members of the Rogina lab and summer rotation students for they always provided extra hands and unwavering encouragement.

The help and guidance of my thesis committee is immeasurable. Dr. James Li and Dr. Gordon Carmichael not only provided access to essential equipment but also put forth challenges that greatly helped in the approach and execution of my project. I would like to acknowledge Dr. William Mohler for his guidance during my graduate studies. He was instrumental in my advancement through the program and also shared my enthusiasm for scientific educational outreach.

The Genetics and Developmental Biology Department encompasses an impressive group of talented people. I was fortunate to work with many of them at various points during my career. The administration is invested and personable, which promotes a welcoming working environment. Despite his many institutional and statewide commitments, Dr. Marc Lalande has always maintained a genuine interest in my future pursuits and contributed greatly to my progress. Dr. Stormy Chamberlain is an incredible individual, and I am lucky to have met her by chance while we were sharing lab space. She has provided motivation, inspiration and friendship,

for which I am extremely thankful.

I would also like to acknowledge my previous mentor, Dr. Ammini Moorthy, for igniting my passion for and desire to teach genetics. She is an inspirational woman and an exceptional educator that taught me the importance of enthusiasm and integrity. We have maintained a valuable friendship over the years, and I hope that can enlighten and excite my future students with the same vigor that she bestowed upon me.

I began my Ph.D. with the promise that I would neither become engulfed in work nor let it cloud my perspective. Thanks to a strong network of friends and family, I am proud to say that I have kept this promise. My parents and younger brother never let me forget the importance of being kind, being humble and working hard. In times of doubt or difficulty my family was a constant source of support, wisdom and good old-fashioned craic. My friends are a constant reminder that good fun always outweighs a bad experiment and distance is just an excuse to explore. Finally, it is essential to thank Matthew Hammond and Diane Gantenhammer. These two people were the ultimate support system, and I truly cannot express how much their love, companionship and faith has helped me over the past few years. The people in my life are what make it worthwhile, and I am eternally grateful to all of them for their unconditional support and encouragement.

The National Institute of Health (AG023088) funded this work. It is with great pride and humility that I submit this dissertation to the Department of Genetics and Developmental Biology at the University of Connecticut in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the field of Biomedical Science.

Indy* Encodes a Physiological Regulator of Intermediary Metabolism that Modulates Longevity in *Drosophila melanogaster

Ryan Patricia Rogers

TABLE OF CONTENTS-*Chapter Listing*

Indy* Encodes a Physiological Regulator of Intermediary Metabolism that Modulates Longevity in *Drosophila melanogaster

Ryan Patricia Rogers

TABLE OF CONTENTS-*Section Listing*

List of Tables

List of Figures

List of Abbreviations

ADP: Adenosine di-phosphate

AMPK: Adenosine monophosphate-activated protein kinase

ap: apical

ATP: Adenosine 5' tri-phosphate

Bgal: Beta galactosidase

bl: basal

cDNA: Complimentary DNA

COI: *Cytochrome c oxidase*

CR: Caloric restriction

D: Delta ligand

DAPI: 4',6-diamidino-2-phenylindole

dPGC-1 : Drosophila Peroxisome Proliferator Gamma Cofactor 1

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dilp: Drosophila insulin like peptide

EB: Enteroblast

EC: Enterocyte cell

EE: Enteroendocrine Cell

esg: escargot

ETC: Electron transport chain

FOXO: Forkhead Box protein O1

GAL4: Yeast transcription fusion protein Gal4

GAPDH: glyceraldehydes 3-phosphate dehydrogenase

GFP: Green fluorescent protein

GstE1: Glutathione S transferase E1

GstD5: Glutathione S transferase D5

HC: High caloric

HFD: High fat diet

IIS: Insulin-IGF1 signaling

Indy: I'm Not Dead Yet

InR: Insulin receptor

ISC: Intestinal stem cell

JNK: Jun-N-terminal Kinase

l(3) neo 18: neo

mIndy: mammalian I'm Not Dead Yet

TOR: target of rapamycin

mtDNA: mitochondrial DNA

mRNA: messenger RNA

NAD: Nicotine amide

NADH : Nicotinamide adenine dinucleotide

ND23: *NADH:ubiquinone reductase 23kD subunit precursor*

ND42: NADH:ubiquinone reductase 42kD subunit precursor

ND75: NADH:ubiquinone reductase 75kD subunit precursor

OXPHOS: oxidative phosphorylation

Pdsw: NADH-ubiquinone oxidoreductase, subunit 10

PBS: Phosphate buffer saline

PBT: 0.1%Triton X-100 in phosphate-buffered saline

pH3: Phosphorylated histone 3

qPCR: Quantitative polymerase chain reaction

RNAi: RNA interference

ROS: Reactive oxygen species

RNA: Ribonucleic acid

SE: Standard error

SEM: Standard error of the mean

SD: Standard deviation

Sir2: Silent Information Regulator 2

SIRT1: Silent Information Regulator T 1

SLC13: solute carrier 13 transporter protein

TCA: Tricarboxylic acid cycle

UAS: Upstream activator sequence

w/v: weight per volume

yw: yellow-white

Chapter 1

Genes, metabolism and aging

1. Introduction

Aging is a complex biological process that has evaded definition due to its progressive nature and molecular complexity. The process of aging cannot be completely halted; therefore, research focuses on the mechanisms that contribute to biological aging and revealing potential avenues for intervention. For instance, reduction of caloric intake has remained the most effective intervention to preserve cellular functional efficiency and promote longevity in a myriad of species (Weindruch et al. 1988). Caloric restriction (CR) is thought to work predominantly through nutrient sensing pathways to alter intermediary metabolism and promote energy balance (Barja, 2013; Guarente, 2008; Lopez-Lluch et al., 2006; Zhu, 2004). The benefits associated with CR are well defined and have been shown to improve aging in multiple systems; however, meeting nutritional requirements under this regimen is challenging outside of a controlled lab environment. For this reason, emphasis has been placed on deciphering the downstream genetic targets of CR, in effort to identify genes involved in mediating longevity.

Indy (*I'm Not Dead, Yet*) encodes a plasma membrane protein that transports Krebs' cycle intermediates across tissues associated with intermediary metabolism (Knauf et al., 2002; Fei et al., 2003; Knauf et al., 2006; Birkenfeld et al., 2011). Reduced *Indy*–mediated transport extends longevity in worms and flies by decreasing the uptake and utilization of nutrients and altering intermediate nutrient metabolism in a manner similar to CR (Rogina et al., 2000; Knauf et al., 2002; Fei et al., 2003; Fei et al., 2004; Neretti et al., 2009; Wang et al., 2009; Rogina & Helfand, 2013).

1.2 Caloric restriction and longevity extension

1.2.1 An overview of aging and CR

Genetic and environmental factors influence the rate of aging and susceptibility to age-related syndromes. Current research focuses on the processes that contribute to biological aging in order to reveal potential avenues for intervention. CR is the most successful intervention to delay the onset of age-related pathologies and extend longevity in a myriad of species (Guarente, 2008; Lopez-Lluch et al., 2006; Nisoli et al., 2005; Rizza et al., 2013). CR is thought to impose a low level of stress that activates multisystem stress responses that protect against age-related functional decline (Ingram et al., 2006). There are many hypotheses surrounding the mechanistic underpinnings of CR; however, definitive support for a central mechanism that is capable of facilitating such diverse effects has yet to be confirmed.

Mutations in certain genes are able to mimic beneficial effects of CR with few biological tradeoffs; therefore studying the molecular genetics of aging will allow researchers to direct and develop novel therapies to combat age-related pathologies. The cumulative nature of aging, coupled with genetic variation, makes it difficult to conduct long-term studies on complex eukaryotes. Investigation of genes involved in the aging process has been made in genetically tractable model organisms such as yeast, worms, flies and mice. These genetic studies have established aging as a genetically regulated process that has a global impact on signal transduction, physiology and other biological processes. Moreover, evolutionary conservation of genes reveals potential points of intervention that can be further investigated in more complex organisms (Arslan-Ergul, 2013).

1.2.2 Nutrient sensing pathways and CR

CR is associated with robust changes in cellular and organ metabolism that are thought to

promote longevity. Nutrient sensing pathways have been implicated in the downstream response to CR due to high evolutionary conservation of molecular components and efficient transduction of stress responses (Dilova et al., 2007; Ingram et al., 2006; Jiang et al., 2009; Lee and Min, 2013; MacNeil et al., 2013; Zhu, 2004). Insulin/IGF-1-like signaling (IIS) and target of rapamycin (TOR) signaling pathways converge to regulate growth and development by responding to the energetic and metabolic status of the body (Dilova et al., 2007; Lee and Min, 2013; Long et al., 2014; Zhu, 2004). Although both of these pathways monitor nutrients, the IIS pathway works through an endocrine signal that monitors the nutrient status of the whole organism, whereas the TOR pathway monitors the intracellular nutrient status.

IIS and TOR signaling pathways promote growth and development by responding to nutrient availability and regulating metabolism; however there is evidence that they are also highly involved in regulating stress response (Dilova et al., 2007). Invertebrate and mammalian model organisms on a calorically restricted diet both exhibit decreased IIS and TOR signaling (Dilova et al., 2007; Lee and Min, 2013; Zhu, 2004), which is thought to represent an innate survival adaptation. Moreover, both pathways are modulated by AMPK, a highly conserved kinase responsible for regulating cellular energy homeostasis. AMPK phosphorylation activity leads to enhanced regulation of specific target genes involved in stress resistance and changes in energy metabolism(Cheng et al., 2010; Long et al., 2014) Genetic manipulation of IIS and TOR pathway activity results in increased oxidative stress resistance and lifespan extension, both of which are also observed in CR and support pathway activity as a downstream response to reduced nutrient availability.

1.3 The *Drosophila* midgut and intestinal stem cells

1.3.1 The Midgut is regenerated by multipotent intestinal stem cells

The *Drosophila* midgut is a key site for nutrient metabolism in flies. Similar in structure to the mammalian intestine (Figure 1.1), this tissue is highly regenerative and separated into distinctive regions based on physiological function (Singh et al., 2012; Buchon et al., 2013; Marianes and Spradling, 2013). Resident, tissue-specific stem cells have been reported in invertebrate and mammalian tissue (Casali and Batlle, 2009). Such cells serve both to maintain normal homeostasis and to respond to injury or disease by repairing or replacing damage tissue (Apidianakis and Rahme, 2011; Casali and Batlle, 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The recent characterization of such regions has provided insight into the variation between morphology and density of basally located intestinal stem cell (ISC) populations. While ISCs reside throughout the entire midgut, they vary regionally in behavior and gene expression, thereby sustaining subregion specialization (Marianes and Spradline, 2013; Buchon et al., 2013).

Despite these differences, the general proliferation patterns of ISCs are the same between individuals. ISCs respond to signals from neighboring cells, injury, pathogens or changes in environmental changes, which results in either symmetric or asymmetric division (O'brien et al., 2011). Symmetric division is associated with non-homeostatic growth in effort to expand organ size to account for metabolic demands (O'brien et al., 2011). Asymmetric division is more common, and results in generation of an immature progenitor enteroblast (EB). Both the ISC and EB retain the transcription factor *escargot (esg)*, thus *esg*-positive cells reflect all undifferentiated cells throughout the midgut. Differntiation is dictated by Notch signaling between the ISC and EB, which results in EB differentiation into either an enterocyte or

enteroendocrine cell(Ohlstein and Spradling, 2006; Micchelli and Perrimon, 2006; Ohlstein and Spradling et al., 2007 and Figure 1.2). Polyploid enterocytes and comprise ~90% of the intestinal cell population, stemming from ISCs with high levels of the Notch ligand Delta (Ohlstein and Spradling et al., 2007; Singh et al., 2012). Conversely, enteroendocrine cells constitute only 10% of midgut cells and can be traced to ISCs with low levels of Delta (Ohlstein and Spradling et al., 2007; Singh et al., 2012). Together these cells coordinate nutrient uptake, utilization and removal thereby modulating nutritional status of the organism.

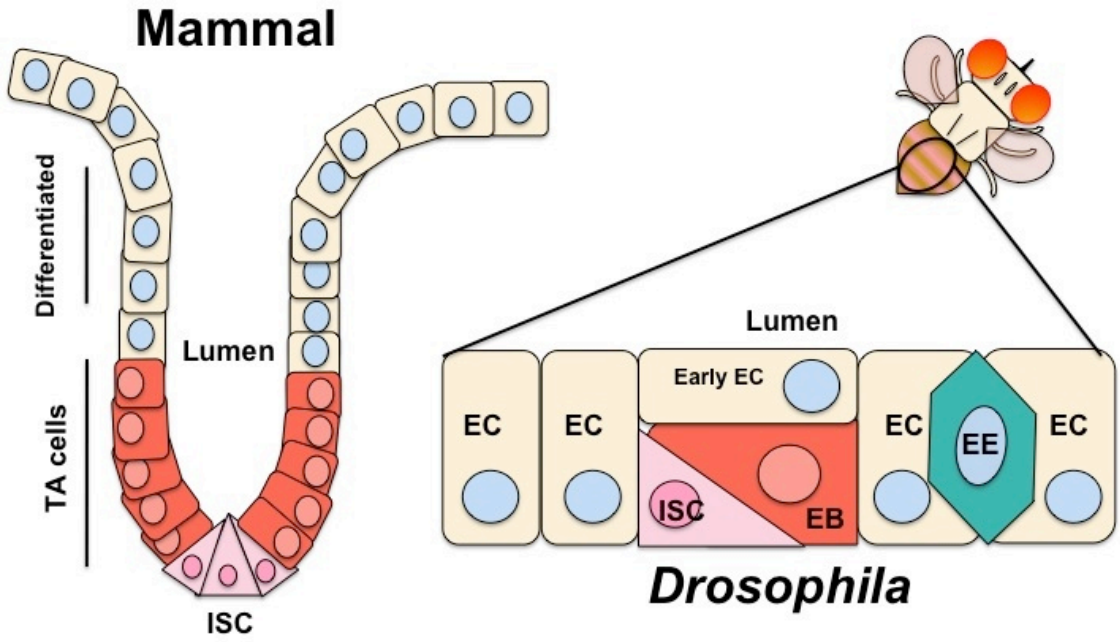

Figure 1.1: The *Drosophila* midgut is structurally similar to the mammalian small intestine. ISCs reside in the basal region of the midgut and divide asymmetrically yielding an identical daughter ISC and an immature progenitor cell known as an enteroblast (EB) in flies or transient amplifying (TA) cells in mammals. ISCs have the ability to divide, but do not directly differentiate. EBs are diploid cells that move toward regions of recent apoptosis to differentiate into either absorptive enterocyte (EC) or secretory enteroendocrine (ee) cells (Adapted from Casali & Batlle, 2009).

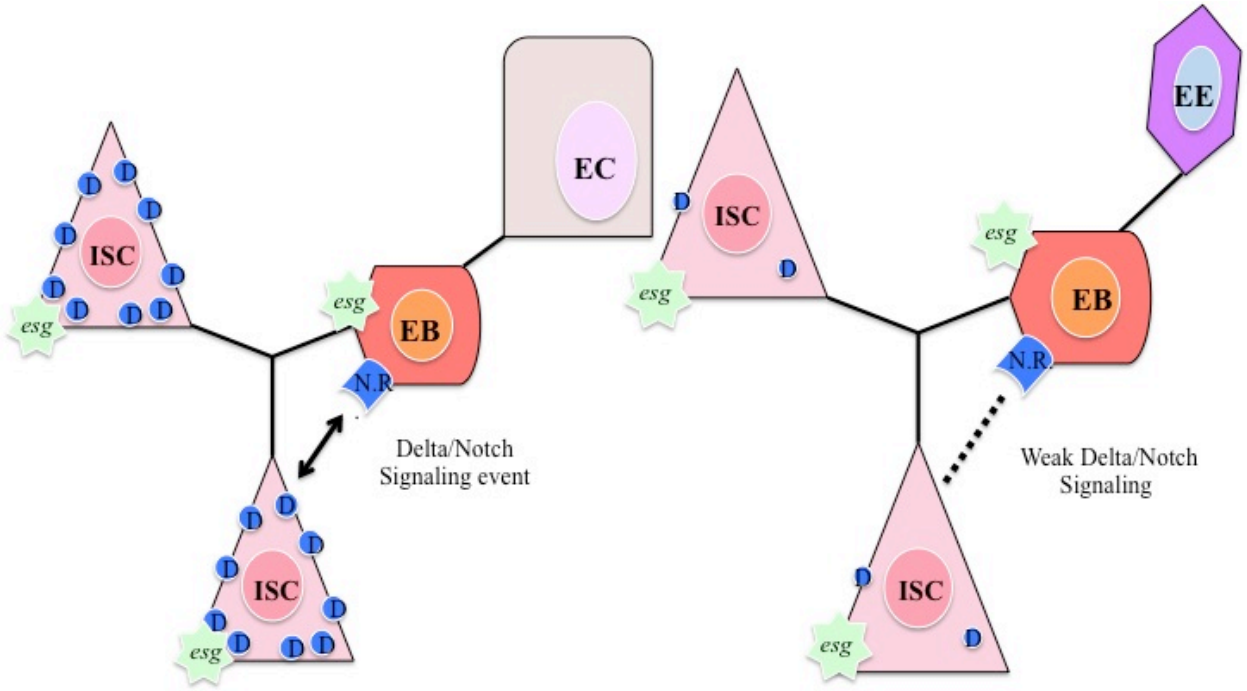

Figure 1.2: Differentiation patterns of Intestinal stem cells (ISCs) in the midgut. ISCs undergo asymmetric division to yield an identical daughter ISC and an immature enteroblast (EB) with differentiation potential. ISCs with Delta rich vesicles upregulate Notch signaling and drive enterocyte (EC) differentiation (left). ISCs with few Delta vesicles have weak Notch signaling and drive enteroendocrine (EE) differentiation (right).

1.3.2 Intestinal Stem Cell regulation and aging

Regulation of ISC proliferation and differentiation is of great importance with respect to organismal health and longevity. As a key site of intermediate metabolism in the fly, the midgut can directly influence health status by modulating nutrient absorption and metabolism. For this reason, sustained tissue homeostasis is directly related to longevity. One of the fundamental aspects of aging is a change in tissue structure and functional efficiency. With respect to the midgut, such changes can be catastrophic to organismal nutrient metabolism and accelerate the onset of mortality.

Age-related accumulation of mitochondrial reactive oxygen species (ROS) reduces the regulation of ISC proliferation patterns (Biteau et al., 2011a; Biteau et al., 2010; Choi, 2008; Hochmuth et al., 2011; Rera et al., 2011). This causes an unfavorable imbalance between ISC division and EB differentiation into mature EC or EE cells. The lack of regulated intestinal homeostasis is marked by an age-related increase in immature and misdifferentiated cell types in aged *Drosophila* midguts (Biteau et al., 2010; Choi, 2008). As a result, large populations of ISCs, known as aggregates, accumulate in the gut as flies age creating a toxic environment that induces premature aging. These conditions impair metabolic tissue function and contribute to the onset of age-related diseases (Biteau et al., 2010; Choi, 2008; Hochmuth et al., 2011; Jasper and Jones, 2010; Kennedy, 2012; Rera et al., 2011; Rogers, 2012).

ISCs in the *Drosophila* midgut are responsive to changes in nutrient availability (CR), alterations in redox environment and manipulation of nutrient sensing pathway components (Biteau et al., 2010; O'brein et al., 2011; Choi et al., 2011). Interestingly, all of these manipulations are intertwined with nutrient availability being the central factor in downstream intermediary metabolic activity and bioenergetic efficiency. ISC have plastic energetic

requirements during proliferation and differentiation in effort to produce maximum energy without generation of harmful byproducts (Panopoulos et al., 2012; Zhang et al., 2012). In that likeness, regulated bioenergetic efficiency is crucial for ISC functional efficiency and disruption via ROS or dietary toxins can have deleterious effects on tissue homeostasis (Jasper et al., 2010; Panopoulos et al., 2012; Zhang et al., 2012). For this reason, interventions, which promote mitochondrial health and bioenergetic efficiency at the cellular level, are associated with preserved intestinal stem cell homeostasis and subsequential healthy aging.

1.3 *I'm Not Dead Yet*, genetic CR

1.3.1 INDY is an intermediate transporter

The *Drosophila Indy* gene encodes a plasma membrane transporter of Krebs cycle intermediates (Knauf et al., 2006; Knauf et al., 2002; Rogina and Helfand, 2013; Rogina et al., 2000). INDY is mainly found in the fat body, midgut and oenocytes where it facilitates bidirectional exchange of Krebs cycle intermediates independent of sodium, potassium and chloride (Knauf et. al 2002; Knauf et. al 2006). Functional studies in *Xenopus* reveal INDY as having a high affinity for citrate, succinate, alpha-ketoglutarate, glutarate, and fumarate uptake, suggesting that it plays an active role in regulation of intermediary metabolism in *Drosophila* (Knauf et. al 2002; Knauf et al. 2006). Moreover, INDY is part of the SLC13 protein family, with high homology to *ceNaDC1 and ceNaDC2* in *C. elegans* and the Na+-coupled SLC13A5 citrate transporter gene in mice (Birkenfeld et al., 2011; Frankel and Rogina, 2012; Knauf et al., 2006; Rogina and Helfand, 2013). INDY proteins differ with respect to transport mechanics between species; however, they all function in dicarboxylate and tricarboxylate flux during intermediary metabolism, suggesting high evolutionary functional conservation.

1.3.2 Indy mutation and longevity

Reduction of *Indy* gene activity in fruit flies, and homologs in worms, extends lifespan by altering energy metabolism in a manner similar to CR (Rogina et al., 2000; Fei et al., 2003; Fei et al., 2004; Neretti et al., 2009; Wang et al., 2009; Birkenfeld et al., 2011; Rogina & Helfand, 2013). *Indy* mutant flies on regular food share many characteristics with CR flies and do not have further longevity extension when aged on a CR diet (Wang et al., 2009; Toivonen et al., 2007; Frankel & Rogina, 2012). Furthermore, the *Indy* mutant mice, *mINDY*$^{-/-}$, on regular chow share 80% of the transcriptional changes obtained with CR mice, supporting a conserved role for INDY in metabolic regulation that mimics CR and promotes healthy aging (Birkenfeld et al., 2011). We postulate that enhanced energetic homeostasis sufficiently creates a state of genetic CR in *Indy* mutants and extends lifespan.

Reduced INDY alters metabolism (CR) with few biological tradeoffs; however, little is known about the physiological effects of INDY reduction or how such changes contribute to fly health and longevity. INDY regulates cytoplasmic citrate, which can also be broken down to malate and transported to the mitochondria to be used in the TCA cycle. Reduction of INDY transport activity thus reduces mitochondrial substrates necessary for energy production and may activate cellular energy sensors to compensate for energy deficits. Activation of AMPK is triggered by altered ADP/ATP ratio as a result of reduced ATP synthesis and observed in CR (Lopez-Lluch et al., 2006; Nisoli et al., 2005; Wallace, 2005). AMPK activation is related to increasing insulin sensitivity, lipid-oxidation and synthesis and mitochondrial biogenesis via the transcriptional co-activator *dPGC-1/spargel*. *dPGC-1/spargel* is the *Drosophila* homolog of mammalian PGC-1, which promotes mitochondrial biogenesis by increasing the expression of genes encoding mitochondrial proteins (Puigserver & Spiegelman, 2003; Gershman et al., 2006).

Upregulation of *dPGC-1* is a hallmark of CR-mediated longevity and has been previously observed in *Indy* mutants (Puigserver & Spiegelman, 2003; Lopez-Lluch et al., 2006; Neretti et al., 2009; Birkenfeld et al., 2011).

The observed increase in *dPGC-1* levels in the *Indy* mutant midgut promotes mitochondrial biogenesis and functional efficiency, representing a protective mechanism activated in response to reduced energy availability. Moreover, *dPGC-1* is involved in modulating antioxidant gene expression, which decreases ROS accumulation and protects against oxidative damage (Guarente, 2008; Lopez-Lluch et al., 2006; Nisoli et al., 2005). As a result, any increase in ROS levels, whether from mitochondrial demise or exposure to external ROS sources, can be readily metabolized to prevent accumulation of oxidative damage. Thus, enhanced ROS detoxification mechanisms induced by *Indy* reduction and subsequent elevation in *dPGC-1* contributes to enhanced health and longevity in mutant flies.

1.4 Mitochondria in aging

1.4.1 Mitochondrial dynamics

Aging is marked by a decline in mitochondrial functional efficiency, which leads to decreased bioenergetic efficiency and increased levels of oxidative damage (Guarente, 2008). The cause of mitochondrial demise is thought to stem from mitochondria themselves, as they are the main source of cellular ROS. Mitochondria conduct several oxidation reactions, called oxidative phosphorylation, to convert nutrients into usable energy in the form of ATP (Hwang, 2012). Oxidative phosphorylation occurs in the mitochondrial matrix along the electron transport chain (ETC). This mode of energy production is more efficient than glycolysis or fermentation in terms of maximum ATP output; however, electron transport becomes less efficient with age and can leak electrons (Nisoli et. al 2005, Chan 2006, Hwang, 2012). Electron leakage can cause

premature oxidative reduction, which damages nearby cells and accelerates the process of age-related decline. Mitochondria exist as dynamic organelles that use metabolic substrate to produce energy. Mitochondrial dynamics were initially thought to represent a quality control mechanism that uses fission and fusion events to transfer cellular components or remove damaged material. However, mitochondrial dynamic properties can also become activated in response to metabolic demands as a form of bioenergetic adaptation (Liesa and Shirihai, 2013).

Given the importance of mitochondrial function in intermediary metabolism and energy balance, optimization of such dynamic properties may represent an important regulatory event that contributes to aging.

Nutrient excess leads to an inhibition of fusion, and interruption of normal mitochondria quality control mechanisms (Figure 1.3). This results in accumulation of dysfunctional mitochondria that cannot properly oxidize nutrients, thereby decreasing bioenergetic efficiency. Conversely, limited nutrient conditions result in increased mitochondrial elongation and cristae conformation (Figure 1.3; Liesa and Shirihai 2013). Changes in the cristae structure may allow for increased nutrient import and subsequent oxidation to increase bioenergetic efficiency when nutrient substrate is scarce (Liesa and Shirihai, 2013). Such hypothesis can be applied to the physiological changes that result from CR. Reduced caloric consumption, without starvation, induces changes in mitochondrial physiology and architecture that are associated with elevated bioenergetic efficiency (Liesa and Shirihai, 2013; Schlicker et al., 2008; Tranah, 2011; Wallace, 2005). Given the relationship between nutrient availability and mitochondrial dynamics, it is possible that interventions interfering with intermediate nutrient metabolism can have substantial effects on energy supply and demand, which in turn influence health and longevity.

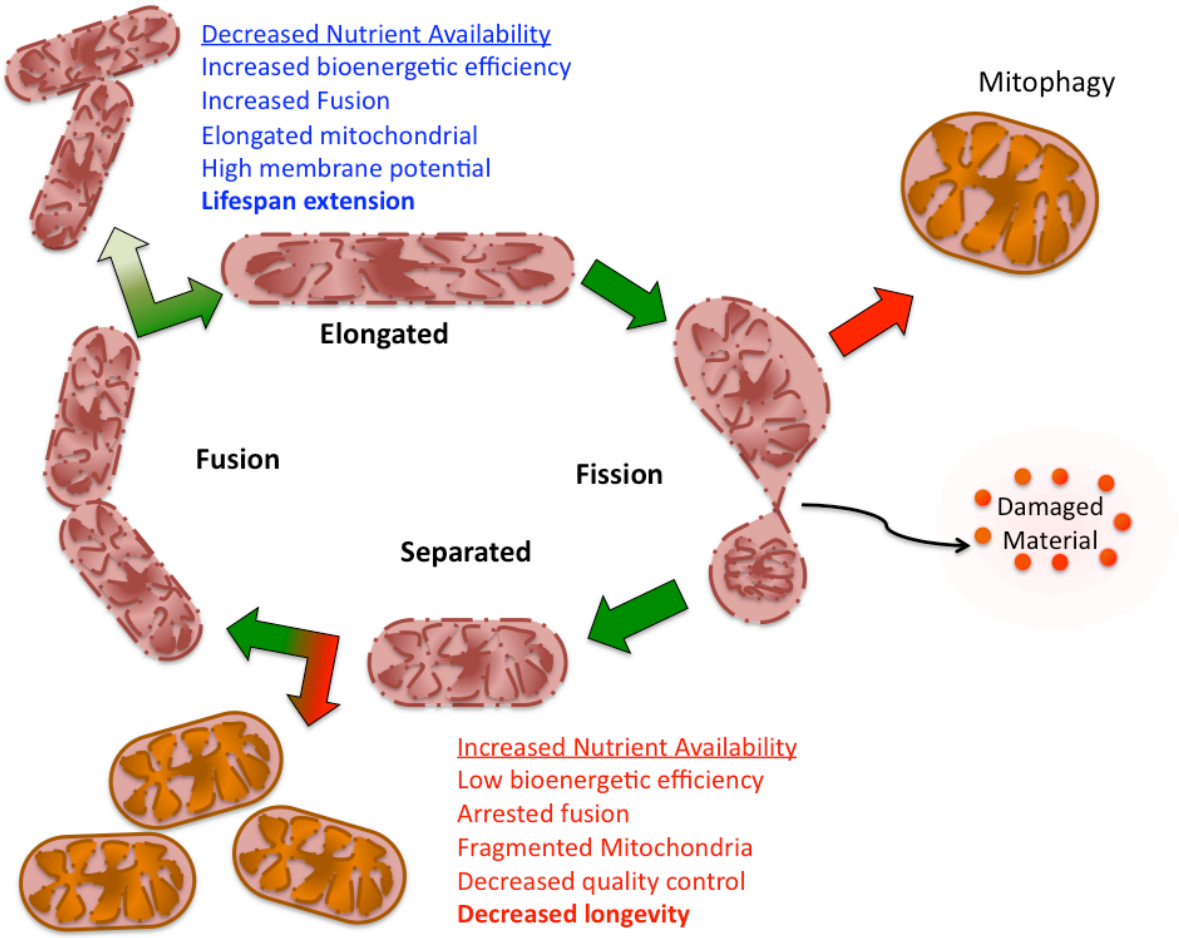

Figure 1.3: Mitochondrial dynamics. Mitochondria alter their structure as a way to maintain quality control. Adaptation to excess nutrient environment interferes with quality control functions and, as a result, affects mitochondrial functional efficiency. High caloric diets inhibit mitochondrial fusion, which leads to low bioenergetic efficiency, increased free radical production and inability to remover damaged material. Such conditions are associated with decreased longevity. Low caloric diets are associated with elongated mitochondria, high mitochondrial membrane potential, increased bioenergetic efficiency and healthy aging (Adapted from Liesa and Shirihai 2013).

1.4.2 Mitochondrial decline with age

Mitochondria pump protons across an electrochemical gradient as electrons are transferred to generate ATP during oxidative phosphorylation(Chan, 2006; Feng et al., 2002; Guarente, 2008). This series of reactions occurs along four respiratory enzyme complexes located in the inner mitochondrial membrane, known as the electron transport chain (ETC). Complexes III and I become less efficient with age, resulting in leakage of electrons and generation of ROS (Copeland, 2009). Low levels of ROS production are inevitable during oxidative phosphorylation and are required for some cellular processes. However, dramatic elevation of free radical production, as a result of decreased ETC functional efficiency, causes cumulative cellular damage that is thought to contribute to aging.

Mitochondrial DNA (mtDNA) is proximally close to the ETC, making it vulnerable to free radical mutation. Since mitochondria lack excision repair machinery, DNA mutations cannot be properly removed and therefore accumulate in the mitochondrial genome (Chan, 2006; Long et al., 2014). Increased mutation frequency in mtDNA is thought to propagate a "vicious cycle," in which mutated mtDNA encode defective ETC transport machinery, increasing the likelihood for additional ROS production, leading to further mtDNA mutation and deleterious changes to mitochondrial complex activity. Recently an inverse correlation between mtDNA damage and maximum longevity was described, suggesting that mitochondrial health is a key determinant of aging (Long et al., 2014).

1.4.3 Mitochondria, CR and sirtuins

Limiting caloric intake decreases the availability of mitochondrial substrates and thereby alters the conversion of metabolic substrates into chemical energy. By reducing input into mitochondria via CR, the rate of oxidative phosphorylation is also reduced, which decreases

ROS emission and oxidative damage. In order to compensate for decreased mitochondrial substrate and energy production, stress response mechanisms are initiated to promote mitochondrial biogenesis. Increasing mitochondrial density results in sustained bioenergetic efficiency without consequential elevation of oxidative damage.

The highly conserved family of sirtuin nicotine amide (NAD)-dependent histone deacetylases are elevated during CR and are associated with regulating downstream expression of other mitochondrial genes (Guarente, 2008; Lopez-Lluch et al., 2006; Someya et al., 2010; Verdin et al., 2010). The relationship between sirtuins and aging was initially studied in yeast by observing the longevity effects related to *Sir2* mutation (Guarente, 2006; Kenyon, 2005). It was found that *Sir2* activity increased during CR and that the *Sir2* gene is essential for the observed extension in life span by CR (Lin et al. 2000, 2002; Anderson et al. 2003). In that likeness, targeted overexpression of *Sir2* yields significant lifespan extension not only in yeast, but also in worms and flies when the homolog is overexpressed. Additionally, manipulation of the mammalian SIRT1 homolog was shown to beneficially influence the rate of aging, suggesting an evolutionary conserved role in longevity (Guarente 2006).

The mechanism of sirtuin-mediated longevity is not completely understood, however analysis of downstream targets provides a potential avenue for exploration. The mitochondrial transcriptional coactivator, Peroxisome Proliferator-activated receptor Gamma Coactivator 1α (PGC-1α), is one of the substrates of the SIR2 ortholog, SIRT1 (Guarente, 2008; Kaeberlein et al., 2005). Energy homeostasis is highly regulated by the PGC-1α family of transcriptional proteins, which promote mitochondrial biogenesis and respiration across species (Guarente, 2008; Lopez-Lluch et al., 2006; Nisoli et al., 2005). Ubiquitous upregulation of *dPGC-1*, the *Drosophila* PGC-1α gene, sufficiently increased mitochondrial activity in aging flies*;* however

extension of lifespan is only observed in a midgut-targeted manner (Rera et al. 2011). Notably, flies overexpressing *dPGC-1* in progenitor cells have prolonged intestinal homeostasis and reduced dysplasia at older ages, suggesting a relationship between tissue homeostasis and energy balance.

1.5. *Indy* as a physiological regulator

CR is an environmental manipulation that decreases the amount of nutrient intake, which reduces the rate of intermediary metabolism and extends longevity. Reduced INDY alters metabolism and extends longevity in a manner similar to CR with few biological tradeoffs; however, little is known about the tissue specific physiological effects of INDY reduction or how such changes contribute to fly health and longevity. Both CR and *Indy* mutations alter the processes involved in converting metabolic products into chemical energy. Mutations in *Indy* gene function are able to mimic beneficial effects of CR with few biological tradeoffs. Rather than decreasing the amount of nutrient intake, the *Indy* mutation limits intermediary metabolism by decreasing intermediate metabolite flux across tissue to achieve a similar metabolic state to that observed in CR. Moreover, *Indy* mutant flies on a regular diet share phenotypes with calorically restricted flies, such as decreased weight gain, decreased insulin signaling, resistance to starvation, increased spontaneous physical activity, and absence of further longevity extension when aged on a CR diet (Wang et al., 2009; Toivonen et al., 2007).

Upregulation of *dPGC-1* is a hallmark of CR-mediated longevity and is thought to represent a response mechanism to compensate for energetic deficits caused by limited nutrient availability (Puigserver & Spiegelman, 2003; Lopez-Lluch et al. 2006). Increases in *dPGC-1* preserve mitochondrial functional efficiency without consequential changes in ROS. Previous analyses of *Indy* mutant flies revealed upregulation of mitochondrial biogenesis mediated by

increased levels of *dPGC-1* in heads and thoraces (Neretti et al. 2009). Moreover, research revealed that aged *Indy* mutants have reduced transcription and enzymatic activity of ETC complex I & III and reduced production of free radicals (Neretti et al. 2009). *Indy* mutant flies display reduced oxidative damage, which is thought to stem from increased efficiency in the ATP/ROS production ratio. Other shared benefits include reduction of oxidative damage and significant lifespan extension (Neretti et al. 2009).

The overall focus of this work investigates a role for *Indy* as a physiological regulator that modulates intermediary metabolism in response to changes in nutrient availability. The effects of INDY reduction in the *Drosophila* midgut became a focal point after previous work highlighted the importance of intestinal tissue homeostasis in healthy aging and longevity. Variation in *Indy* mRNA levels in a diet-dependent manner suggests that nutrient levels modulate INDY-mediated transport. Further, *dPGC-1* is characterized as a potent mediator of the downstream regulatory effects of INDY reduction. This finding accounts for the observed changes in *Indy* mutant mitochondrial physiology, oxidative stress resistance and reduction of ROS levels. Longevity studies support a role for *dPGC-1* as a downstream effector of *Indy* mutations as shown by overlapping longevity pathways and absence of lifespan extension without wild-type levels of *dPGC-1*. Our findings show that *Indy* mutations affect intermediary metabolism to preserve energy balance in response to altered nutrient availability, which by affecting the redox environment of the midgut promotes healthy aging.

Chapter 2

Indy is a metabolic regulator of longevity

2.1. Background and significance

Single gene mutations are able to mimic the beneficial effects of CR without significant biological tradeoffs. A genetic screen using P element insertional mutagenesis showed near doubling of lifespan in *Drosophila* when one copy of the *Indy* gene activity was decreased in multiple genetic backgrounds (Rogina and Helfand, 2013; Rogina et al., 2000). Using the reporter protein ß-galactosidase (ßgal), it was determined that *Indy* is robustly expressed in tissue associated with intermediate metabolism, such as the midgut, fat body and oenocytes, with low level expression in other tissues (Knauf et al., 2002; Rogina et al., 2000). Genomic and cDNA sequences predicted a 572–amino acid protein with 34% identity and 50% similarity to human and rat renal sodium dicarboxylate cotransporters. Functional studies using *Xenopus* revealed a function for INDY as a membrane protein that transports Krebs' cycle intermediates with a high affinity for citrate (Knauf et al., 2006; Knauf et al., 2002).

The functional properties of INDY as a dicarboxylate cotransporter in tissue associated with intermediate metabolism strongly suggest a role for INDY in intermediary metabolism regulation. *Indy* expression in head and thorax fluctuates in response to nutrient availability and decreased INDY-mediated transport alters nutrient metabolism and energy production similar to CR, suggesting that it may function in the downstream response to CR (Knauf et al., 2006; Knauf et al., 2002; Marden et al., 2003; Rogina et al., 2000; Wang et al., 2009). Reduced *Indy* expression causes similar physiological changes in flies, worms and mice suggesting its regulatory role is conserved (Birkenfeld et al., 2011; Fei, 2004; Rogina et al., 2000). The present work attempts to define a role for INDY as a physiological regulator of mitochondrial function

and related metabolic pathways, modulating nutrient flux in response to nutrient availability.

2.2 Rationale

Genetic interventions that conserve intermediary metabolism and subsequent mitochondrial energetic capacity have been shown to promote healthy aging and longevity (Biteau et al., 2008; Biteau et al., 2011; Rera et al., 2011; Hochmuth et al., 2011). The *Drosophila Indy* gene is involved in intermediary metabolite transport, with robust expression in the midgut. INDY reduction is thought to induce a state metabolically similar to CR and extends longevity in flies (Rogina, 2000; Marden et al., 2003; Wang et al. 2009). Previous analysis of *Indy* mutant heads and thoraces show broad changes in nutrient metabolism and energy balance (Wang et al., 2009, Neretti et al., 2009); however, little is known about the tissue specific physiological effects of INDY reduction or how such changes contribute to health and longevity. Given that the *Drosophila* midgut has robust INDY activity (Knauf et al., 2002), and maintenance of intestinal tissue homeostasis is related to longevity (Biteau et al. 2010), it would be important to determine a midgut-specific regulatory role for INDY in the context of aging. The goal of the current work aims to identify the following: a) a relationship between *Indy* mRNA expression and aging in midgut tissue, b) the relationship between *Indy* mRNA and nutrient availability in the midgut, and c) similarities between *Indy* reduction and CR to solidify *Indy* mutation as a mimetic of environmental CR.

2.3 Results

2.3.1 Indy^{206} flies have reduced Indy mRNA and protein expression at all ages.

The *Indy*^{206} mutant fly line has a P-element insertion in the *Indy* gene that results in optimal reduction of *Indy* mRNA and the greatest extension of longevity (Rogina 2000 & Rogina

and Helfand 2013). Prior to beginning studies, it was first necessary to eliminate any influence of genetic background by backcrossing flies from the *Indy*^{206} line for ten generations into the *yellow-white (yw)* genetic background. This line has a LacZ reporter construct in the *Indy* gene region; therefore ßgal reflects regions of *Indy* expression (Figure 2.1). Consistent with literature, *Indy* is predominantly found in the oenocytes, fat body and midgut with low-level expression in other tissues (Knauf et al., 2002; Rogina and Helfand, 2013; Rogina et al., 2000) All references to *Indy* mutant flies refer to the *yw* genetically backcrossed strain unless otherwise noted and the denotation of refers to *Indy*^{206}/*Indy*^{206} or *Indy*^{206}/+ homozygous and heterozygous flies, respectively.

In order to establish adequate reduction of *Indy* transcript in the midgut of the *Indy*^{206} mutant fly line, mRNA levels were measured in young and old flies using quantitative polymerase chain reaction (qPCR). Midgut tissue isolated from control *yw,* heterozygous *Indy*^{206}/+ and homozygous *Indy*^{206}/*Indy*^{206}flies reveal significant reduction of *Indy* transcript in young and old male and female flies (Figure 2.2A, 2.2B).

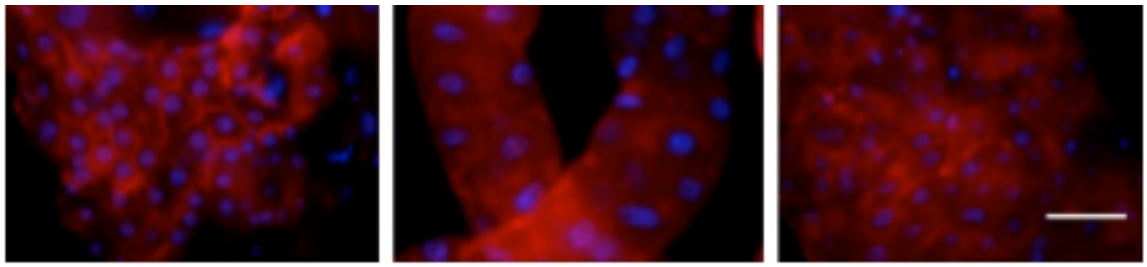

Figure 2.1: *Indy* is expressed in tissue related to intermediary metabolism in flies. Control female fat body (left), malpighian tubules (center) and midgut (right) at 20 days viewed with the 40x oil immersion objective and stained with anti-INDY antibodies (Red) and DAPI (Blue). Scale bar represents 1μm.

2.3.2 Aging increases Indy mRNA levels in the midgut of control flies

In order to identify a relationship between *Indy* expression and aging in midgut tissue, *Indy* mRNA levels in control *yellow-white* (*yw*) flies were measured using qPCR at 10 and 40 days. *Indy* transcript levels increase by approximately 89% in female *yw* flies, whereas $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutants show decreased *Indy* mRNA levels at all ages (Figure 2.2A). In order to determine whether changes in *Indy* were a consequence of aging, flies were exposed to 20 mM paraquat in order to induce free radical production and mimic age-related accumulation of oxidative damage (Choi et al., 2008; Jasper & Jones, 2010). Following overnight exposure, *Indy* mRNA and protein levels were significantly increased in young control *yw* flies mirroring those observed in aged genetic controls (Figure 2.2D-2.2E).

2.3.3 Indy levels change in response to nutrient availability

CR is associated with increased longevity, enhanced nutrient sensing and decreased intermediary metabolism. Since INDY functions in the transport of intermediate metabolites and reduction of transport induces many phenotypes reminiscent of CR, it is hypothesized that *Indy* mutations extend longevity by genetically regulating intermediary metabolism and limiting dicarboxylate transport. *Indy* mRNA levels in female *yw* control, $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ flies aged on a standard food, high caloric (HC) and CR diet were measured in midgut tissue isolated from male and female flies at 20 days. Interestingly, *Indy* mRNA levels nearly doubled in *yw* flies on a HC diet, and are similar to those observed in aged flies (Figure 2.3). *yw* flies aged on a diet with 30% reduction of caloric content demonstrate a 50% reduction in transcript at levels similar to those observed in $Indy^{206}$/+ flies on a regular diet (Figure 2.3). Diet-induced changes in *Indy* mRNA levels support a role for *Indy* as a physiological regulator, whose expression changes to modulate intermediate metabolism in response to nutrient availability.

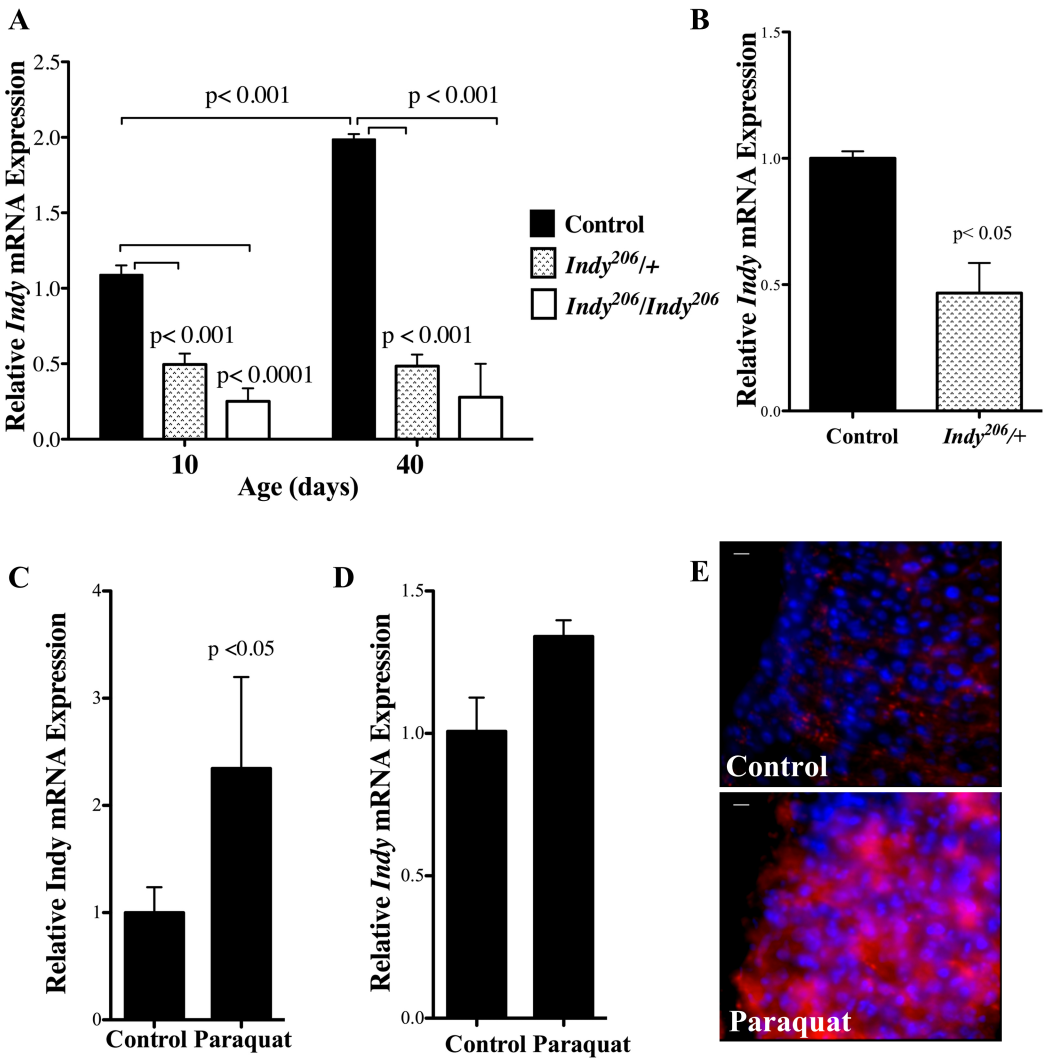

Figure 2.2 There is an age-related increase in *Indy* levels. (A) *Indy* mRNA levels in female control (*yw*), *Indy*^{206}/+ and *Indy*^{206}/*Indy*^{206} midguts at 10 and 40 days determined by qPCR. Controls show an age-related increase in *Indy* mRNA, which is absent in the mutant midgut **(B)** *Indy* mRNA is reduced in male *Indy*^{206}/+ midguts at 40 days compared to *yw* controls. **(C)** *Indy* mRNA levels in the midgut of *yw* female and **(D)** male flies following overnight exposure to 20mM paraquat at 20 days determined by qPCR. There is a paraquat-induced increase in *Indy* mRNA in the midgut of controls. **(E)** Immunofluorescence images of control and 20mM paraquat treated female midguts at 20 days stained with anti-INDY antibodies. (n=3, 25 guts per replicate. p<0.05, p<0.001, p<0.0001, t test, error bars represent SEM).

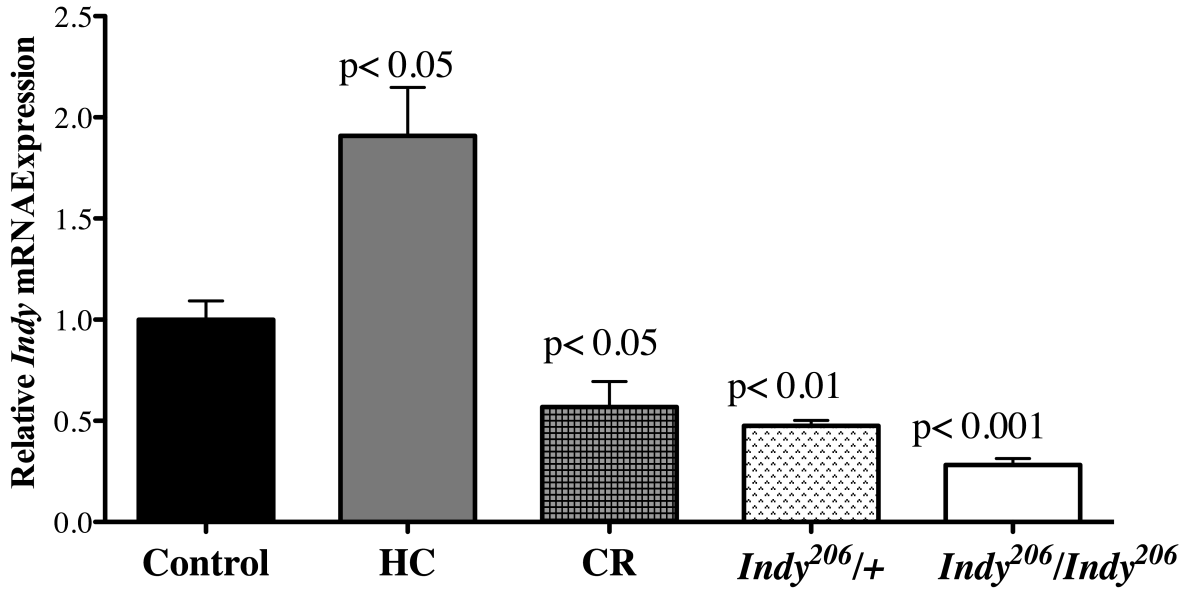

Figure 2.3: *Indy* levels change in response to nutrient availability and *Indy* mutation. *Indy* mRNA levels in control (*yw)* flies on a regular diet, after overnight exposure to paraquat, HC and CR, and $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutant flies at 20 days. A HC diet significantly increases and CR significantly reduces *Indy* transcript levels in the midgut of control female flies (n=3, 25 guts per replicate, $p<0.01$, $p<0.05$, $p<0.001$, error bars represent SEM).

2.3.4 Indy reduction alters nutrient sensing and insulin signaling in the midgut

Augmentation of nutrient sensing pathway activity is a hallmark of CR-mediated longevity. AMPK is a master metabolic switch that regulates metabolic energy homeostasis and stress resistance in response to nutrient availability. Previous studies show that both CR and genetic overexpression of AMPK extend longevity in multiple model systems, presumably by initiating downstream stress responses to promote survival during limited nutrient availability (Long et al., 2014; Wang et al., 2009; Zhu, 2004). Consistent with findings in *mIndy*$^{-/-}$ mice (Birkenfeld et al., 2011), long-lived *Indy*^{206}/+ mutant flies show significantly increased levels of *AMPK* mRNA at 40 days (Figure 2.4A). These findings support the hypothesis that *Indy* mutation induces a metabolic state similar to CR, which in turn activates cellular stress response pathways.

One of the pathways critically affected by AMPK signaling Inulin IGF-1 signaling (IIS) pathway. AMPK is responsible for regulating intracellular fatty acid oxidation; therefore AMPK has been proposed as a positive regulator of insulin sensitivity (Quiang et al., 2007). In order to investigate whether *Indy* mutation affected IIS, the levels of *Insulin receptor (InR)* and *Drosophila insulin like peptide 3 (dilp3)* mRNA levels were measured in aged *Indy* mutant midgut tissue using qPCR. Analysis of *Indy* mutant midgut tissue shows significant reduction of *InR* mRNA, which is universally associated with reduced IIS activity and enhanced insulin sensitivity (Figure 2.4B). *dilp3* mRNA is also reduced in *Indy* mutant midgut tissue, which is of particular interest due to the fact that *dilp3* has implications for ISC proliferation and intestinal growth (Obrien et al., 2011 and Figure 2.4C). Moreover, *dilp3* mRNA levels remain low when mutant flies are aged on a HC diet, suggesting protection against diet-induced IIS deregulation (Figure 2.4C).

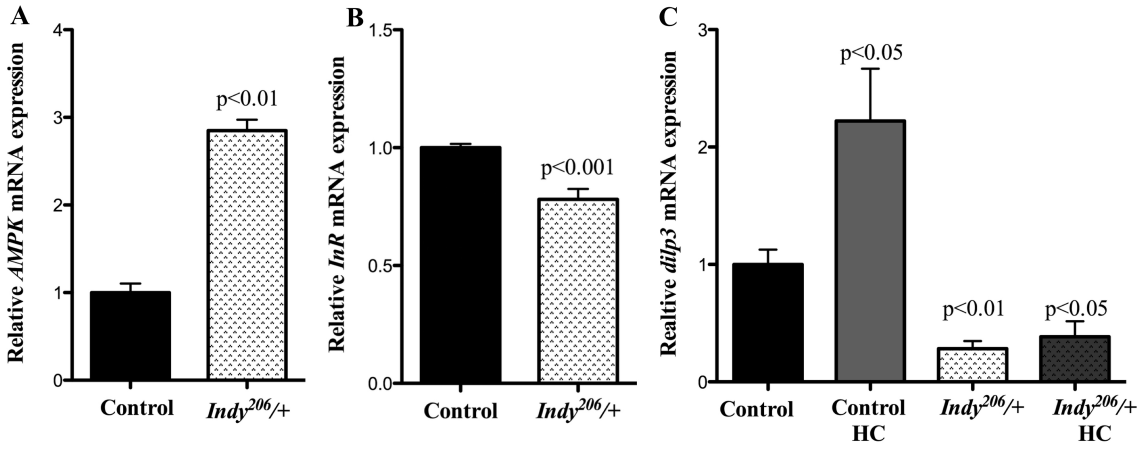

Figure 2.4: *Indy* mutant flies have decreased IIS in the midgut. (A) *AMPK* mRNA levels in the midgut of control (*yw*) and *Indy*^{206}/+ female flies at 40 days determined by qPCR. **(B)** *InR* mRNA levels in the midgut of control (*yw*) and *Indy*^{206}/+ female flies at 40 days determined by qPCR. **(C)** *dilp3* mRNA levels in the midgut of control, HC control, *and Indy*^{206}/+ *and* HC *Indy*^{206}/+ female flies at 40 days determined by qPCR. (n=3, 25 guts per replicate. t test, error bars represent SEM).

2.4 Discussion

Reduction of *Indy* gene activity in fruit flies, and homologs in worms, extends lifespan by altering energy metabolism in a manner similar to CR (Rogina et al., 2000; Fei et al., 2003; Fei et al., 2004; Neretti et al., 2009; Wang et al., 2009; Birkenfeld et al., 2011; Rogina & Helfand, 2013). As such, detangling the relationship between *Indy* mutation and CR in the context of healthy aging would provide valuable insight to the downstream CR responses related to nutrient sensing and energy balance. Given the high genetic evolutionary conservation of *Indy* from bacteria to mammals, it is likely that the mechanism underlying *Indy*-mediated longevity is conserved in humans and INDY may represent a novel point of intervention to delay mortality and extend lifespan. The data support a role for INDY as a physiological regulator that senses changes in nutrient availability and alters intermediary metabolism to energy balance while meeting tissue-specific requirements.

The results from qPCR analysis of young and aged *Indy* mutant midgut tissue show an age-associated increase in *Indy* mRNA that can be replicated by manipulations that accelerate aging such as increasing the caloric content of food or exposing flies to paraquat. Conversely, CR decreases *Indy* mRNA in control midgut tissues, consistent with previous findings in fly muscle and mouse liver (Wang et al., 2009; Birkelfeld et al., 2011). Diet-induced variation in midgut *Indy* expression suggests that INDY regulates intermediary metabolism by modifying citrate transport to meet tissue or cell-specific bioenergetic needs. Specifically, as a plasma membrane transporter, INDY can regulate cytoplasmic citrate, thereby affecting fat metabolism, respiration, and via conversion to malate, the TCA cycle.

The influence of *Indy* mutation on nutrient sensing is still largely unclear; however, the relationship between *Indy* expression and nutrient availability strongly suggest that INDY

activity modulates downstream nutrient sensing activities. Previous work shows reduced *dilp2, dilp3* and *dilp5* mRNA levels in $Indy^{206}$/+ heads and thoraces at levels similar to those obtained from CR flies, suggesting protection against age-related insulin resistance. Moreover, the same study also showed localization of forkhead box protein O1 (FOXO1) to the nucleus in thoracic tissue. Such a localization event is associated with initiation of stress response mechanisms that promote oxidative stress resistance, insulin sensitivity, and longevity extension (Wang et al., 2009).

Reduction of the ATP/ADP ratio activates AMPK, which in turn modifies several downstream signaling pathways, such as inhibiting IIS. Activation of AMPK and subsequent preservation of insulin sensitivity is associated with healthy aging and lifespan extension; therefore increased *AMPK* coupled with reduced *InR* and *dilp3* in mRNA in *Indy* mutant midgut tissue likely reflects enhanced nutrient sensing in response to decreased metabolite concentrations. Support for this hypothesis stems from the previous study showing reduced uptake of substrates in $mINDY^{-/-}$ mutant mice leads to reduced biochemical energy, which results in the activation of AMPK and subsequent preservation of insulin sensitivity in aged tissue (Birkenfeld et al., 2011).

The finding that $Indy^{206}$/+ midgut tissue has reduced *dilp3* and *InR* expression is of particular interest in terms of diet-induced tissue-specific gene expression changes related to aging. Reduction of *Drosophila* insulin like peptide 3 (*dilp3*) is associated with decreased ISC proliferation and intestinal growth, both of which are associated with prolonged intestinal health and longevity. A recent publication highlights *dilp3* as a potent regulator of midgut homeostasis that fluctuates in response to nutrient availability (O'brien et al. 2011). The study shows significant reduction of *dilp3* and striking changes in physical appearance in the midgut when

nutrients are scarce with the opposite effect when flies are reared on a HC diet. These data, coupled with the observed reduction of *dlip3* in *Indy* mutant midgut tissue on normal and HC diets, further support a role for INDY as a physiological regulator of intermediary metabolism in response to nutrient availability.

2.5 Materials and methods

2.5.1 Fly strains and maintenance

The $Indy^{206}$ line was obtained from Tim Tully (Boyton & Tully, 1992). Approximately 20 females and 20 males flies are kept together in each vial and passed to fresh vials every 2 days for aging studies. Flies were collected within 24 hours after eclosion and maintained in plastic vials containing standard food medium and kept in a humidified, temperature-controlled incubator with 12/12-h on/off light cycle at 25 °C. All strains were backcrossed 10x to *yw* background and reared on food containing 25 mg/mL tetracycline for 3 generations to eliminate *Wolbachia*, followed by several generations in tetracycline-free food.

2.5.2 Quantitative PCR (q-PCR)

Total RNA was isolated from the midguts of 3 biological replicates with more than 25 flies in each replicates using Trizol as described (Wang et al 2009). qPCR was performed following cDNA synthesis and changes in gene expression patterns were determined using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). Gene specific TaqMan primers for *Indy* were obtained from the Invitrogen and *ankryn* was used as an endogenous control. All experiments were run in triplicate.

2.5.3 Antisera

Fixed tissue was incubated overnight with mouse anti-gal 1:500 (Invitrogen); or rabbit anti-INDY 1:300 (Knauf et al., 2002) primary antibodies diluted in PBT [0.1%Triton X-100 in phosphate-buffered saline (PBS)] at 4°C. Following washing and blocking, tissue was incubated with the goat anti-rabbit Cy3 1:300 (Jackson) or goat anti-mouse FITC 1:300 (Jackson) secondary antibodies and DAPI 1:1000 (Invitrogen) diluted in PBT and 2% donkey serum for 1 hour at room temperature.

2.5.4 Immunofluorescence and Imaging

Midguts were dissected from flies at 10, 20 and 40 days, fixed in 4% paraformaldehyde and stained as described (Rera et al., 2011). Following washing, samples were mounted and imaged using a Leica camera attachment and LAS V4.1 software on a Lecia microscope, or a Zeiss LSM 780 confocal system mounted on an inverted Axio Observer Z1 microscope. Images were analyzed using Adobe Photoshop or Image J. Variability between different regions of the gut was reduced by quantifying images from the same designated region for each genotype in a 0.06 x 0.02 cm area.

2.5.5 Statistical analysis

Significance was determined using a two-tailed, unpaired t-test from at least three independent experiments. Error bars represent SEM. P values less than 0.05 were considered statistically significant and p values are specifically indicated in each figure.

Chapter 3

Indy reduction promotes *dPGC-1* mediated mitochondrial biogenesis and function

3.1. Background and significance

Genetic interventions that conserve mitochondrial energetic capacity have been shown to maintain a favorable redox state and regenerative tissue homeostasis (Biteau et al., 2008; Biteau et al., 2011; Rera et al., 2011; Hochmuth et al., 2011). *dPGC-1/spargel* is the *Drosophila* homolog of mammalian PGC-1, a transcriptional co-activator that promotes mitochondrial biogenesis by increasing the expression of genes encoding mitochondrial proteins (Puigserver & Spiegelman, 2003; Gershman et al., 2006). *dPGC-1* is a downstream target of *Sir2,* which is activated during CR and associated with healthy aging in yeast, worms, flies and mammals (Guarente, 2006). Upregulation of *dPGC-1* is a hallmark of CR-mediated longevity and is thought to represent a response mechanism to compensate for energetic deficits caused by limited nutrient availability (Puigserver & Spiegelman, 2003; Lopez-Lluch et al., 2006). Previous analyses of long-lived *Indy* mutant flies revealed upregulation of *dPGC-1* and subsequent elevation of mitochondrial biogenesis in heads and thoraces (Neretti et al., 2009), which highlight another, key similarity between CR and *Indy* mutant flies.

dPGC-1 transcriptional activity decreases as a consequnce of normal aging, thus compromising the rate of mitochondrial biogenesis and regulation of ROS detoxification (Rera et. al 2011). Such changes negatively affect mitochondrial physiology and morphology, which cause an imbalance in the ATP/ROS production ratio resulting in deleterious phenotypes and accelerated aging (Guarente, 2006; Rera et. al 2011). This phenotype is particularly detrimental in the fly midgut, which facilitates nutrient uptake, waste removal and response to bacterial infection. This work aims to characterize the relationship between *Indy* and *dPGC-1* activity with

respect to changes in mitochondrial biogenesis, physiology and ROS modulation in the aging *Drosophila* midgut.

3.2 Rationale

Mitochondrial physiology and function are responsible for maintaining the ATP/ROS production ratio and, when damaged, result in deleterious phenotypes and accelerated aging. Recently, *dPGC-1* upregulation in stem and progenitor cells of the digestive tract was shown to preserve ISC proliferative homeostasis and extend lifespan (Rera et al., 2011). The *Drosophila* midgut is regenerated by multipotent ISCs, which replace damaged epithelial tissue in response to injury, infection or changes in redox environment (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006; Ohlstein & Spradling, 2007; Jasper & Jones, 2010; Hochmuth et al., 2011). Low levels of ROS maintain stemness, self-renewal and multipotency in ISCs; whereas, age-associated ROS accumulation induces continuous activation marked by ISC hyper-proliferation and loss of intestinal integrity (Hochmuth et al., 2011). *Indy* mutations mimic CR in *Drosophila* with respect to metabolic and energetic efficiency, showing increased mitochondrial biogenesis and reduced ROS production in head and thorax (Neretti et al., 2009). We aimed to investigate the relationship between *Indy* and *dPGC-1* activity with the goal of characterizing the downstream effects on mitochondrial biogenesis, physiology and ROS levels in the aged *Indy* mutant midgut.

3.3 Results

3.3.1 Indy reduction is associated with increased dPGC-1 levels in the midgut

The observation that *Indy* levels decrease in response to CR (Figure 2.3), posed the question of whether INDY reduction is sufficient to upregulate *dPGC-1* in the fly midgut and

rescue age-associated decline in expression levels. Since *Sir2* is known to interact with and activate *dPGC-1* in response to limited nutrient availability, it was important to first assess whether *Indy* mutantion was capable of inducing a similar change. *Sir2* mRNA levels were significantly increased in $Indy^{206}$/+ mutant midgut tissue at 40 days, suggesting that reduced INDY activates downstream stress response mechanisms to compensate for decreased nutrient availability (3.1A).

dPGC-1 activity increases mitochondrial biogenesis in response to CR and decreases as a consequence of normal aging (Guarente, 2008; Hwang, 2012; Lopez-Lluch et al., 2006; Puigserver, 2003; Rera et al., 2011).In contrast to the age-related decline observed in control flies between 20 and 40 days, there are significantly higher *dPGC*-1 mRNA levels at all ages in both $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutant females (Figure 3.1B). A similar increase is also observed in $Indy^{206}$/+ male flies at age 40, suggesting sustained *dPGC-1* activity in *Indy* mutant midgut tissue (Figure 3.1B, 3.1C).

In *Drosophila,* spacial and temporal regulation of gene expression can be conducted using an inducible GeneSwitch Gal4 expression system (Osterwalder et al., 2001; Poirier et al., 2008). This system allows for targeted tissue-specific expression of upstream activating sequence (UAS) effector lines induced by yeast GAL4 protein that becomes active in the presence of the steroid RU486 (mifepristone). The GeneSwitch system uses flies from the same generation, with the only variable being implementation of RU486, thus alowing for precise genetic controls and eliminating any potential interference of genetic background (Figure 3.2).

To further examine the relationship between *Indy* and *dPGC-1* mRNA levels, the *TIGS2*-geneswitch driver (*TIGS2-GS*) was employed to drive gut specific *Indy*-RNAi mediated silencing. Using qPCR, it was determined that targeted reduction of *Indy* mRNA was sufficient to increase

dPGC-1 mRNA levels in the midguts of *TIGS2-GS; Indy*-RNAi male and female flies at 20 days (Figure 3.3A-3.3D). While the reduction of *Indy* was not significant in either sex, the observed increased in *dPGC-1* activity suggests that even a slight variation in *Indy* levels is capable of altering downstream gene expression. Moreover, the data support an inverse relationship between *Indy* and *dPGC-1* mRNA levels in the midgut.

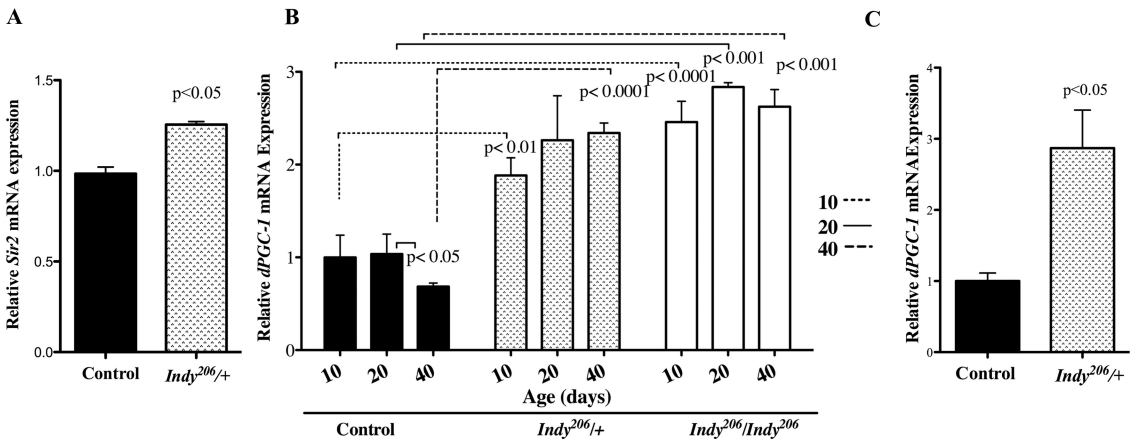

Figure 3.1 *Indy* mutant flies have increased *dPGC-1* mRNA. (A) *Sir2* mRNA levels in female *Indy*^{206}/+ mutant midgut tissue at 40 days. There is a significant increase in *Sir2* mRNA ($p<0.01$, t test, n=25 guts per replicate). **(B)** *dPGC-1* mRNA levels in female control *yw*, *Indy*^{206}/+ and *Indy*^{206}/*Indy*^{206} mutant midguts at 10, 20 and 40 days. There is a significant age-related decrease in *dPGC-1* mRNA in control flies by 40 days ($p <0.05$, t test) that is absent in heterozygous and homozygous *Indy*^{206} mutants, which show increased *dPGC-1* mRNA levels compared to controls at all ages ($p<0.01$, $p<0.001$, $p<0.0001$, t test. n=3, 25 guts per replicate, error bars represent SEM). **(C)** *dPGC-1* mRNA levels in the midgut of heterozygous *Indy*^{206}/+ males at 40 days. There is a significant increase in *dPGC-1* mRNA levels in *Indy*^{206}/+ male midgut tissue ($p<0.05$, n=3, 25 guts per replicate).

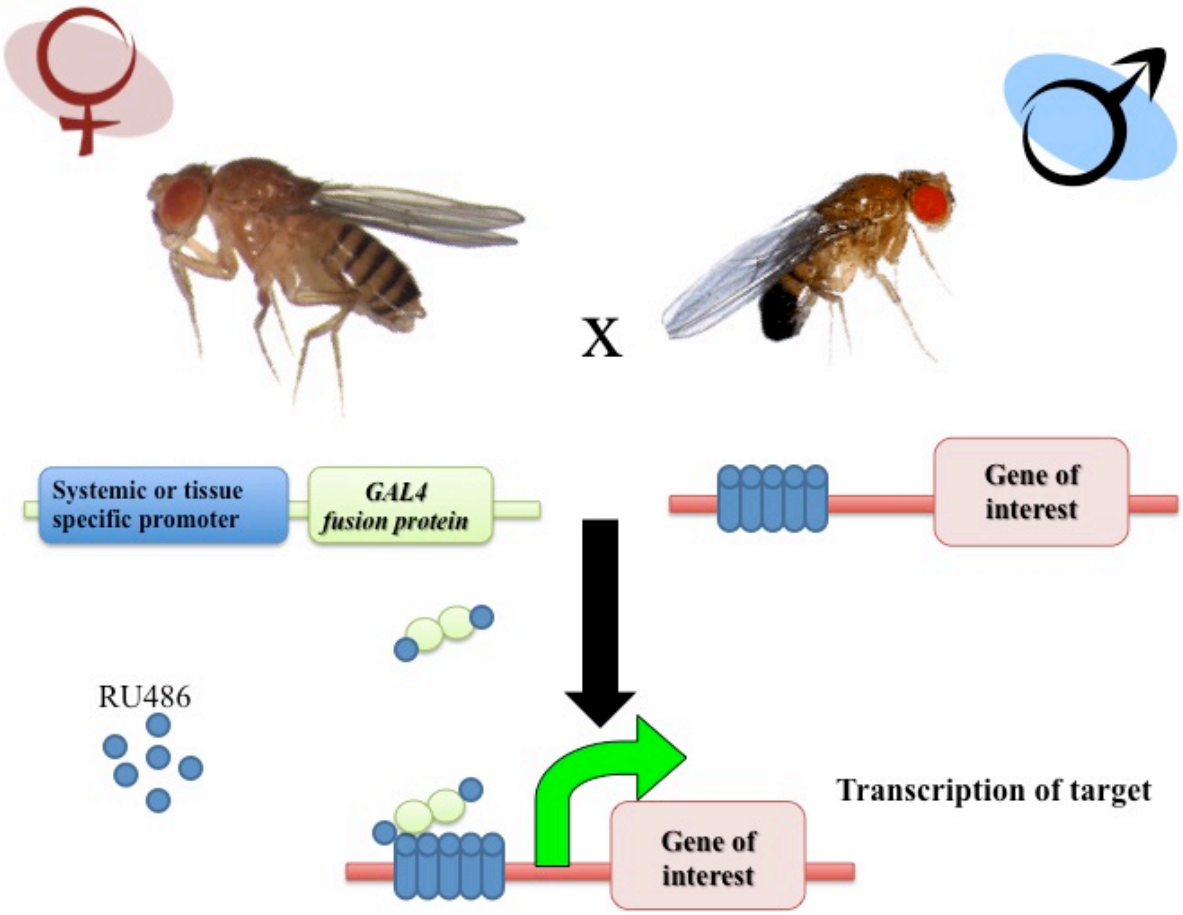

Figure 3.2 The *Drosophila* gene switch system for spatial and temporal gene expression. The Gene- Switch system is inactive without the RU486 ligand. Upon ingestion of the Ru486 ligand, and subsequent interaction between GAL4 and RU486, the GAL4 fusion protein is capable of forming chimeric dimers on the Upstream Activation Sequence (UAS), and thus activating temporal expression of the target gene.

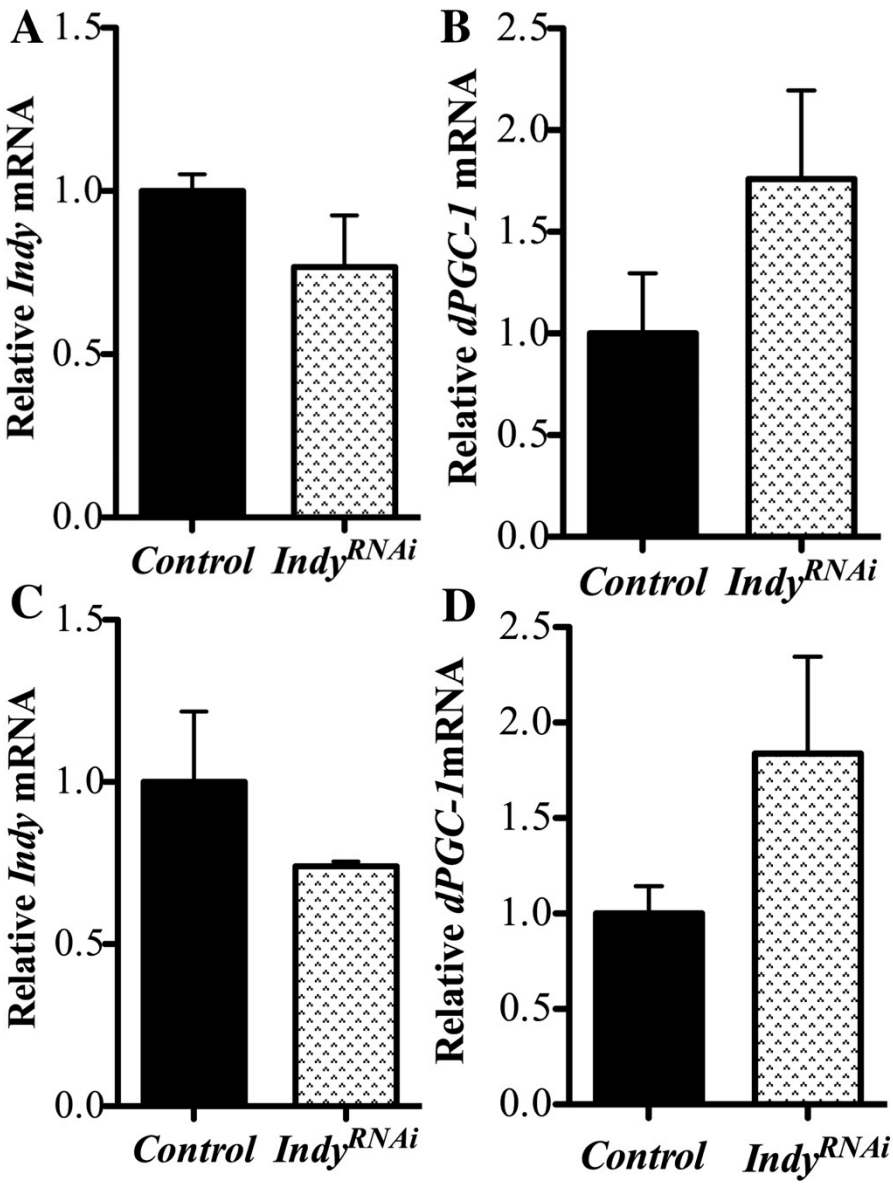

Figure 3.3: Small decreases in *Indy* result in increased *dPGC-1* mRNA. (A) *Indy* mRNA levels in the midgut of female *TIGS2-GS*;*Indy*^{9981} RNAi lines at 20 days measured by qPCR. **(B)** *dPGC-1* mRNA levels in the midgut of female *TIGS2-GS*;*Indy*^{9981} RNAi lines at 20 days measured by qPCR. **(C)** *Indy* mRNA levels in the midgut of male *TIGS2-GS*;*Indy*^{9981} RNAi lines at 20 days measured by qPCR. **(D)** *dPGC-1* mRNA levels in the midgut of male *TIGS2-GS*;*Indy*^{9981} RNAi lines at 20 days measured by qPCR. *Indy*^{RNAi} flies show a small increase in *dPGC-1* mRNA compared to control flies suggesting that targeted depletion of *Indy* in the midgut increases *dPGC-1* transcription (n=3, 25 guts per replicate). Error Bars represent SEM.

3.3.2 Reduced Indy increases dPGC-1 mediated mitochondrial biogenesis

dPGC-1 is a master regulator of mitochondrial biogenesis and ROS detoxification in cells (Barja, 2013; Guarente, 2008; Hwang, 2012; Puigserver, 2003; Rera et al., 2011). In order to determine whether *dPGC-1* upregulation in the midgut of *Indy* mutant flies was sufficient to increase mitochondrial biogenesis, mitochondrial density was measured by calculating the ratio of mitochondrial DNA to nuclear DNA in the midgut of $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ mutant flies. Mitochondrial DNA content was determined by the ratio of the mitochondrial gene, *cytochrome oxidase subunit I (COI)*, to a nuclear gene *GAPDH* (Neretti et al., 2009). Observation of *Indy* mutant midgut tissue revealed significantly increased mitochondrial copy number compared to controls at 40 days (Figure 3.4). In order to determine whether the observed increase in mitochondrial DNA copy number in *Indy* mutant flies depends on increased *dPGC-1* levels, double mutant flies with $Indy^{206}$ and a hypomorphic *dPGC-1* allele $(Indy^{206}/dPGC\text{-}1^{KG08646})$ were generated. At 40 days these flies have mitochondrial DNA copy numbers strikingly similar to those observed in control flies, suggesting that two copies of the wild-type *dPGC-1* gene are needed for increased mitochondrial biogenesis in aged *Indy* mutant flies (Figure 3.4).

To further investigate the effects of *Indy* mutations on mitochondrial physiology, electron microscopy and the point counting method were employed to visualize and quantify changes in density and size. Since enterocyte cells comprise 90% of the midgut epithelia, mitochondria were quantified in these cells to represent averge density and size throughout the tissue. Consistent with the recently published findings observed in in liver of $mIndy^{-/-}$ mice (Birkenfeld et al. 2011), electron micrographs of $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ mutant midgut tissue show a clear increase in mitochondrial density by 20 days (Figure 3.5A, 3.5B). Moreover, individual mitochondria are

significantly smaller in size compared to controls at 20 days, indicating preserved mitochondrial plasticity and protection against age-related functional decline (Figure 3.5C and Tranah 2011).

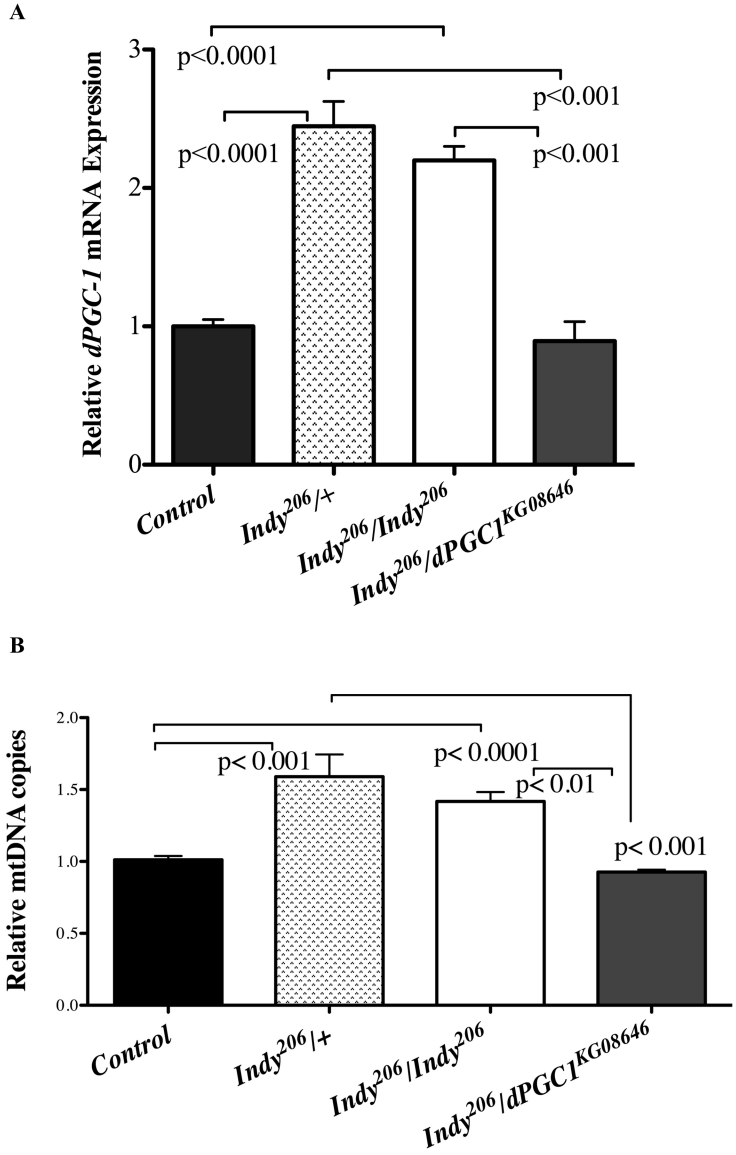

Figure 3.4: *Indy* mutants have increased mitochondrial DNA. (A) *dPGC-1* mRNA levels in female control *yw*, $Indy^{206}$/+, $Indy^{206}/Indy^{206}$ mutant and $Indy^{206}$; $dPGC\text{-}1^{KG08646}$ hypomorph midguts at 40 days. There is a significant increase in *dPGC-1* mRNA in *Indy* mutant flies ($p < 0.001$; $p < 0.0001$, t test) that is absent in hypomorphic mutants, which show *dPGC-1* mRNA levels similar to controls (t test. n=3, 25 guts per replicate, error bars represent SEM). **(B)** Mitochondrial (*COI)* and nuclear (*GAPDH*) DNA ratio determined by qPCR. $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutant females show significant increases in mitochondrial DNA copy number, compared to *yw* control or $Indy^{206}/dPGC\text{-}1^{KG08646}$ mutant flies ($p < 0.01$, $p < 0.001$, $p < 0.0001$, n=3, 25 guts per replicate).

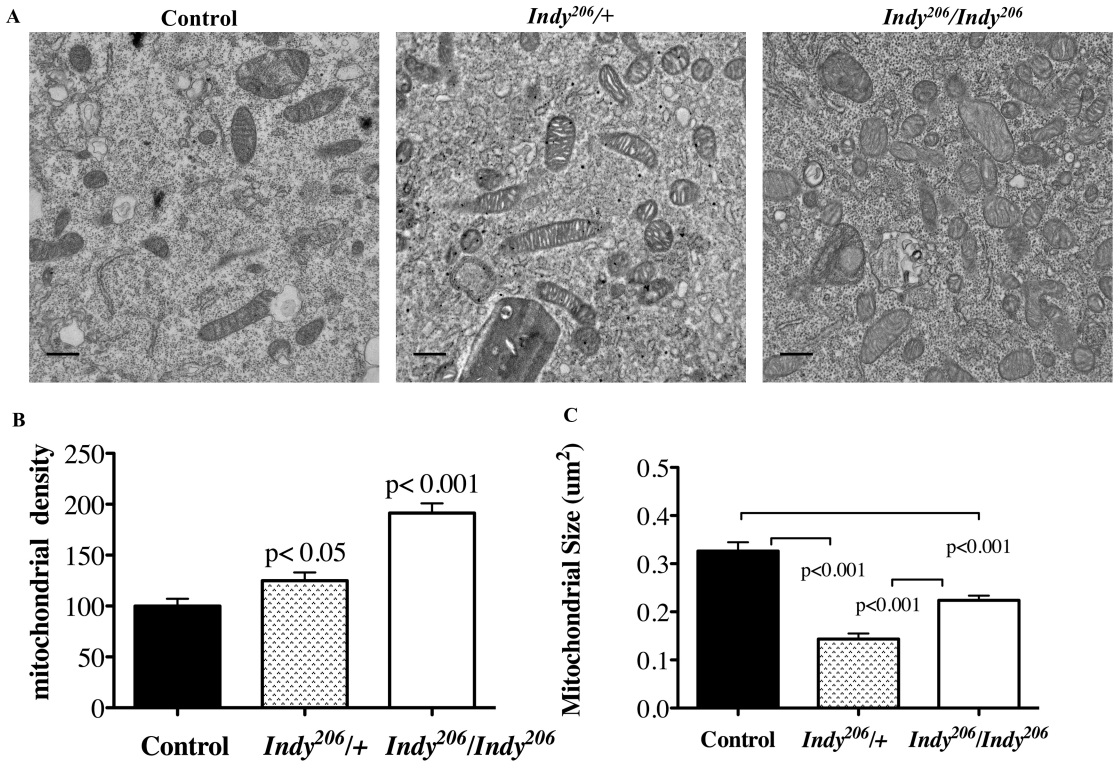

Figure 3.5: *Indy* mutants have increased mitochondrial biogenesis in the midgut. (A) Electron micrographs of control, $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ midguts at 20 days imaged at 10,000x. Scale bar represents 1µm. **(B)** Mitochondrial density (mitochondrial number/counted cell volume X100) as assessed by point counting in enterocytes using Image J. There is a significant increase in mitochondrial number in $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutant midgut tissue at 20 days ($p<0.05$, $p<0.001$, t test. $n> 25$ cells per guts). **(C)** $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutants have significantly smaller mitochondria at 20 days in midgut tissue assessed by point counting in Image J. ($p<0.001$, $n>25$ cells per gut).

3.3.3 Indy mutants have preserved mitochondrial activity in the midgut

The efficiency of mitochondrial oxidative metabolism is an indication of mitochondrial functional status and can be assesed by observing changes in mitochondrial ETC gene expression. In addition to higher mitochondrial biogenesis, $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ females have increased mitochondrial ETC complex I gene expression. While *l(3)neo18* and *Pdsw* mRNA levels are elevated in both $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$, significant increases in *ND23, ND42*, *ND75* mRNA levels were also observed in the midgut of $Indy^{206}/Indy^{206}$ mutant flies by 20 days (Figure 3.6A, 3.6B). Furthermore, the mRNA levels of ETC *Cytochrome C oxidase I (COI),* a complex IV component, were also significantly increased in aged $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ female flies (Figure 3.6A).

Changes in mitochondrial membrane potential are associated with changes in the electrochemical gradient between the inner and outer mitochondrial membranes, which affects permeability of ions and small molecules. High membrane potential is associated with preserved mitochondrial physiology and efficiency; whereas decreased mitochondrial function has a negative impact on the physiological function of tissues, and organismal aging (Barja, 2013; Scarpulla, 2011; St-Pierre et al., 2006; Tranah, 2011; Wang et al., 2012) In order to determine the status of mitochondrial membrane potential, the cationic dye JC-1 can be used to measure mitochondrial membrane potential at a cellular level (Rera et al., 2011). High mitochondrial membrane potential allows positively charged JC-1 to enter the mitochondrial matrix and form fluorescent aggregates emitting red light at 568nm. Likewise, low mitochondrial membrane potential can be distinguished by the presence of monomeric green staining in the cellular cytoplasm (Rera et al., 2011). Therefore, membrane potential can be determined by the ratio of red: green fluorescence. Using this approach, the status of mitochondrial membrane potential in aged *Indy* mutant midguts was found to be significantly greater in both $Indy^{206}$/+ and

Indy^{206}/*Indy*^{206} mutants compared to controls (Figure 3.7A, 3.7B). Moreover, the red:green ratio for *Indy*^{206}/*dPGC-1*^{KG08646} double mutant flies is strikingly similar to that observed in controls suggesting that *dPGC-1* mediates changes in mitochondrial physiology observed in *Indy* mutants (Figure 3.7A, 3.7B).

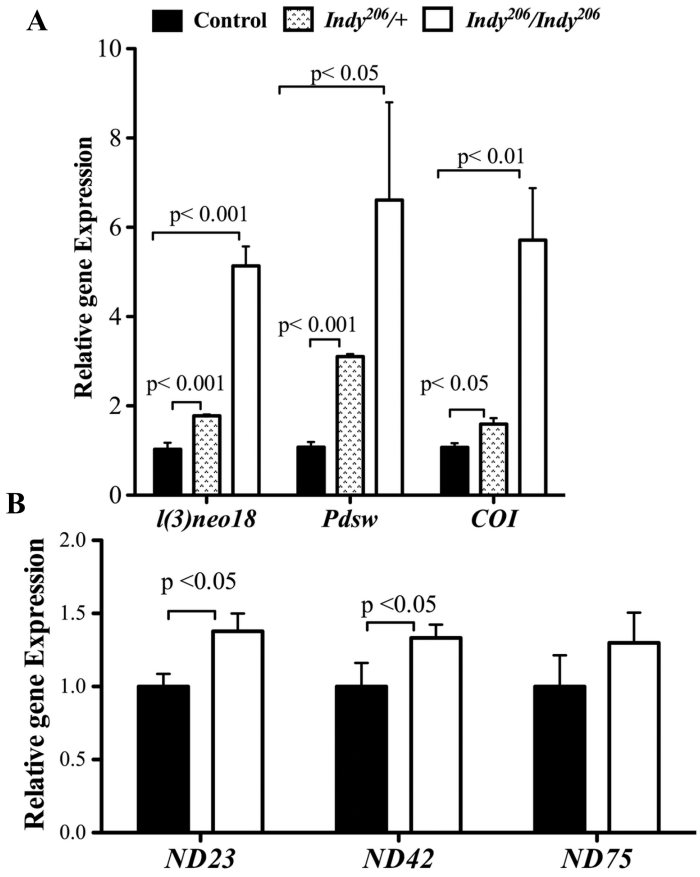

Figure 3.6: *Indy* mutant flies have increased mitochondrial ETC complex activity in the midgut. (A) Quantification of complex I mRNA for components: *l (2) neo*, *Pdsw* encoding components of and Cytochrome C oxidase encoding a component of complex IV are increased in the midgut of $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutant flies determined by qPCR. ($p<0.05$, $p<0.01$, $p<0.001$, n=3, 25 guts per replicate. **(B)** Quantification of Complex I mRNA, *ND23, ND42* and *ND75* in the midgut of control and homozygous $Indy^{206}/Indy^{206}$ mutant flies determined by qPCR. *ND23* and *ND42* are significantly increased in *Indy* mutant at 20 days ($p<0.05$, n=3, 25 guts per replicate).

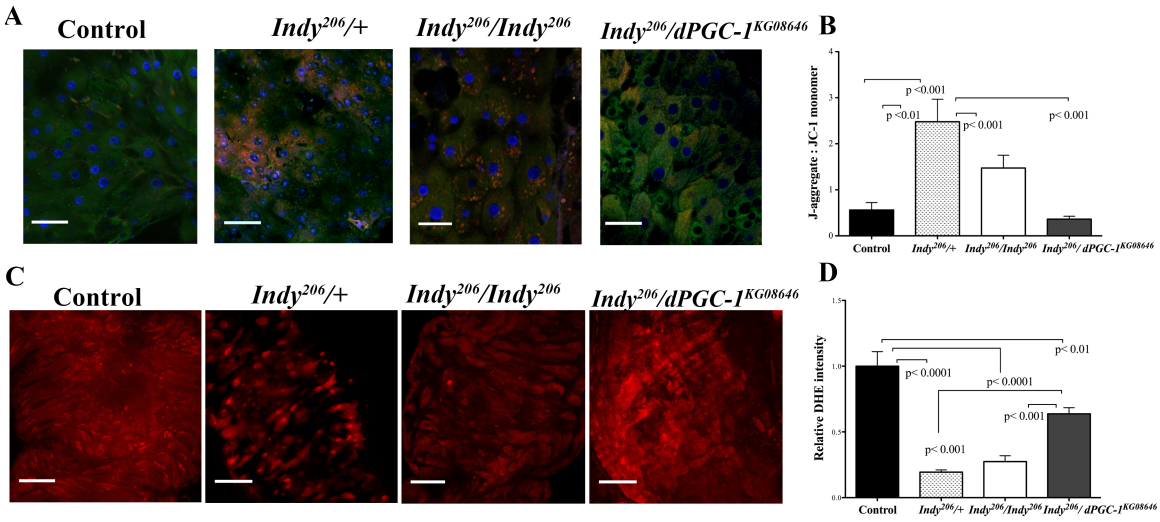

Figure 3.7: *Indy* mutants have increased mitochondrial membrane potential and reduced ROS. **(A)** Visualization of JC-1 dye in female control *yw,* $Indy^{206}$/+, $Indy^{206}/Indy^{206}$ and $Indy^{206}/dPGC\text{-}1^{KG08646}$ mutant flies at 40 days. *Indy* mutant flies show increased mitochondrial membrane potential compared to controls and $Indy^{206}/dPGC\text{-}1^{KG08646}$ mutants. Scale bar represents 1µm **(B)** Ratio of red JC-aggregates to green JC-1 monomer. $Indy^{206}$/+ and $Indy^{206}/Indy^{206}$ mutant flies have increased mitochondrial membrane potential shown by increased red: green JC-1 ratio compared to control and $Indy^{206}/dPGC\text{-}1^{KG08646}$ mutants ($p<0.01$, $p<0.001$, $n>10$ guts per genotype, compared by Mann-Whitney U test **(C)** Positive DHE staining for ROS in compressed Z-stack of female control, $Indy^{206}$/+, $Indy^{206}/Indy^{206}$ and $Indy^{206}/dPGC\text{-}1^{KG08646}$ mutant midgut flies at 40 days. Scale bar represents 1µm. **(D)** Mean DHE intensity for in compressed Z-stack of female control, $Indy^{206}$/+, $Indy^{206}/Indy^{206}$ and $Indy^{206}/dPGC\text{-}1^{KG08646}$ mutant midguts at 40 days. ($p<0.001$, $p<0.0001$, $n>15$ guts per genotype; Scale bar represents 1µm).

3.3.4 Indy mutants have reduced ROS and increased resistance to oxidative stress

Increased levels of mitochondrial ROS production and oxidative damage are associated with decreased mitochondrial function and considered hallmarks of aging across species (Wallace, 2005; Lopez-Lluch et al., 2006; St-Pierre et al., 2006; Hwang et al. 2012). Dihydroethidium (DHE) fluoresces at 568nm when it reacts with superoxide and was used to measure changes in the total redox environment in the midgut of female $Indy^{206}$ mutants, $Indy^{206}/dPGC\text{-}1^{KG08646}$ double mutants and control flies at 40 days. Both $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ mutants had significantly decreased red fluorescence when compared to controls indicating lower levels of ROS (Figure 3.7C, 3.7D). $Indy^{206}/dPGC\text{-}1^{KG08646}$ show partial protection against ROS accumulation as shown by intermediate levels of DHE intensity, which is likely due to the effects of *Indy* reduction on the wild-type copy of *dPGC-1* (Figure 3.7C, 3.7D).

There is evidence that *dPGC-1* is a potent regulator of ROS metabolism and is required for the induction of several ROS-detoxifying enzymes(Rera et al., 2011; Rogers, 2012; St-Pierre et al., 2006). ROS-detoxification factors *Glutathione S transferase E1* (*GstE1*) and *Glutathione S transferase D5* (*GstD5*) mRNA were significantly increased in $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ mutant midgut tissue at 40 days compared to *yw* controls (Figure 3.8A). Levels were not significantly altered in $Indy^{206}/dPGC\text{-}1^{KG08646}$ flies compared to controls, suggesting that both copies of wild type *dPGC-1* are necessary to modulate ROS detoxification in *Indy* mutants.

A common phenotype associated with increased longevity is increased oxidative stress resistance. Longevity studies using 20 mM paraquat show male and female $Indy^{206}$ mutant flies as having increased oxidative stress resistance compared to *yw* controls at 20 days (Figures 3.8B, 3.8C and Table 3.1). It is important to note that heterozygous $Indy^{206}/+$ flies have higher resistance to paraquat than homozygous $Indy^{206}/Indy^{206}$ flies, $Indy^{206}/+$ mutants. This observation

is consistent with previously published longevity studies (Wang et al., 2009), which show increased longevity in heterozygous *Indy*^{206}/+ flies. It can be speculated that this disparity in longevity between heterozygous and homozygous *Indy* mutants may reflect the differences in mitochondrial physiology and ROS levels found in *Indy*^{206}/+.

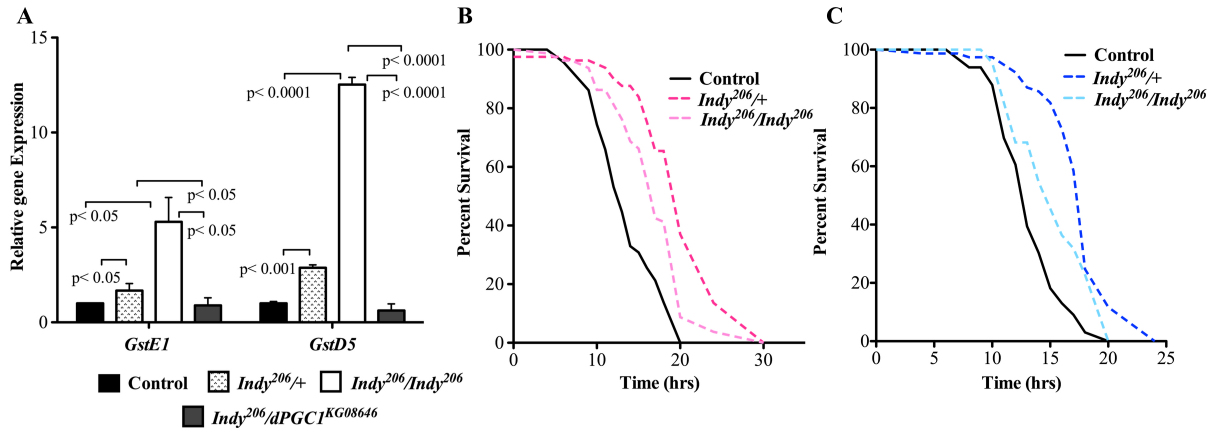

Figure 3.8: *Indy*^{206} mutants have increased resistance to oxidative stress. (A) Levels of *GstE1* and *GstD5* mRNA in female control *yw*, *Indy*^{206}/+ and *Indy*^{206}/*Indy*^{206} and *Indy*^{206}/*dPGC-1*^{KG08646} mutant midgut tissue determined by qPCR. There is a significant increase ($p<0.05$, $p<0.001$, $p< 0001$ n=3, 25 guts per replicate) in levels of both gene mRNA levels in *Indy*^{206} mutant midgut tissue at 20 days compared to control and *Indy*^{206}/*dPGC-1*^{KG08646}. Survival curves for female (B) and male (C) control *yw*, *Indy*^{206}/+ and *Indy*^{206}/*Indy*^{206} flies on 20mM paraquat. *Indy*^{206} mutants have increased resistance to oxidative stress compared to control.

Gender	Genotype	N	Median Lifespan (% Change)	X^2	*p*	Maximal Lifespan (% Change)
F	*yw*	95	15.1	-	-	21
F	$Indy^{206}/+$	85	19.8 (31.3)	62.2	p< 0.0001	26.8 (27.6)
F	$Indy^{206}/Indy^{206}$	85	17.1 (13.2)	24.3	p< 0.0001	24.4 (16.1)
M	*yw*	80	13.5	-	-	19.9
M	$Indy^{206}/+$	80	19.4 (43.7)	25.9	p< 0.0001	24.4 (22.6)
M	$Indy^{206}/Indy^{206}$	70	16.1 (19.2)	1.81	p=0.1787	22.1 (11.05)

Table 3.1: *Indy* mutants have increased resistance to oxidative stress. The median and maximal lifespan of female (F) and male (M) *yellow-white (yw)* control, heterozygous ($Indy^{206}/+$) and homozygous ($Indy^{206}/Indy^{206}$) *Indy* mutant flies in *yw* background. N: number of flies in each lifespan. Median and Maximal Life spans are in hours. Long-rank analyses were performed using the JMP 10 program.

3.4 Discussion

One of the hallmarks of CR-mediated longevity extension is increased mitochondrial biogenesis mediated by *dPGC-1* (Nisoli et al., 2005; Lopez-Lluch et al., 2006; Guarente, 2008). Increased *dPGC-1* levels and mitochondrial biogenesis have been described in the muscle of *Indy* mutant flies (Neretti et al., 2009), the liver of *mIndy*$^{-/-}$ mice (Birkenfeld et al., 2011), and here it is described in the midgut of *Indy* mutant flies. Such a finding is of particular interest due to the recent attention directed toward the role of mitochondrial bioenergetics in preserving intestinal tissue homeostasis and the resulting extension of lifespan in flies.

One possible mechanism for these effects is related to reduction of INDY transport activity. Reduction of INDY transport decreases the amount of free citrate in the cellular cytoplasm, which in turn leads to a scarcity of substrate for mitochondrial oxidative phosphorylation. These conditions cause an increase in the ADP/ATP ratio and subsequent activation of stress response pathways, activating AMPK, which initiates downstream *dPGC-1* synthesis and ultimately inducing a state metabolically similar to CR. Therefore, it can be argued that the observed increase in *dPGC-1* levels in *Indy* mutant midguts represents a protective mechanism activated in response to reduced energy availability.

As discussed earlier (Figure 1.3), limited nutrient availability alters mitochondrial morphology and physiology in effort to promote nutrient oxidation and bioenergetic efficiency. Mitochondria in the *Indy* mutant are consistent with such a bioenergetic adjustment, showing regions of small densely populated mitochondria. The observed increase in *dPGC-1* levels in *Indy* mutant midgut therefore appears to promote mitochondrial biogenesis and functional efficiency, representing a protective mechanism activated in response to reduced energy availability. Increased mitochondrial number disperses the workload among the mitochondrial

population and decreases stress on individual organelles. Reduced individual workload decreases the amount of ETC activity per mitochondria, thereby decreasing the likelihood of free radical emission, accumulation of oxidative damage and onset of mortality.

Recent studies (Liesa and Shirihai, 2013) show that healthy mitochondria continually alter their architecture, through fission and fusion, in response to energy demands and as a way to remove damaged material (Figure 1.3). Analysis of mitochondrial physiology in the *Indy* mutant midgut shows upregulation of respiratory proteins, maintenance of mitochondrial membrane potential and increased mitochondrial biogenesis, all of which are signs of enhanced mitochondrial health (Wallace, 2005). This is particularly beneficial in the fly midgut, which facilitates nutrient uptake, waste removal and response to bacterial infection. *Indy* mutant flies have striking increases in the steady-state expression of the *GstE1* and *GstD5* ROS detoxification genes. As a result, any increase in ROS levels, whether from mitochondrial demise or exposure to external ROS sources can be readily metabolized to prevent accumulation of oxidative damage. Thus, enhanced ROS detoxification mechanisms induced by *Indy* reduction and subsequent elevation in *dPGC-1* contributes to preservation of ISC functional efficiency, and may be a contributing factor to the long-lived phenotype of *Indy* mutant flies.

3.5 Materials and Methods

3.5.1 Fly strains and maintenance

Flies were collected within 24 hours after eclosion and maintained in plastic vials containing standard food medium and kept in a humidified, temperature-controlled incubator with 12/12-h on/off light cycle at 25 °C. All strains were backcrossed 10x to *yellow-white (yw)* background and reared on food containing 25 mg/mL tetracycline for 3 generations to eliminate *Wolbachia*, followed by several generations in tetracycline-free food. *yellow-white (yw)* and y^{1}*;P{SUPorP*

Spargel^{KG08646}*ry*^{506}*/TM3,Sb*^{1}*Ser*^{1} *(#14965)* flies were obtained from the Bloomington Stock Center at Indiana University. The *TIGS-2* Gene-Switch driver line was provided by Scott Pletcher (Osterwalder et al., 2001; Poirier et al., 2008) and the UAS-*Indy*^{RNAI} (*w*^{1118}*;P{GD2712}v9981)* line was obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007). Flies requiring gene-switch induction were grown on food containing 200 mM RU486 and controls on EtOH. Approximately 20 females and 20 males flies are kept together in each vial and passed to fresh vials every 2 days for aging studies.

3.5.2 Quantitative PCR (qPCR)

Total RNA was isolated from the midguts of 3 biological replicates with more than 25 flies in each replicates using Trizol as described (Wang *et al*., 2009). qPCR was performed following cDNA synthesis and changes in gene expression patterns were determined using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). All experiments were run in triplicate. Gene specific TaqMan primers for *Indy, dPGC-1, Pdsw, l (3) neo18, COI, ND23, ND42, ND75, GstD5 and GstD1* were obtained from the Invitrogen. *Ankryn* was used as an endogenous control.

3.5.3 Mitochondrial DNA Measurement

Total DNA from the midguts of more than 25 flies was isolated at 40 days using the Invitrogen DNA blood and tissue isolation kit (Life technologies). DNA copy number was determined using qPCR as described above. Mitochondrial DNA content was determined by the ratio of the mitochondrial gene for *COI* to a nuclear gene, *GAPDH* (Neretti *et al*., 2009). *Rpl32* was used as an endogenous control.

3.5.4 Electron Microscopy and Mitochondrial Quantification

Flies were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer as described (Ohlstein & Spradling, 2006). A minimum of 15 electron micrographs of midgut sections of each sample were taken at 10,000-15,000x, using an unbiased sampling method. Images were processed and analyzed in Adobe Photoshop. Post-fixation was conducted for 1 hr in 1% osmium tetroxide-0.8% potassium ferricyanide. Samples were stained in block with 1% aqueous uranyl acetate, dehydrated in a graded ethanol series, and embedded in Spurr low-viscosity epoxy resin. Thin sections of areas containing midgut were stained with uranyl acetate and lead citrate, and examined in a Hitachi H7650. Mitochondria were counted by using the point counting method by using a grid system to count the number of mitochondria present in a given image relative to cytoplasmic volume (Birkenfeld *et al*., 2011). Size was determined by measuring the grid overlays per mitochondria and expressed relative to cytoplasmic volume.

3.5.5 JC-1 Analysis

JC-1 analysis was performed as described (Rera et al., 2011). Whole midguts were dissected from female flies at 40 days directly into 5 μM JC-1 (Molecular Probes) in DMSO containing 1:1000 DAPI (Invitrogen) and incubated in the dark for 30 minutes at room temperature. Midguts were washed 2 times for 5 minutes each and mounted in PBS. Images were taken of midguts approximately 300 mm from the anterior pylorus in the 568 nm channel using the Zeiss 780 combined confocal/FCS/NLO system and analyzed in Image J. Mean pixel intensities for JC-1 monomer and aggregates were averaged, and significant differences between means were determined with a Mann-Whitney U test.

3.5.6 Dihydroethidium Staining

ROS levels were assessed in live whole midgut tissue as described (Rera et al., 2011; Hochmuth *et al*., 2011). Tissue was dissected directly in Schneider's medium and incubated for 7 minutes in 60 mM dihydroethidium (DHE) (Invitrogen Molecular Probes) in Schneider's medium and 1:1000 4',6-diamidino-2-phenylindole (DAPI) nuclear stain in 0.1% PBT 2% donkey serum. Midguts were washed in Schneider's medium at room temperature, mounted in 70% glycerol and imaged using a Zeiss 780 combined confocal/FCS/NLO system, mounted on an inverted Axio Observer Z1. 2 mm Z stacks of regions 200–500 mm anterior to the pylorus were measured for mean signal intensity at 568 nm in Image J. Pixel intensities of Z stacks, spanning from the basal to apical cell layers, for at least 15 midguts per genotype were used for each of the quantifications.

3.5.7 Oxidative Stress Resistance Studies

Oxidative stress resistance studies were conducted by keeping 20 flies in a vial containing filter paper soaked with 300 ml of 20 mM paraquat following initial starvation for 6 hours. The number of dead flies was counted hourly during the day and twice overnight until no flies remained alive. Stress resistance data were analyzed by long-rank tests using the JMP 10 program. Total number of flies per experiment is listed in Table 1.

3.5.8 Statistical analysis

Significance was determined using a two-tailed, unpaired t-test from at least three independent experiments and expressed as P values, with the exception of oxidative stress resistance studies which were analyzed by long-rank tests using the JMP 10 program and JC-1 aggregation analysis, which used the Mann-Whitney U test because of unpaired data. Error bars represent SEM, t test, P values are specifically indicated in each figure.

Chapter 4

A gutsy way to extend longevity

A commentary on: Modulation of longevity and tissue homeostasis by the *Drosophila* PGC-1 homolog

by Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W. S., Lo, T. J., Jones, D. L., and Walker, D. W. (2011). *Cell Metabol.* 14, 623–634.

Published Frontiers in Genetics: 13 June 2012 doi: 10.3389/fgene.2012.00108

Aging is a complex biological process characterized by declining physiological functions. One of the most prominent age-associated declines is a loss of mitochondrial function. Progressive loss of mitochondrial activity and biogenesis negatively affects longevity whereas preservation of mitochondrial biogenesis results in life span extension (Cho et al., 2011; Guarente, 2008). For instance, beneficial effects and longevity extension associated with dietary restriction are closely related to increased mitochondrial biogenesis in a variety of species (Guarente, 2008). The PGC-1 family of transcription coactivators promotes mitochondrial biogenesis through coactivation of nuclear transcription factors (Scarpulla, 2011). Together, they induce expression of genes encoding mitochondrial proteins to enhance mitochondrial activity. The recent publication by Rera et al. (2011) offers additional evidence for the role of mitochondrial biogenesis in aging. They found an age related decrease in the levels of *dPGC-1* (*Drosophila PGC-1* or *spargel)* mRNA and investigated if an increase in *dPGC-1* levels in the whole body or in a tissue specific manner could affect fly longevity. While overexpression of *dPGC-1* in the whole body increases mitochondrial activity, it also decreases fly life span. However, overexpression of *dPGC-1* specifically in the *Drosophila* midgut pro- motes intestinal homeostasis and extends fly longevity. Their findings indicate a key role for mitochondrial

biogenesis in intestinal stem cell (ISC) homeostasis and longevity and provide the link between two important areas of aging research, ISCs, and mitochondrial biogenesis.

Midgut maintenance and homeostasis has recently emerged as an important determinant of fly life span (Biteau et al., 2010). The *Drosophila* midgut is maintained by multipotent ISC activity. ISCs undergo asymmetric division, giving rise to an identical daughter ISC and an immature enteroblast (EB) with differentiation potential (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). In old flies, ISCs hyper-proliferate, but ISC daughter cells do not differentiate, which results in the accumulation of misdifferentiated ISC daughter cells, a phenotype thought to con- tribute to gut aging. For instance, genetic or environmental manipulations that prevent tissue maintenance have been associated with accumulation of ISCs, irregular ISC proliferation and differentiation patterns, and shorter lifespan (Biteau et al., 2010). Likewise, genetic manipulations that pre- serve ISCs homeostasis extend longevity (Biteau et al., 2010). Rera et al. (2011) demonstrated that the age related decline in mitochondrial activity observed in the midgut epithelia may be a key component in the loss of ISCs homeostasis. The authors showed that increasing levels of *dPGC-1,* specifically in the immature cells and their progeny in the midgut is sufficient to extend longevity. The transcription factor *escargot* (esg) is a marker for ISCs and EBs. They used the *esgGal4/dPGC-1/UAS* and the *5691GeneSwitch/dPGC-1* UAS system, to drive expression of *dPGC-1* in *esg-positive* cells. *dPGC-1* overexpression in *esg-positive* cells resulted in preservation of mitochondrial membrane potential and increased activity of mitochondrial complexes I and II.

dPGC-1 and tissue homeostasis

Age-associated hyper-proliferation and accumulation of misdifferentiated cellular aggregates in the midgut is associated with increased exposure to mitochondrial free radicals. Prolonged

exposure to oxidative stress triggers persistent ISC proliferation in an attempt to restore damaged tissue. When the rate of proliferation exceeds the ability
for faithful differentiation, it results in accumulation of misdifferentiated cells (Biteau et al., 2010). Upregulation of *dPGC-1* activates ROS-detoxifying enzymes, which reduce age related oxidative damage in the midgut (Rera et al., 2011). Consequently, *esg* targeted *dPGC-1* overexpression resulted in a significant decrease in both proliferation and cellular misdifferentiation in aged flies when compared to controls.

Aged flies often exhibit irregular intestinal tissue architecture or deterioration due to impaired ISC function, which fails to replace recent regions of apoptosis (Biteau et al., 2010). *esgGAL4* driven expression of *dPGC-1* was sufficient to maintain intestinal integrity. Long-lived flies overexpressing *dPGC-1* in the gut ISCs have decreased levels of triglycerides but have the same fecundity, weight, food consumption, and response to hyperoxia and starvation as control flies. These results suggest that longevity effects of *dPGC-1* are independent from the stress resistance and reproduction.

Concluding thoughts

The studies conducted by Rera et al. successfully demonstrate a new role for *dPGC-1* and mitochondrial biogenesis in ISC homeostasis and longevity. How does it all work? *dPGC-1* overexpression increases levels of anti-oxidative enzymes to decrease oxidative damage and conserve ISC homeostasis. Preserved ISC homeostasis is required for gut maintenance, which is necessary for normal energy supply and/or prevention of microbial or toxin over- load. All of these factors may contribute to delaying the onset of age related phenotypes. As Zhou et al. (Zhou et al., 2011) suggested, it is also possible that an unknown factor/s released by PGC-1 overexpression in ISCs/EBs regulates longevity directly or through affecting other longevity

pathways. This work highlights the complex influence of *dPGC-1* on aging at the cellular level as well as at the organismal level. Overall the data presented by Rera et al. (2011) links two major components of aging and has uncovered an attractive target for the modulation of aging.

Received: 17 May 2012; accepted: 25 May 2012; published online: 13 June 2012. Citation: Rogers RP and Rogina B (2012) A gutsy way to extend longevity. Front. Gene. 3:108. doi: 10.3389/ fgene.2012.00108

This article was submitted to Frontiers in Genetics of Aging, a specialty of Frontiers in Genetics.

Chapter 5

Indy mutations preserve ISC homeostasis and intestinal integrity

5.1 Background and significance

The fly midgut facilitates nutrient uptake, waste removal and response to bacterial infection. Populations of somatic multipotent stem cells, termed intestinal stem cells (ISCs) reside in the basal region of the midgut and participate in complex signaling processes to replenish crucial mature midgut cell types (Biteau et al., 2010; Choi, 2008; Jasper and Jones, 2010; Kennedy, 2012; Micchelli and Perrimon, 2006). Interruption of the ISC regenerative response can lead to hyperproliferation and damage the intestinal epithelia (Biteau et al. 2010; Rera et al., 2011; Hochmuth et al., 2011). Loss ISC homeostasis is associated with decreased intestinal integrity and accelerated mortality.

Genetic interventions that conserve mitochondrial energetic capacity have been shown to maintain regenerative tissue homeostasis by augmenting mitochondrial bioenergetic efficiency (Biteau et al., 2008; Biteau et al., 2011; Rera et al., 2011; Hochmuth et al., 2011). Robust INDY expression in the midgut suggests that transporting metabolites across the midgut epithelia is one of the main functions of INDY (Knauf et al., 2002; Knauf et al., 2006). Reduction of INDY-mediated transport leads to an altered metabolic state, similar to CR, which activates downstream stress response pathways, and upregulates *dPGC-1* activity (Wang et al. 2009; Neretti et al., 2009; Figure 3.1). Increases in *dPGC-1* preserve mitochondrial functional efficiency without consequential changes in ROS. Recently, *dPGC-1* upregulation in stem and progenitor cells of the digestive tract was shown to preserve intestinal stem cell (ISC) proliferative homeostasis and extend lifespan (Rera et al., 2011), leading to the hypothesis that one mechanism underlying *Indy*-mediated longevity may be due to preserved ISC homeostasis.

5.1.2 ISC proliferation and homeostasis

The *Drosophila* midgut is a pseudostratified epithelium maintained by small populations of basally located intestinal stem cells (ISCs) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs maintain midgut epithelial tissue homeostasis in the adult fly via highly regulated proliferation and differentiation patterns, similar in function to mammalian ISCs (Casali and Batlle, 2009). Asymmetric division of an ISC yields one identical quiescent progenitor cell and an immature enteroblast cell (EB) with differentiation potential (Figure 1.2). ISCs and EBs both express the *escargot* (*esg*) transcription factor on their surface, but can be distinguished by the additional presence of the Notch ligand, Delta, on the ISC surface. Upregulation of Notch signaling between EB and ISC will direct differentiation into a mature enterocyte cell (EC). Weak Notch signaling drives enteroendocrine (EE) differentiation (Ohlstein and Spradling, 2007).

Age-related accumulation of mitochondrial reactive oxygen species (ROS) reduces the regulation of ISC proliferation patterns (Biteau et al., 2011a; Biteau et al., 2010; Choi, 2008; Hochmuth et al., 2011; Rera et al., 2011). This causes an unfavorable imbalance between ISC division and EB differentiation into mature EC or EE cells (Figure 1.2). The lack of regulated intestinal homeostasis is marked by an age-related increase in immature and misdifferentiated cell types in aged *Drosophila* midguts (Biteau et al., 2010; Choi, 2008). As a result, large populations of ISCs, known as aggregates, accumulate in the gut as flies age creating a toxic environment that induces premature aging. These conditions impair metabolic tissue function and contribute to the onset of age-related diseases (Biteau et al., 2010; Choi, 2008; Hochmuth et al., 2011; Jasper and Jones, 2010; Kennedy, 2012; Rera et al., 2011; Rogers, 2012).

5.2 Rationale

Recently, *dPGC-1* upregulation in stem and progenitor cells of the digestive tract was shown to reduce midgut ROS levels, preserve ISC proliferative homeostasis and extend lifespan in flies (Rera et al., 2011). Low levels of ROS maintain stemness, self-renewal and multipotency in ISCs; whereas, age-associated ROS accumulation induces continuous activation marked by ISC hyper-proliferation and loss of intestinal integrity (Hochmuth et al., 2011). This phenotype suggests a relationship between energy homeostasis and ISC proliferation in aging *Drosophila*. Based on the finding that *Indy* mutant flies have reduced free radical production and oxidative damage it is possible that enhanced mitochondrial physiology in the midgut likely preserves the intestinal redox environment and promotes ISC proliferative homeostasis. These experiments examine the effects of enhanced mitochondrial physiology in *Indy* mutant flies on midgut homeostasis.

5.3 Results

5.3.1 Indy is expressed in the midgut

Indy is robustly expressed in the basolateral membrane of the midgut and functions in the transports Krebs cycle intermediates across the epithelia and into tissue associated with intermediate metabolism (Rogina, 2000, Knauf et al., 2002). The midgut is maintained by *esg*-expressing populations of ISCs that divide and differentiate in order to maintain tissue homoestasis. Using $Indy^{206}$ and *esgLacZ* flies which both contain βgal reporter constructs in the respective region of expression, the spacial relationship between *Indy* expression and *esg*-positive undifferentiated midgut cells was determined. Whole mount X-gal staining revealed INDY to surround the midgut membrane in a comb-like pattern (Figure 5.1A); whereas, *esg*-positive cells presented in a punctate pattern through out the entirety of the midgut (Figure 5.1B).

In order to further characterize the cellular localization of INDY, midguts were analyzed via confocal microscopy following immunostaining for INDY and the architectural marker *armadillo* (Biteau et al., 2010). INDY staining is most prominent in lateral images and is replaced by *armadillo* in medial sections (Figure 5.2 left, middle). Cross sections reveal basally localized INDY and *armadillo* localization immediately apical surrounding cell populations above the lumen (Figure 5.2 right). These findings suggest that INDY is localized to the outermost membrane of the midgut and may function in transporting metabolites into basally located cell populations.

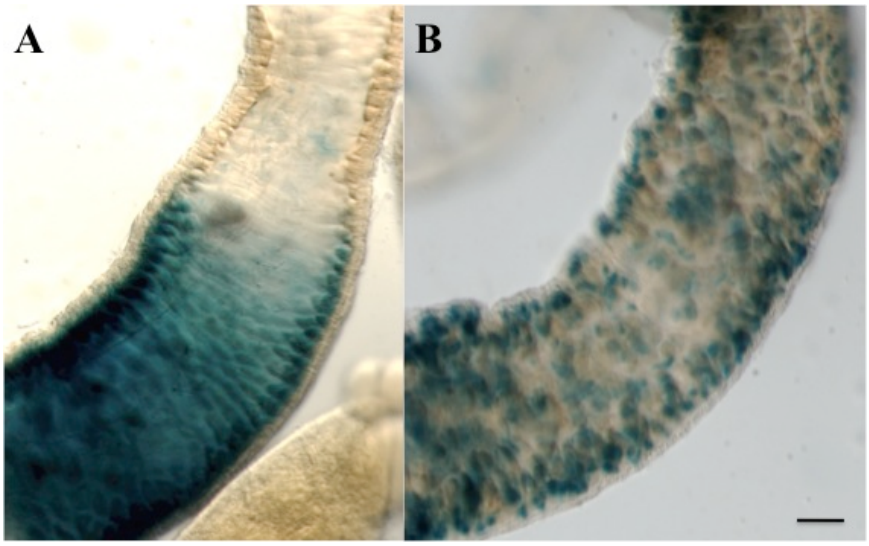

Figure 5.1: *Indy* and *escargot* are expressed in the midgut. (A) Whole mount X-gal staining of $Indy^{206}/Indy^{206}$ midgut tissue shows regions of *Indy* expression. **(B)**Whole mount X-gal staining for *escargot*-positive cells shows punctate positive staining through out the midgut (Images acquired at 40x, scale bar represents 2µm).

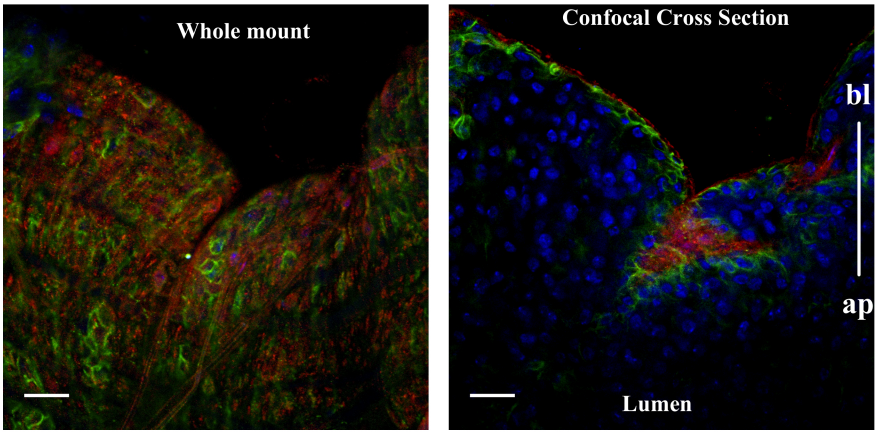

Figure 5.2: INDY is localized to the basolateral plasma membrane of the midgut. Positive staining for INDY (red) is prominent in the outer plasma membrane of the midgut (left) in control *yw* flies. Confocal cross-section of the midgut shows basally localized INDY, which is retained to the plasma membrane of the tissue. Armadillo (green, cell junctions) and DAPI (blue, nuclear) staining show cellular architecture. Scale bar represents 1um (bl: basal; ap: apical).

5.3.2 Characterization of Indy^{YC0030} mutant flies for ISC studies

While the *Indy*^{206} mutant strain is most used for studying the beneficial effects of *Indy* mutations, other lines have been developed to expand the breadth of analysis. The effect of decreased *Indy* expression on intestinal homeostasis was examined using the *Indy*^{YC0030} mutant fly line, which has a fluorescent protein (GFP) tag inserted in the *Indy* gene region (Quinones-Coello *et al*., 2007). In order to reduce genetic background interference, *Indy*^{YC0030} mutant flies were backcrossed into the *yw* genetic background for 10 generations. qPCR analysis shows reduced *Indy* transcript in the midgut of *Indy*^{YC0030}/+ flies at levels similar to those observed in *Indy*^{206}/+ mutants, confirming significant reduction (Figure 5.3A, 5.3C). Longevity studies were employed to determine whether this strain of mutants exhibited characteristic lifespan extension of *Indy* mutant flies. Both male and female flies show significant longevity extension, with a 58.3% and 42.2% increase in median life span, respectively (Figure 5.3B, 5.3D, Table 5.1). Together these data support the *Indy*^{YC0030} fly line as a comparable model to the most commonly studied *Indy*^{206} mutant strain.

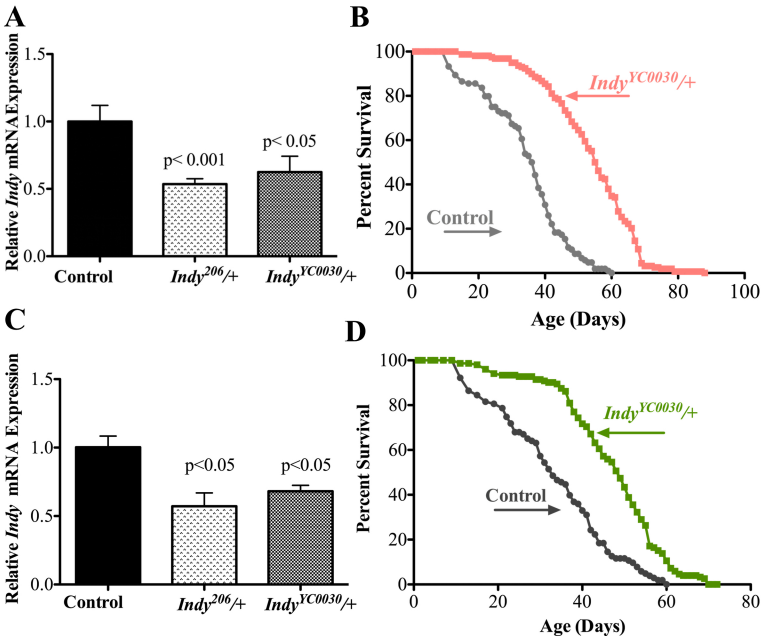

Figure 5.3: Characterization of *Indy*$^{YC0030/+}$ heterozygous mutant flies. (A) *Indy* mRNA levels in the midgut of control (*yw)*, *Indy*^{206}/+ and *Indy*^{YC0030}/+ female flies aged 20 days determined by qPCR. There is a significant ($p<0.001$, $p<0.05$, n=3, 25 guts per replicate) decrease in *Indy* mRNA levels in the midgut of *Indy*^{206}/+ and *Indy*^{YC0030}/+. Error Bars represent SEM. **(B)** Life-span curves of control (gray) and heterozygous *Indy*^{YC0030}/+ females (pink). A 58.3% increase in median survival was observed in heterozygous *Indy*^{YC0030}/+ females. **(C)** Endogenous *Indy* mRNA levels in the midgut of control (*yw*), *Indy*^{206}/+ and *Indy*^{YC0030}/+ male flies aged 20 days determined by qPCR. There is a significant ($p<0.05$, n=3, 25 guts per replicate) decrease in *Indy* mRNA levels in the midgut of *Indy*^{206}/+ and *Indy*^{YC0030}/+. Error Bars represent SEM. **(D)** Life-span curves of control (gray) and heterozygous *Indy*^{YC0030}/+ males (green). A 46.8% increase in median survival was observed in heterozygous *Indy*^{YC0030}/+ males.

Gender	Genotype	N	Median Lifespan (% Change)	X^2	*p*	Maximal Lifespan (% Change)
F	*yw*	160	34.1	-	-	53.5
F	$Indy^{YC0030}$/+	180	54 (58.3)	148.5	p< 0.0001	72 (34.5)
M	*yw*	145	32.9	-	-	55.6
M	$Indy^{YC0030}$/+	160	46.8 (42.2)	61.2	p<0.0001	64.8 (16.4)

Table 5.1. Characterization of heterozygous $Indy^{YC0030}$ /+ mutant fly longevity. The median and maximal lifespan of female (F) and male (M) *yellow-white (yw)* control and heterozygous *Indy* ($Indy^{YC0030}$/+) mutant flies in *yw* background. N: number of flies in each lifespan. Median and maximal lifespans are in days. Long-rank analyses were performed using the JMP 10 program.

5.3.3 Indy mutations preserve ISC homeostasis

The number of undifferentiated cells in control *yw* and *Indy*^{YC0030} mutant flies was assessed using flies that express ßgal under an *esg* reporter (*esgLacZ).* Both the ISC and the progenitor EB express the transcription factor *escargot (esg);* therefore *esg*-positive cells represent undifferentiated cells (Figure 4.1, Micchelli & Perrimon*,* 2006; Ohlstein & Spradling, 2006). *esgLacZ* flies were backcrossed for 10 generations into *yw* background and made heterozygous to represent a control cohort with endogenous levels of *Indy* and a ßgal reporter. Similarly, *esgLacZ* flies were crossed to *Indy*^{YC0030} mutant flies to generate a population of flies with reduced *Indy* and the *esg* reporter. The presence of the ßgal reporter allowed for immunofluorescence labeling and visual quantification of midgut tissue at various ages. Quantification of ßgal-positive cells in aging male and female control *esgLacZ*/+ and *esgLacZ*;*Indy*^{YC0030}/+ midgut tissue show significantly fewer ßgal-positive cells in *Indy*^{YC0030}/+ flies compared to control flies at 40 days, suggesting protection against age-related accumulation (Figures 5.4A, 5.4B and 5.5A, 5.5B).

Phosphorylation of histone 3 (pH3) occurs during mitosis and marks active cell proliferation (Fabienne, 2001) ISCs are the only known dividing cells in the gut epithelia; therefore, pH3-positive cells represent dividing ISCs. Analysis of *Indy*^{YC0030}/+ mutant midgut tissue shows significantly fewer pH3-positive cells compared to control *esgLacZ*/+ flies at 40 days (Figures 5.45C, 5.4D and 5.5B). Moreover, the number of pH3-positive cells is steady from 20-40 days in *Indy* mutant flies, indicating preserved ISC proliferative homeostasis (Figure 5.4E).

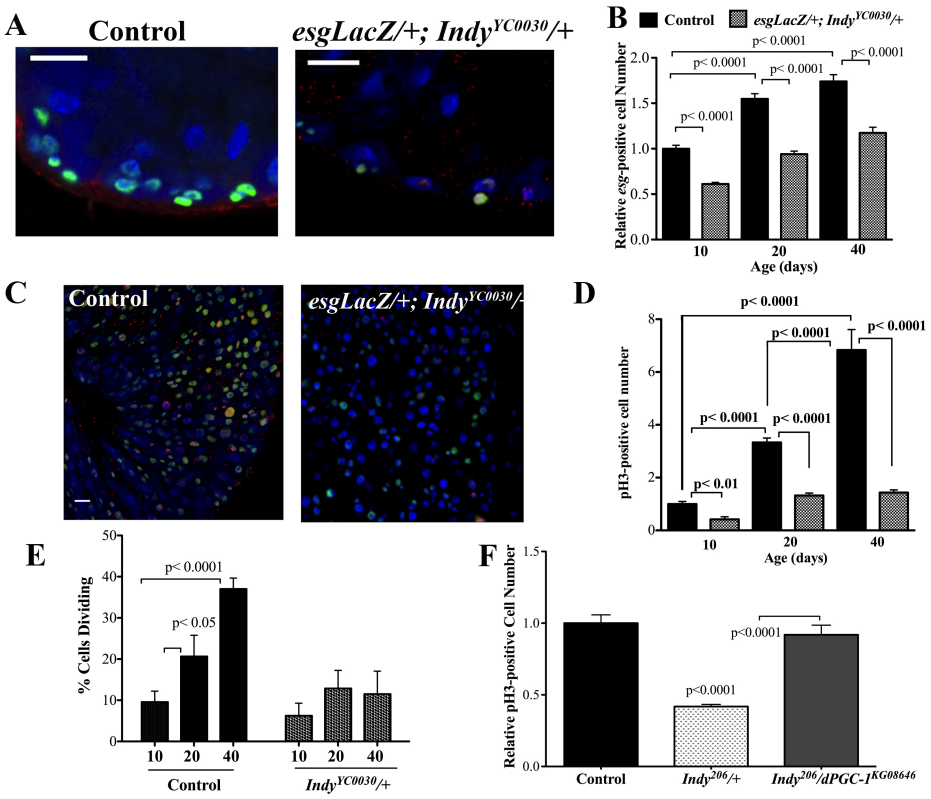

Figure 5.4: *Indy* mutations preserve ISC homeostasis (A) Immunostaining for INDY (red) DAPI (blue nuclear) and β-galactosidase (green) in the midgut of female control (*esgLacZ; yw*) and *Indy* mutant (*esgLacZ;Indy*^{YC0030}/+) flies at 20 days at 40X. Scale bar represents 1µm. **(B)** Quantification shows reduced *esg*-positive cells in *esgLacZ;Indy*^{YC0030}/+ mutant female midguts throughout lifespan ($p<0.0001$, $n>20$). **(C)** Immunostaining for β-galactosidase activity (green), nuclear (blue) and pH3-positive cells (red) in control (*esgLacZ;yw)* and *esgLacZ/+; Indy*^{YC0030}/+ mutant midgut tissue at 40 days. β-gal- positive cells represent ISC/EB populations and pH3-positive cells represent dividing cells. Scale bar represents 1µm. **(D)** Quantification of pH3-positive cells shows increased cell division in female *esgLacZ;yw* midguts throughout lifespan that is largely absent in *esgLacZ;Indy*^{YC0030}/+ mutant females ($p<0.01$, $p<0.0001$, $n>20$). Error bars represent SEM. **(E)** Quantification of dividing cells in the midgut determined by the ratio of pH3-positive cells compared to *Bgal*-positive cells shows consistent proliferations rates *esgLacZ;Indy*^{YC0030}/+ mutant females throughout lifespan ($p<0.05$, $p<0.0001$, $n>20$). Error bars represent SEM. **(F)** Quantification of pH3-positive cells in the midgut of control (*yw*), *Indy*^{206}/+ mutant and *Indy*$^{206/+}$*/dPGC-1*^{KG08646} male flies at 40 days. There are reduced dividing cells in the midgut of *Indy* mutant flies ($p<0.0001$, $n>15$). Error bars represent SEM.

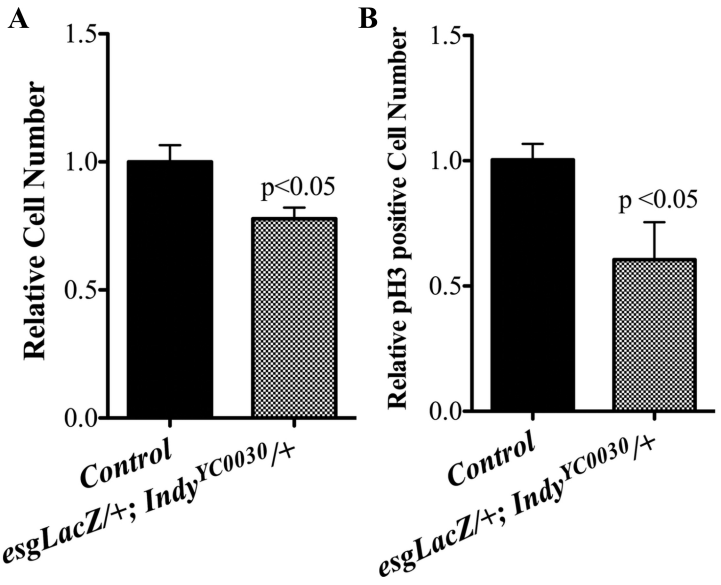

Figure 5.5: Reduced INDY modulates intestinal homeostasis. (A) Immunostaining of a control (*esgLacZ/yw*) male midgut at 40 days imaged at 40x (left) and enlarged (80x). *esg*-positive cells (green) and INDY (red) are expressed in the midgut. Scale bar represents 1μm. **(B)** Quantification of *esg*-positive cells in the midgut of control (*esgLacZ/yw*) and *Indy* mutant (*esgLacZ;Indy*^{YC0030}/+) male flies at 40 days. There are reduced ISC/EBs in the midgut of *Indy* mutant flies ($p<0.05$, $n>15$).

Upregulation of *dPGC-1* in progenitor cells of the midgut decreases ISC hyperproliferation and preserves intestinal integrity (Rera et al., 2011). Our finding that *Indy* mutant flies have significant upregulation of *dPGC-1* transcript in the midgut and reduced pH3-positive cells suggest that these two phenotypes may be related. Using double mutant $Indy^{206}/dPGC\text{-}1^{KG08646}$ flies, we determined that two copies of *dPGC-1* are required to maintain low numbers of pH3-positive cells in aged $Indy^{206}$ mutants (Figure 5.4F). Unlike $Indy^{206}/+$ mutants, double mutant $Indy^{206}/dPGC\text{-}1^{KG08646}$ midguts have significantly more pH3-positive cells, suggesting *dPGC-1* mediates downstream effects of *Indy* reduction (Figure 5.4F).

5.3.4 Indy mutations preserve intestinal integrity

ISCs maintain intestinal architecture by replacing damaged cells that comprise the barrier between lumen and hemolymph in the midgut. Age-related accumulation of aggregates can cause regions of disjoined cells, which is associated with luminal leakage into the hemolymph and decreased intestinal integrity. This condition decreases proper nutrient absorption and accelerates the onset of mortality (Rera et al., 2011). Electron micrographs of control female midguts show such a phenotype; however, $Indy^{206}/Indy^{206}$ midguts clearly retain cellular architecture (Figure 5.6A and 5.6B).

To assess the state of intestinal integrity in aged *Indy* mutants, 2.5% w/v non-absorbable FD&C blue dye #1 was added to fly food and the percentage of flies displaying total tissue staining was quantified (Rera *et al*., 2011). About 40% of aged controls show loss of intestinal integrity illustrated by total staining throughout the body after feeding (Figure 5.6C and 5.6D). In contrast, only about 10% of $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ mutant flies were completely blue, with most retaining blue dye in the digestive tract and proboscis similar to young flies (Figure 5.6D).

In order to determine the role of *dPGC-1* in preserving intestinal integrity of *Indy* mutant flies, the same assay was employed to measure the presence of blues staining in $Indy^{206}/dPGC\text{-}1^{KG08646}$ flies. Approximately 30% of $Indy^{206}/dPGC\text{-}1^{KG08646}$ flies exhibit total tissue staining, representing an intermediate phenotype (Figure 5.6D). While there are fewer $Indy^{206}/dPGC\text{-}1^{KG08646}$ blue flies in the population compared to controls, there are still more than $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ flies, which suggests that *dPGC-1* must be downstream of *Indy* and is requred to fully preserve intestinal integrity. Altogether, analysis of intestinal integrity at the cellular and functional levels provides evidence that decreased *Indy* activity preserves ISC proliferative homeostasis and intestinal integrity; however, full benefits are only achieved if *dPGC-1* is present and functional.

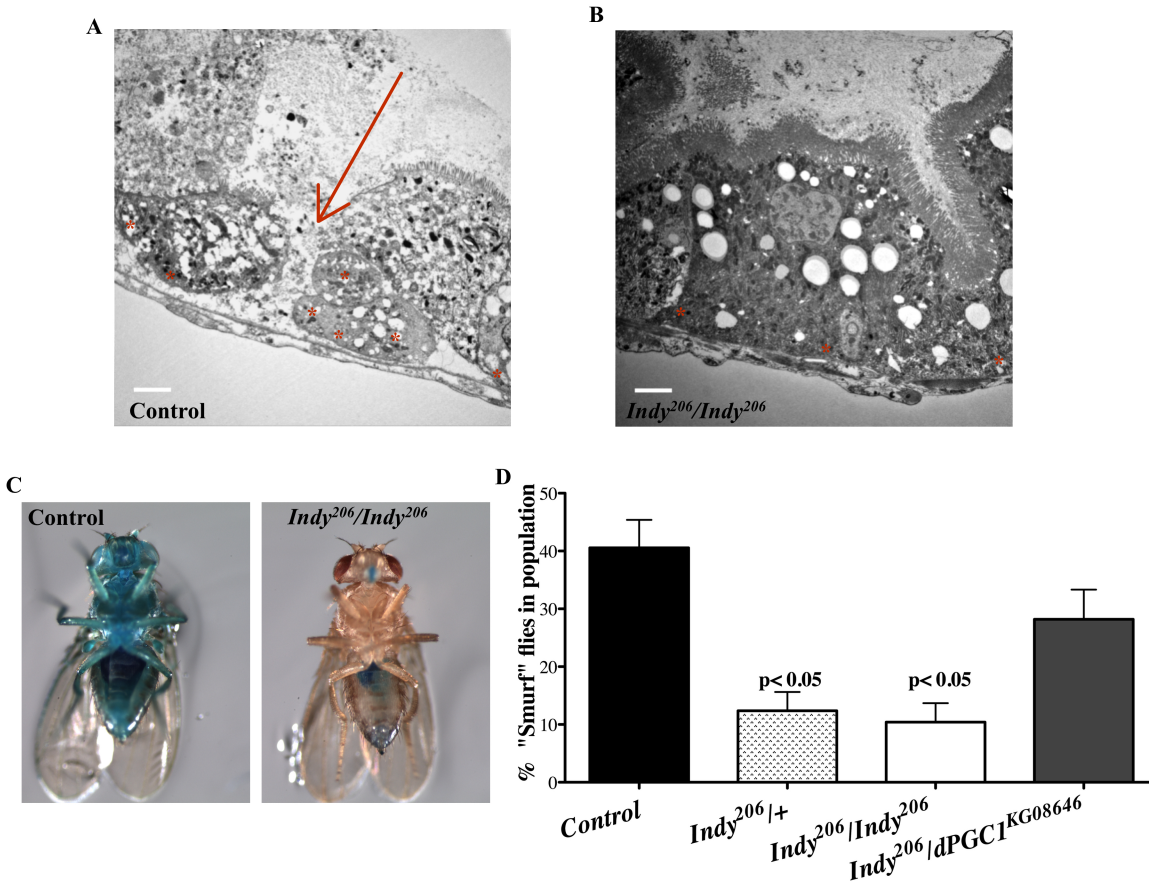

Figure 5.6: *Indy* mutations preserve intestinal integrity. (A) Electron micrograph of control *yw* and **(B)** $Indy^{206}/Indy^{206}$ midgut imaged at 1000x. Arrow shows damaged tissue and asterisks mark ISCs. (A-B Scale bar represents 1 µm). **(C)** Female control and $Indy^{206}/Indy^{206}$ flies fed food containing 2.5% w/v FD&C blue dye for 150 minutes at 40 days. Blue coloring throughout body indicates loss of intestinal integrity. **(D)** Quantitative analysis of blue staining. Control and $Indy^{206}/dPGC\text{-}1^{KG08646}$ flies have a significantly higher number of blue flies compared to $Indy^{206}/+$ and $Indy^{206}/Indy^{206}$ mutants ($p<0.01$, $n>50$).

5.4 Discussion

It was recently reported that intestinal homeostasis correlates with longevity in flies with altered Jun-N-terminal Kinase (JNK), IIS and *dPGC-1* activity (Beebe et al., 2010; Biteau and Jasper, 2011; Kennedy, 2012; Rera et al., 2011). The data presented here support the addition of *Indy* to this list of genes as a potential regulator of ISC homeostasis and longevity extension. The physiological changes induced as a result of *Indy* mutation promote redox homeostasis, which in turn delays the onset of age-related ISC hyperproliferation and midgut dysplasia. The relationship between ISC regulation and longevity suggests that maintaining healthy intestinal function and integrity is an important determinant of fly life span.

The relationship between nutrient availability and ISC activity serves as a useful model to understand the effects of *Indy* mutation on ISC homeostasis. The rate of midgut ISC proliferation has been shown to dramatically increase when food becomes available in newly eclosed flies, driving growth, while the opposite was observed during starvation (O'Brien et al., 2011). Similarly, nutrient availability affects ISC proliferation in adult flies as illustrated by the effects of CR on stem cell quiescence and activation (Biteau et al., 2011a; Biteau et al., 2010; Jasper and Jones, 2010). CR is thought to trigger an evolutionarily adaptive response to changes in the environment, allowing energy allocation toward somatic maintenance to ensure survival (Jasper and Jones, 2010). These findings connect the observed changes in *Indy* mutant intermediary metabolism to those related to ISC proliferation.

Plasticity in energy metabolism allows stem cells to match the energetic demands of self-renewal and differentiation as demonstrated by constant shifts between glycolysis and oxidative phosphorylation (Panopoulos et al., 2012; Zhang et al., 2012). Pluripotent cells favor glycolysis to accelerate the rate of energy production and provide access to partially oxidized substrates

from the TCA to be used as building blocks for biosynthesis (Folmes et al., 2012). CR is known to decrease glycolysis, which is consistent with reports of slower tissue expansion during development and decreased ISC hyperproliferation in aged tissue (O'brien et al., 2011; Choi et al., 2009; Biteau et al., 2010). As a plasma membrane transporter INDY can mimic CR by decreasing the availability of cytoplasmic citrate and impeding conversion into malate for use in the TCA. Such conditions could decrease the available energy needed to initiate ISC proliferation and therefore preserving tissue function during aging.

Another downstream effect of *Indy* mutation is related to the downstream changes in mitochondrial biogenesis and ROS detoxification. As described earlier, *dPGC-1* mRNA expression is significantly increased at all ages in $Indy^{206}$ mutant flies and is associated with elevated ROS-detoxification gene expression (Figure 3.1 and 3.7). Low levels of intracellular ROS are required for ISC stemness and pluripotency, whereas increased levels of ROS signals proliferation (Hochmuth et al., 2010). Enhanced modulation of ROS as a result of preserved mitochondrial bioenergetic efficiency together with elevated ROS-detoxification genes promotes intestinal regenerative homeostasis by preventing ROS-induced hyperproliferation and allowing ISC division to occur in response to natural stimuli such as injury, apoptosis or bacterial infection. Analysis of *Indy* mutant architecture is consistent with such a phenotype as marked by consistent rates of ISC proliferation throughout lifespan, and reduced *esg*-positive cell aggregates in aged tissue. Such conditions promote intestinal integrity, tissue function and are thought to contribute significantly to the long-lived phenotype of *Indy* mutant flies.

5.5 Materials and Methods

5.5.1 Fly strains and culture

The *Indy*^{206} line was obtained from Tim Tully (Boyton & Tully, 1992). The *Indy*^{YC0030} line was obtained from Lynn Cooley (Quinones-Coello *et al*., 2007). The *esgLacZ (y*^{1}*w*^{67c23}*; esgLacZ/Cyo) (#10359), y*^{1}*; P {SUPor-P} SpargelKG08646ry506/TM3, Sb1Ser1 (#14965)* and *yellow-white (yw)* flies were obtained from the Bloomington Stock Center at Indiana University. Flies were collected within 24 hours after eclosion and maintained in plastic vials containing standard food medium and kept in a humidified, temperature-controlled incubator with 12/12-h on/off light cycle at 25 °C. All strains were backcrossed 10x to *yellow-white (yw)* background and reared on food containing 25-mg/mL tetracycline for 3 generations to eliminate *Wolbachia*, followed by several generations in tetracycline-free food.

5.5.2 X-gal staining and imaging:

Flies aged 20 days were dissected to separate abdomen and fixed in 1% gluteraldehyde. Following fixation flies were washed and incubated with X-gal substrate solution and covered overnight at 37°C. Flies were washed and stored at 4°C in 70% glycerol until further analysis of βgal expression. High power images were obtained using Leica S40/0.45 microscope and captured via the Leica camera attachment and analyzed in Adobe Photoshop.

5.5.3 Longevity Studies

Lifespan studies were performed using 10 groups of 25 male and 25 female flies, which were collected within 24 hours following eclosion as described above. Flies were transferred to fresh food every other day for the first 30 days and then every day until no flies remained alive. The

number of dead flies was scored after each passage. Longevity data were censored for early mortality (1-9 Days) and analyzed by long-rank tests using the JMP 10 program.

5.5.4 Quantitative PCR (qPCR)

Total RNA was isolated from the midguts of 3 biological replicates with more than 25 flies in each replicates using Trizol as described (Wang *et al*., 2009). qPCR was performed following cDNA synthesis and changes in gene expression patterns were determined using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). Gene specific TaqMan primers for *Indy* were obtained from the Invitrogen. All experiments were run in triplicate and *ankryn* used as an endogenous control.

5.5.5 Electron Microscopy

Flies were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer as described (Ohlstein & Spradling, 2006). A minimum of 15 electron micrographs of midgut sections of each sample were taken at 10,000-15,000x, using an unbiased sampling method. Images were processed and analyzed in Adobe Photoshop. Post-fixation was conducted for 1 hr in 1% osmium tetroxide-0.8% potassium ferricyanide. Samples were stained in block with 1% aqueous uranyl acetate, dehydrated in a graded ethanol series, and embedded in Spurr low-viscosity epoxy resin. Thin sections of areas containing midgut were stained with uranyl acetate and lead citrate, and examined in a Hitachi H7650.

5.5.6 Immunostaining, Quantification of ISCs/EBs and pH3+ Cells

Midguts were dissected from flies at 10, 20 and 40 days, fixed in 4% paraformaldehyde and stained as described (Rera *et al*., 2011). Following washing, samples were mounted and imaged using the Leica camera attachment using LAS V4.1 software, or the Zeiss 780 combined

confocal/FCS/NLO system, mounted on an inverted Axio Observer Z1. Fixed tissue was incubated overnight with mouse anti-gal 1:500 (Invitrogen); rabbit anti-pH3 1:300 (Invitrogen) or rabbit anti-INDY 1:300 (Knauf et al., 2002) primary antibodies diluted in PBT [0.1%Triton X-100 in phosphate-buffered saline (PBS)] at 4°C. Following washing and blocking, tissue was incubated with the goat anti-rabbit Cy3 1:300 (Jackson) or goat anti-mouse FITC 1:300 (Jackson) secondary antibodies and DAPI 1:1000 (Invitrogen) diluted in PBT and 2% donkey serum for 1 hour at room temperature. Images were analyzed using Adobe Photoshop or Image J. Variability between different regions of the gut was reduced by quantifying images from the same designated region for each genotype in a 0.06x 0.02cm area. Cells were counted, values averaged and standard deviation calculated separately.

5.5.7 Intestinal Integrity

Quantification of intestinal integrity was done as described (Rera et al., 2011). More than 50 female flies were transferred to standard lab food containing 2.5% w/v FD&C blue dye #1 for 150 minutes beginning at 7am. The percentage of blue flies per population was quantified and represented as mean averages ± SE values.

5.5.8 Statistical analysis

Significance was determined using a two-tailed, unpaired t-test from at least three independent experiments and expressed as P values, with the exception of longevity studies. Error bars represent SEM, t test, P values are specifically indicated in each figure.

Chapter 6

The mechanism for *Indy* mutant longevity extension

6.1 Background and significance

It is interesting to consider the tissue-specific requirements for *Indy* gene function during aging. P-element insertional mutagenesis results in reduction of *Indy* gene function in all tissue where it is normally expressed and significant lifespan extension in flies, however the effects on health and homeostasis may vary between tissues. Robust INDY expression in the midgut suggests that transporting metabolites across the midgut epithelia is one of the main functions of INDY; therefore downstream changes in activity and signaling interactions may have a larger influence on longevity. Further support comes from findings that intestinal homeostasis correlates with lifespan in a number of different genotypes including flies with altered Jun-N-terminal Kinase (JNK), IIS and *dPGC-1* activity (Beebe et al., 2010; Biteau et al., 2008; Biteau and Jasper, 2011; Biteau et al., 2011b; Biteau et al., 2010; Cordero and Sansom, 2012; Jiang et al., 2009; Rera et al., 2011).

Progress in ISC research has provided a bridge between energy metabolism and tissue homeostasis in flies that is believed to strongly influence aging. Decreased *Indy* expression and restricted upregulation of *dPGC-1* in midgut stem and progenitor cells extends lifespan in flies (Rogina et al., 2000; Rogina & Helfand, 2013; Rera et al., 2011). Moreover, these two genotypes share other similarities with respect to mitochondrial physiology, ROS-detoxification and intestinal architecture, suggesting a shared mechansim for mediating longevity extension in flies. The goal of this study is to characterize the relationship between *Indy* mutation, tissue-specific effects of *Indy* and the downstream effects on *dPGC-1* in the context of longevity.

6.2 Rationale

Reduced INDY alters metabolism and extends longevity in a manner similar to caloric restriction (CR) with few biological tradeoffs; however, little is known about the tissue specific physiological effects of INDY reduction or how such changes contribute to fly health and longevity. Here emphasis is placed upon the effects of INDY reduction in the *Drosophila* midgut due to the importance of intestinal tissue homeostasis in healthy aging and longevity. Genetic reduction of *Indy* expression via insertional mutagenesis results in increased midgut expression of *dPGC*-1, which is accompanied by increased mitochondrial biogenesis and reduced reactive oxygen species (ROS). Consistently, reports showing elevated *dPGC-1* in the midgut yield similar phenotypes and longevity extension to those observed in *Indy* mutant flies. These physiological changes in the *Indy* mutant midgut preserve intestinal stem cell (ISC) homeostasis and are associated with healthy aging, leading to the hypothesis that *dPGC-1* is a likely mediator of downstream beneficial effects in the context of healthy aging.

If *Indy* and *dPGC-1* longevity pathways overlap, we would not expect that overexpresson of *dPGC-1* in *esg*-positive cells of $Indy^{206}$ mutant flies would further extend longevity of *Indy* mutant flies. This is a key experiment because it will provide insight into the mechanism for *Indy*- mediated lifespan extension and highlight tissue-specific requirements for INDY activity.

6.3 Results

6.3.1 Does tissue-specific Indy manipulation have an effect on longevity?

Indy reduction is associated with increased longevity in many genetic backgrounds (Rogina and Helfand, 2013; Wang et al., 2009); however tissue specific effects of *Indy* reduction have yet to be fully explored. INDY is robustly expressed in tissue associated with intermediary fly metabolism (Figure 2.1); therefore we examined the effect of targeted INDY rescue in

metabolic tissue on *Indy* mutant fly lifespan. *Indy*^{3044} mutant flies have reduced *Indy* expression as a result of P-element insertion; however, the P element insertion contains GAL4 binding sites, which allows for spatial and temporal manipulation of *Indy* expression (Figure 6.1). These flies were crossed to several GeneSwitch drivers corresponding to the fat body (*S106-GS*), oenocyte (*promE800GS (2-2b)/TM3),* and the midgut (*TIGS2-GS*), in effort to restore INDY levels in a tissue specific manner (Luc Poirier, 2008). Interestingly longevity studies showed no change in longevity between controls and tissuee-specific *Indy* mutants (Figure 6.2). These data suggest that the mechanism for *Indy*-mediated longevity may be related to a more specific group of cells or global reduction in all tissue. However, there is a possibility that lack of extension may be due to inefficient GAL4 driver activity.

The effects of INDY reduction in the *Drosophila* midgut are of particular interest due to the robust expression pattern and importance of intestinal tissue homeostasis in healthy aging and longevity. The *escargotGal4* driver line was crossed to *Indy*^{3044} mutant flies to target INDY expression to the progenitor cells of the digestive tract. Although *esgGal4* is also expressed in stem cells of malpighian tubules, the testis and in salivary glands, expression in the midgut is the focus due to its importance in healhy aging (Biteau *et al*., 2010). To avoid any effects of genetic background, all flies were backcrossed to *yw* background for 10 generations. These *Indy* mutant flies have reduced levels of INDY and when crossed to the GAL4 driver line; therefore, in principle, INDY levels should be restored to that of wild type (Figure 6.3). Since *Indy* reduction is associated with increased longevity, it was expected that restoration would return longevity patterns to those observed in wild-type flies and thereby isolate a tissue-specific role for INDY in longevity. Progenitor cell-targeted *Indy* restoration in ISCs and EBs resulted in reduced maximum lifespan.

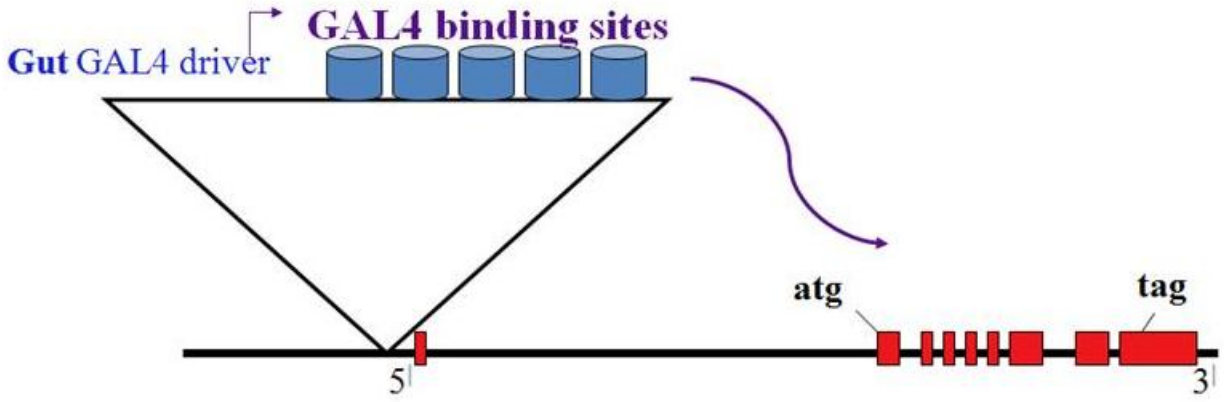

Figure 6.1: Structure of the *Indy*^{3044} P-element. *Indy*^{3044} flies have reduced INDY as a result of P-element of insertional mutagenesis in the *Indy* gene region. The P-element contains GAL4 binding sites, which allows for manipulation of INDY levels in a spatial and temporal manner.

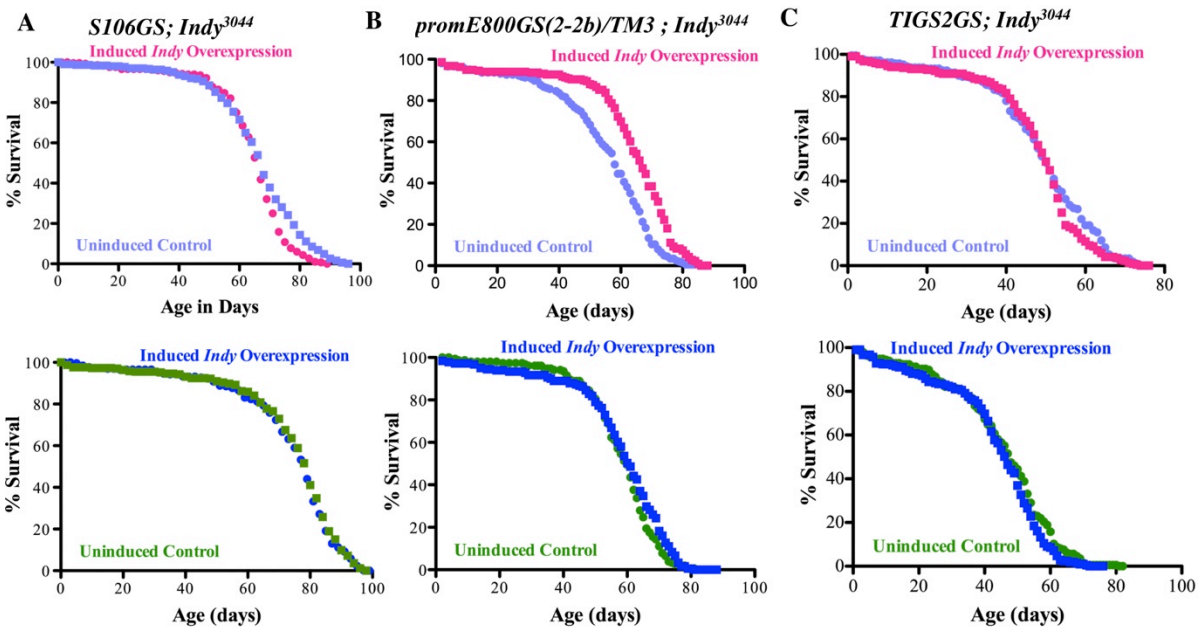

Figure 6.2: There is no change in longevity when INDY is restored in fat body, oenocytes or the midgut. (A) Female (top) and male (bottom) *S106GS;Indy*^{3044} flies show no significant change in longevity when INDY is restored in a fat-body specific pattern. **(B)** Female (top) and male (bottom) *promE800GS (2-2b)/TM3;Indy*^{3044} flies show no significant change in longevity when INDY is restored in an oenocytes specific pattern. **(B)** Female (top) and male (bottom) **(C)** *TIGS2-GS;Indy*^{3044} flies show no significant change in longevity when INDY is restored in a midgut-specific pattern. Together these data suggest that longevity is related to ubiquitous *Indy* reduction.

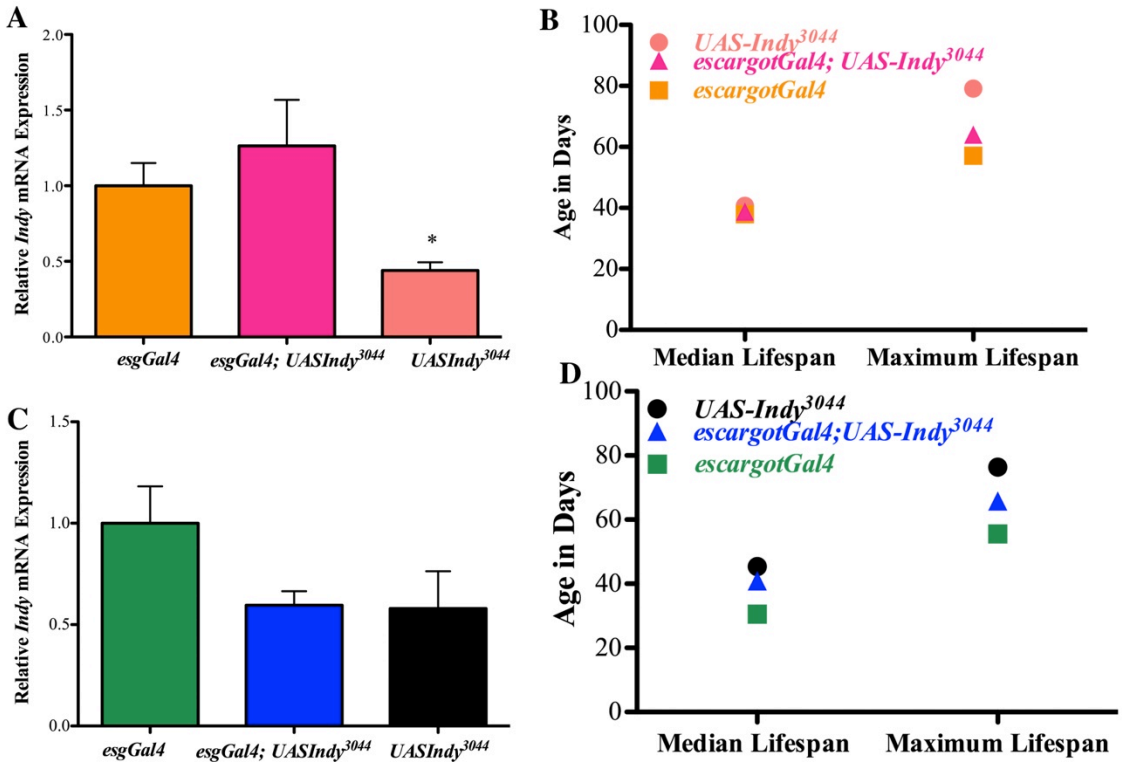

Figure 6.3: Progenitor cell-targeted *Indy* overexpression decreases maximum lifespan in flies. (A) *Indy* mRNA levels in the midgut of female and **(C)** male control (*esgGal4/yw)*, *esgGal4; UASIndy3044*$^{/+}$ and *UASIndy*^{3044} flies aged 20 days determined by qPCR. There is a significant ($p<0.05$, n=3, 25 guts per replicate) decrease in *Indy* mRNA levels in the midgut of *UASIndy*^{3044} Error Bars represent SEM. **(B)** Average median and maximum life spans of *esgGal4/yw*, *esgGal4; UASIndy3044*$^{/+}$ and *UASIndy*^{3044} female and **(D)** male flies. There is no difference in median longevity; however, flies experience non-significant reduction of maximum longevity when I*ndy* is rescued in progenitor cells of the digestive tract.

Noteably, median lifespan is not significantly different between *Indy* mutant flies (*Indy*^{3044}) and flies with *Indy* overexpression restricted to the progenetor cells (*esg;Indy*^{3044}) (Figure 6.3). This could represent a delay in onset of beneficial effects on lonevity; however age-related changes *Indy* mRNA induction levels must be further analyzed to rule out a dosage effect. Since there were no significant changes when INDY was manipulated in various tissue or cell-types, it is likely that the mechanism for *Indy*-mediated longevity is related to the effect of global augmentation of intermediary metabolism and individual changes in tissue activity comprise a combinatoral effect rather than a singular mechanism.

6.3.2 Indy-longevity is mediated by dPGC-1

Decreased *Indy* expression and restricted upregulation of *dPGC-1* in midgut stem and progenitor cells extends lifespan in flies (Rogina et al., 2000; Rogina & Helfand, 2013; Rera et al., 2011). As described above, *Indy* mutants have significantly increased *dPGC-1* mRNA levels in the midgut throughout lifespan (Figure 3.1); therefore it was of interest to investigate whether these two longevity pathways share the same or a similar mechanism. The *esgGAL4/UASdPGC-1*(*Spargel*^{EY05931}*)* system was used to overexpress *dPGC-1* in stem and progenitor cells of the digestive tract (Brand & Perrimon, 1993; Rera et al., 2011). The first 9 days of longevity studies were censored following eclosion to reduce the influence of early, non-age associated death on longevity studies. Female and male *esgGal4;UAS-dPGC1* flies have increased median lifespan by 19.9% and 35.1%, respectively, compared to control *esgGal4/yw* flies (Figure 6.4, Table 3). If *Indy* and *dPGC-1* longevity pathways overlap, overexpresson of *dPGC-1* in *esg*-positive cells of *Indy*^{206} mutant flies (*esgGal4;Indy*^{206}*;UAS-dPGC-1)* is not expected to further extend the longevity of *Indy* mutant flies. While these flies have median lifespan extension of 27.7% and

40.6%, in females and males, respectively, compared to controls, they do not experience additional increase in lifespan compared to *esgGal;UAS-dPGC-1* (Figure 6.5, Table 3).

Flies heterozygous for the $Indy^{206}$ allele with one copy of the *UAS-dPGC-1* construct ($Indy^{206}$*;UAS-dPGC-1*) experience lifespan extension compared to the survivorship of *UAS-dPGC-1/yw* flies with 22.5% and 23.5% increases in female and male flies respectively, (Figure 6.5, Table 4). Furthermore longevity extension was not observed in double mutant $Indy^{206}$*/dPGC-1*^{KG08646} flies compared to genetic controls (UAS-*dPGC-1/yw)*, suggesting that the longevity extension observed in $Indy^{206/+}$ mutant flies is most likely mediated by increased levels of *dPGC-1* (Figure 6.5, Table 3). This conclusion is supported by findings that *dPGC-1* mRNA levels found in the midgut of *Indy* mutant flies are the same as those found in flies overexpressing *dPGC-1* in *esg*-positive cells in *Indy* mutants or flies with wild type INDY (Figure 6.6). Together, the data support a model for *Indy*-mediated longevity that is mediated by downstream activation of *dPGC-1* to mediate changes in mitochondrial physiology.

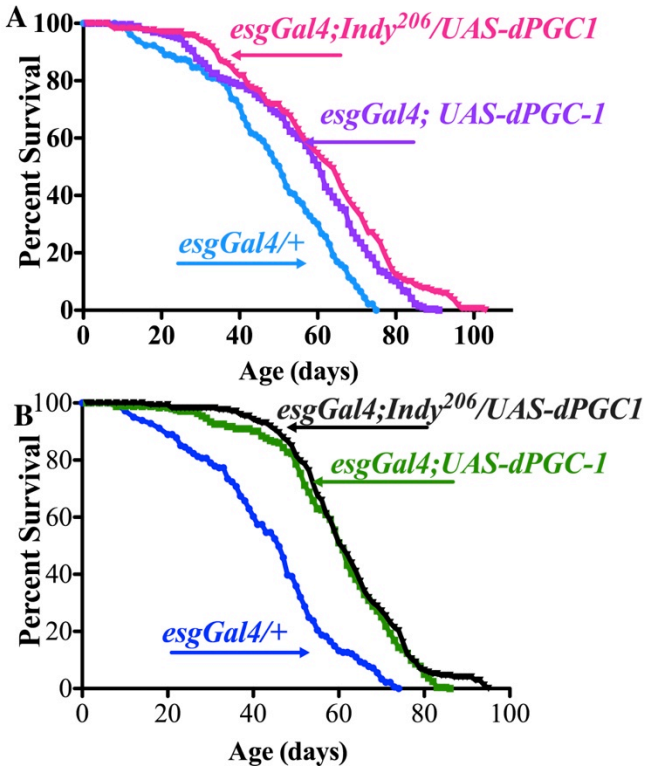

Figure 6.4: *Indy* and *dPGC-1* longevity pathways overlap. (A) Lifespan curves of female *esgGal4;Indy^{206}/UAS-dPGC1* (magenta), *esgGal4; UAS-dPGC-1* (purple) and genetic controls (*esgGal4/+*) (blue) flies. *esgGal4; UAS-dPGC-1* females overexpressing *dPGC-1* in *esg*-positive cells and *Indy* mutant females with *dPGC-1* overexpression in the *esg*-positive cells have 19.9%, and 27.7% increase in median longevity compared to genetic controls (*esgGal4/+*) flies, respectively. **(B)** Lifespan curves of male *esgGal4;Indy^{206}/UAS-dPGC1* (black), *esgGal4; UAS-dPGC-1* (green) and genetic controls (*esgGal4/+*) (blue). *esgGal4; UAS-dPGC-1* males overexpressing *dPGC-1* in *esg*-positive cells, and *Indy* mutant male with *dPGC-1* overexpression in the *esg*-positive cells have 35.1%, and 40.6% increase in median longevity compared to genetic controls (*esgGal4/+*), respectively.

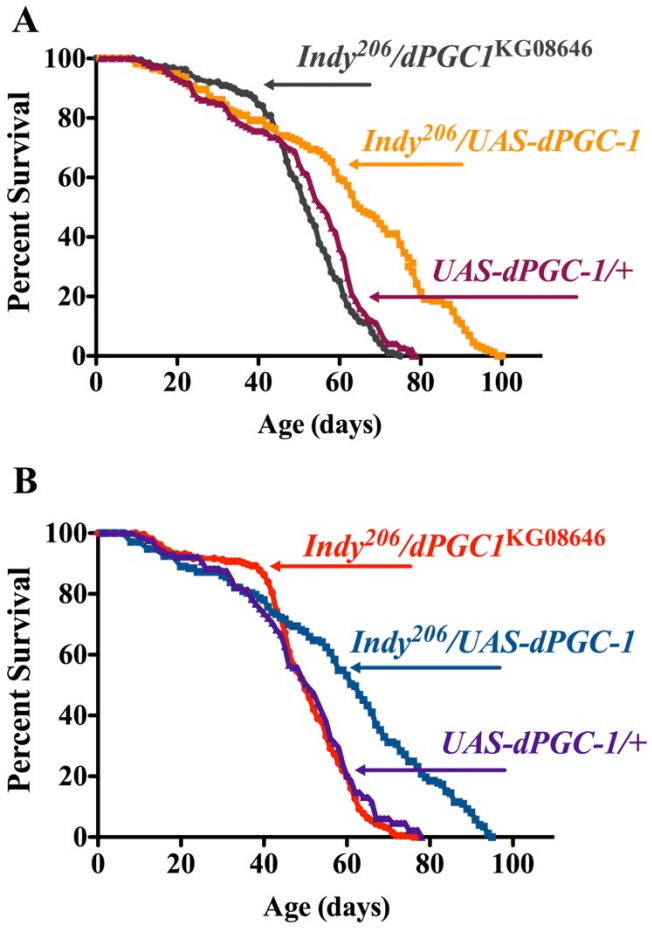

Figure 6.5: *dPGC-1* is required for longevity extension in *Indy* mutant flies (A) Lifespan curves of female *Indy*^{206} mutants with a hypomorphic allele for *dPGC-1 (Indy*^{206}*/dPGC-1*^{KG08646}) (gray), *Indy*^{206}*; UAS-dPGC-1* (orange) and genetic controls (UAS-*dPGC-1/yw*) (maroon). *Indy*^{206}*/dPGC-1*^{KG08646} flies show similar longevity compared to controls. **(B)** Lifespan curves of male *Indy*^{206} mutant with hypomorphic allele for *dPGC-1 (Indy*^{206}*/dPGC-1*^{KG08646}) (red), *Indy*^{206}*; UAS-dPGC-1* (blue) and genetic controls (*UAS-dPGC-1/yw*) (green). *Indy*^{206}*/dPGC-1*^{KG08646} flies show similar longevity compared to controls.

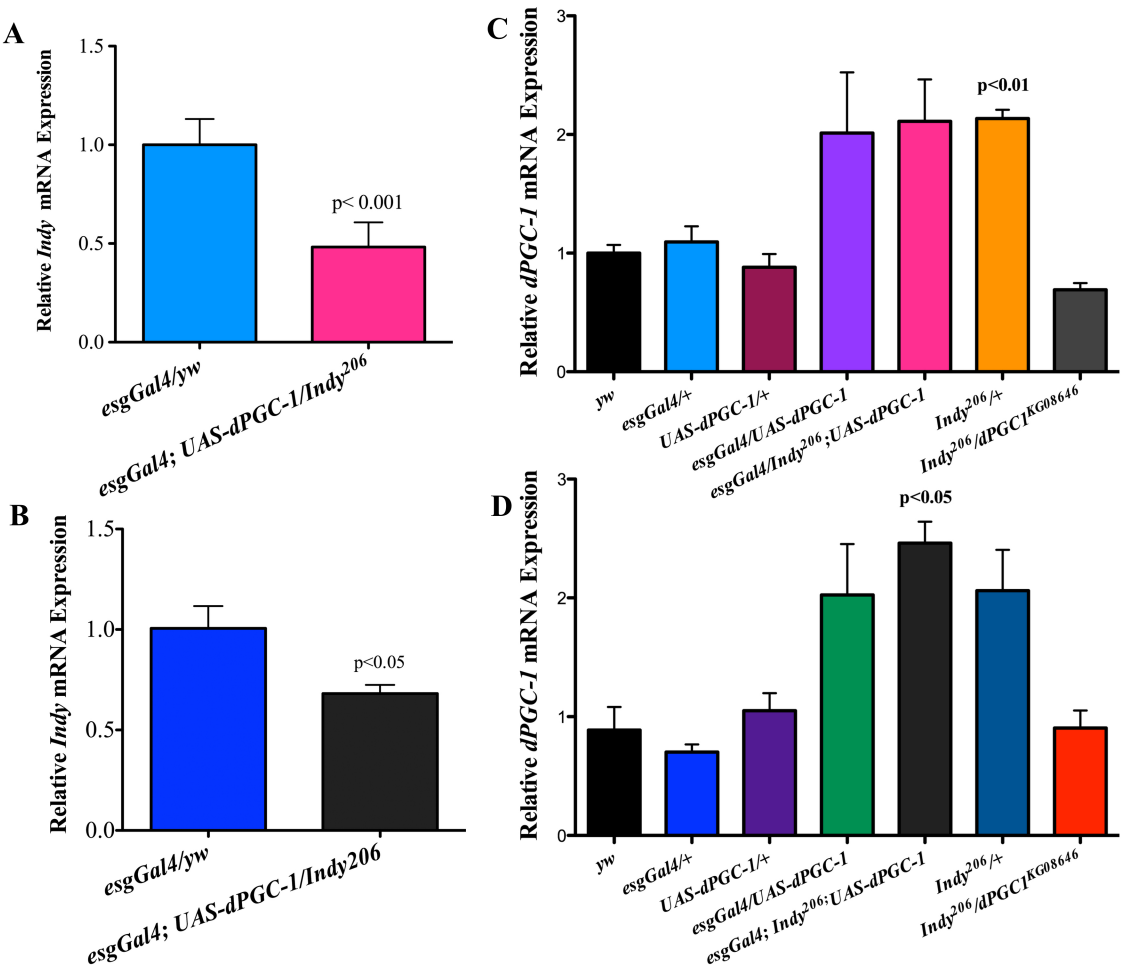

Figure 6.6: *Indy* mutation and *dPGC-1* overexpression increase *dPGC-1* mRNA in the midgut. (A) Relative *Indy* mRNA levels measured by qPCR. *Indy* is significantly decreased in *esgGal4;Indy^{206}/UAS-dPGC-1* females and **(B)** males at 20 days compared to control ($p<0.001$, $p<0.05$, n=3, 25 guts per replicate). **(C)** Relative *dPGC-1* mRNA levels in the midguts of females and males **(D)** measured by qPCR. *dPGC-1* is increased in *esgGal4; UAS-dPGC-1, Indy^{206}; UAS-dPGC-1 and esgGal4;Indy^{206}/UAS-dPGC-1* and decreased in *Indy^{206}/dPGC-1^{KG08646}*, compared to control *esgGal4/+* ($p<0.05$, $p<0.001$ n=3, 25 guts per replicate) but not between groups. Error bars represent SEM.

Gender	Genotype	N	Median Lifespan (% Change)	X^2	*p*	Maximal Lifespan (% Change)
F	*esgGal4/+*	182	48.4	-	-	71.95
F	*esgGal4; UAS-dPGC-1*	262	58.1 (19.9)	49.6	p< 0.001	85.1 (18.3)
F	*esgGal4/+;Indy^{206}/UAS-dPGC-1*	170	61.8 (27.7)	67.73	p< 0.001	93.2 (29.6)
M	*esgGal4/+*	180	44.7	-	-	70.3
M	*esgGal4; UAS-dPGC-1*	243	60.4 (35.1)	110.9	p<0.001	81.1 (15.5)
M	*esgGal4/+;Indy^{206}/UAS-dPGC-1*	173	62.9 (40.6)	106.0	p<0.0001	86.7 (23.3)

Table 6.1: *Indy* and *dPGC-1* pathways overlap to extend longevity. The median and maximal lifespan of female (F) and male (M) control *esgGal4/+*: heterozygous flies with the e*sgGal4* driver in *yellow white* background. *esgGal4; UAS-dPGC-1*: *escargotGal4* driver possess one copy of *UAS-dPGC-1* construct overexpressing *dPGC-1* in *esg*-positive midgut progenitor cells. *esgGal4/+;Indy^{206}/UAS-dPGC-1*: *esgGal4* driver crossed to *UAS-dPGC-1* construct overexpressing *dPGC-1* in *esg*-positive midgut progenitor cells in *Indy^{206}* mutant background. All values are compared to either male or female *esgGal4/+* control groups to determine the percent increase in median and maximal lifespan. *N*: number of flies used in the experiment. Median and maximal lifespan are in days. Data are censored for 0-9 days. Long-rank analyses were performed using the JMP 10 program.

Gender	Genotype	N	Median Lifespan (% Change)	X^2	*p*	Maximal Lifespan (% Change)
F	*UAS-dPGC-1/+*	150	51.5	-	-	73.3
F	*Indy^{206};UAS-dPGC-1*	173	63.1 (22.5)	58.48	p< 0.001	95.9 (30.8)
F	*Indy^{206}/UAS-dPGC-1^{KG08646}*	188	51.8 (0.58)	5.19	p=0.022	70.1 (-4.3)
M	*UAS-dPGC-1/+*	145	48.5	-	-	71.3
M	*Indy^{206};UAS-dPGC-1*	173	59.9 (23.5)	84.21	p<0.0001	92.9 (30.2)
M	*Indy^{206}/UAS-dPGC-1^{KG08646}*	205	49.2 (1.4)	1.21	p=0.27	66.7 (-6.5)

Table 6.2: *dPGC-1* is required for *Indy* mutant longevity extension. The median and maximal lifespan of female (F) and male (M) control *UAS-dPGC-1/+*: heterozygous flies with the *UAS-dPGC-1* construct in *yellow white* background. *Indy^{206}; UAS-dPGC-1: UAS-dPGC-1* construct in *Indy^{206}* mutant background. *Indy^{206}/UAS-dPGC-1^{KG08646}*: *Indy^{206}* crossed to *dPGC-1^{KG08646}* flies hypomorph for the *dPGC-1*. All values are compared to either male or female *UAS-dPGC-1/+* control groups to determine the percent increase in median and maximal lifespan. *N*: number of flies used in the experiment. Median and maximal lifespan are in days. Data are censored for 0-9 days. Long-rank analyses were performed using the JMP 10 program.

6.4 Discussion

Previous studies using *Xenopus* oocytes suggest that INDY facilitates the exchange of metabolites and expression patterns in metabolic tissue support a role in intermediary metabolism (Rogina et al., 2000; Knauf et al., 2002; Knauf et al., 2006). Reduced *Indy* expression dramatically extends lifespan in multiple genetic backgrounds when mRNA is decreased to an optimal level (Rogina et al., 2000; Rogina and Helfand 2013). P-element insertion in the *Indy* gene locus results in down regulation of INDY-transport activity and lifespan extension. Similarly, *Indy* transcript is also reduced in the heads, thoraces and midgut during CR (Wang et al., 2009; Figure 2.4), suggesting that INDY may function as a downstream physiological regulator of CR by augmenting intermediary nutrient transport.

Flies have specialized organs whose principle function is in the utilization, uptake and storage of nutrients during intermediary metabolism. The localization of INDY in such tissue and role in Krebs cycle intermediate transport led to the hypothesis that manipulation of transport activity would differentially affect fly longevity. Longevity studies show no significant changes in longevity compared to *Indy* mutants when *Indy* is returned to the mutant fat body or midgut; however, female flies do show insignificant extension when INDY is increased in the oenocytes. Oenocytes metabolize fatty acids in a similar way to the mammalian liver and are required for depleting stored lipid from the fat body during fasting (Gutierrez et al., 2007). When nutrients are scarce, falling amino-acid levels trigger lipid release from the fat body and subsequent uptake from oenocytes. Marginal longevity extension when oenocytes have wild type INDY levels suggest an important role during lipid metabolism, in that lipids are an important source of energy stores during CR or fasting and INDY transport is required to sustain bioenergetic requirements.

CR induces global changes in downstream gene expression that differentially affect tissue function and benefit longevity. Mitochondrial transcriptional coactivator *dPGC-1* is upregulated during CR and studies in long-lived *Indy* mutant flies and mice show similar phenotypes, suggesting a key role in longevity extension (Lopez-LLuch et al 2006; Neretti et al. 2009; Birkenfeld et al., 2011). It was recently published that targeted overexpression of *dPGC-1* in the progenitor cells of the *Drosophila* midgut resulted in significant lifespan extension, whereas ubiquitous overexpression had detrimental effects on longevity (Rera et al., 2011). While longevity studies overexpressing INDY in progenitor cells of the *Indy* mutant digestive tract show no change in median lifespan and insignificant reduction of maximum longevity analysis of *Indy* mutant midgut tissue reveal elevated *dPGC-1* transcript, increased mitochondrial biogenesis and preservation of ISC proliferation patterns (Figures 3.1, 3.2,3.3,5.3, 5.4). These phenotypes are consistent with those reported with *esgGal4*-driven *dPGC-1* in the progenitor cells; which suggests *dPGC-1* as a potent mediator of downstream beneficial effects.

Several lines of evidence indicate that INDY and *dPGC-1* are part of the same regulatory network in the midgut, in which *dPGC-1* functions as a downstream effector of INDY. The similarity between *dPGC-1* mRNA levels and survivorship of flies overexpressing *dPGC-1* in *esg*-positive cells and *Indy* mutant flies suggests that *Indy* and *dPGC-1* interact to extend lifespan. This is further supported by the lack of additional longevity extension when *dPGC-1* is overexpressed in *esg*-positive cells of *Indy* mutant flies. Moreover, hypomorphic *dPGC-1* flies in an *Indy* mutant background are similar to controls with respect declines in mitochondrial activity, ROS-detoxification and life span. Together, these data suggest that *dPGC-1* must be present to mediate the downstream physiological benefits and lifespan extension of *Indy* mutant flies.

There are some physiological differences between the effects of *Indy* mutations and *dPGC-1* overexpression in *esg*-positive cells (Rera et al., 2011). While *Indy* mutant flies are less resistant to starvation and more resistant to paraquat, a recent report showed that overexpressing *dPGC-1* in *esg*-positive cells has no effect on resistance to starvation or oxidative stress (Wang *et al*., 2009; Rera et al., 2011). Additionally, mice lacking skeletal muscle PGC-1a were found to lack mitochondrial changes associated with CR but still showed other CR-mediated metabolic changes (Finley et al., 2012). In the fly INDY is predominantly expressed in the midgut, fat body and oenocytes, though there is also low level expression in the malpighian tubules, salivary glands, antenae, heart and female follicle cell membranes. Thus, the effects of INDY on intermediary metabolism and longevity could be partially independent from *dPGC-1* or related to changes in tissues other than the midgut.

6.5 Materials and Methods

6.5.1 Fly Strains and maintenance

The $Indy^{YC0030}$ line was obtained from Lynn Cooley (Quinones-Coello *et al*., 2007). The *esgGal4* (y^1w; *esgGAL4/Cyo)(#26816), esgLacZ* (y^1w^{67c23}; *esgLacZ/Cyo) (#10359), UAS-dPGC-1 (yw; Spargel* ^{EY05931}*) (#2009*), y^1; P *{SUPor-P} SpargelKG08646* ry^{506}/ *TM3, Sb1Ser1 (#14965)* and *yellow-white (yw)* flies were obtained from the Bloomington Stock Center at Indiana University. W; promE800GS (2-2b)/TM3 GS driver line was generously donated from the Jasper lab. The $Indy^{206}$ line was obtained from Tim Tully (Boyton & Tully, 1992). The *TIGS-2* Gene-Switch driver line was provided by Scott Pletcher and the UAS-$Indy^{RNAi}$ (w^{1118}*; P {GD2712} v9981)* line was obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007). Flies requiring gene-switch induction were grown on food containing 200 mM RU486 and controls on EtOH.

Approximately 20 females and 20 males flies are kept together in each vial and passed to fresh vials every 2 days for aging.

6.5.2 Lifespan Studies

Lifespan studies were performed using 10 groups of 25 male and 25 female flies, which were collected within 24 hours following eclosion and maintained in plastic vials containing standard, high or low calorie food medium and kept in a humidified, temperature-controlled incubator with 12/12-h on/off light cycle at 25 °C. Flies were transferred to fresh food every other day for the first 30 days and then every day until no flies remained alive. The number of dead flies was scored after each passage. Longevity data were censored for early mortality (1-9 Days) and analyzed by long-rank tests using the JMP 10 program.

6.5.3 Quantitative PCR (qPCR)

Total RNA was isolated from the midguts of 3 biological replicates with more than 25 flies in each replicates using Trizol as described (Wang *et al*., 2009). qPCR was performed following cDNA synthesis and changes in gene expression patterns were determined using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). All experiments were run in triplicate. Gene specific TaqMan primers for *Indy, dPGC-1, Pdsw, l (3) neo18, COI, ND23, ND42, ND75, GstD5 and GstD1* were obtained from the Invitrogen. *Ankryn* was used as an endogenous control for all experiments.

6.5.4 Statistical analysis

Significance was determined using a two-tailed, unpaired t-test from at least three independent experiments and expressed as P values, with the exception of longevity studies, which were

analyzed by long-rank tests using the JMP 10 program Error bars represent SEM, t test, P values are specifically indicated in each figure.

Chapter 7

I'm (still) *Not Dead Yet:* general discussion and future directions

7.1 General Discussion

CR is the most successful intervention in delaying the onset of age-related pathologies in multiple systems; however, meeting nutritional requirements under this regimen is challenging outside of a controlled lab environment. For this reason, genetic interventions that successfully mimic CR have surfaced as an alternative approach to promote healthy aging. The *Drosophila I'm Not Dead Yet* (*Indy*) gene was discovered more than a decade ago and homologs have since been identified and characterized in worms and mice (Birkenfeld et al., 2011; Fei, 2004; Fei et al., 2003). Functional characterization of the INDY protein and characterization of the mutant phenotype (Knauf et al., 2002; Rogina et al., 2000), led to the hypothesis that *Indy* mutations could induce a state of genetic CR by altering intermediary metabolism. The expression patterns, physiological properties, and lifespan extending effects of INDY support this hypothesis and reveal a role for INDY as a physiological regulator that modulates intermediate transport in response to nutrient availability.

The initial goal of this project was to identify a tissue-specific mechanism for mediating *Indy*-mutant lifespan extension. Given the variation in bioenergetic requirements and the previous characterization of INDY as an intermediate metabolite transporter, it was hypothesized that manipulation of INDY transport would have a tissue-specific effect on fly longevity. After conducting multiple longevity studies with many different tissue-specific *Gal4* or *GS* drivers in effort to decrease or upregulate INDY, it became clear that there was no single tissue responsible for mediating *Indy* mutant longevity. While these results were disappointing, they are consistent with the hypothesis that *Indy* mutations induce a genetic state of CR. CR yields reduction of *Indy*

activity in heads, thoraces and midgut (Wang et al, 2009; Rogers and Rogina 2014 *unpublished*); which suggests that longevity is most likely contingent upon cumulative downstream changes in cellular and tissue physiology.

Indy levels decrease as a result of CR, suggesting that INDY functions as a physiological regulator that modulates energy balance in response to changes in nutrient availability. A reduction of intracellular metabolite concentrations redirects nutrient sensing pathways toward survival by activating AMPK, which induces mitochondrial biogenesis via *dPGC-1*. Increased *dPGC-1* levels and mitochondrial biogenesis have been described in the muscle of *Indy* mutant flies (Neretti *et al*., 2009), the liver of *mIndy*$^{-/-}$ mice (Birkenfeld *et al*., 2011), and now the present study shows consistent results in the midgut of *Indy* mutant flies. *dPGC-1* mediated changes in mitochondrial physiology are known to have a beneficial effect on the intestinal redox state; therefore this finding presented a new approach to elucidating the mechanism for *Indy* mutant lifespan extension.

As a plasma membrane transporter, INDY can modulate intracellular metabolite concentrations and regulate nutrient metabolism. Mitochondrial malfunction is observed in subjects with age-associated insulin resistance, suggesting a connection between insulin signaling and mitochondrial functional efficiency. Glucose and lipid metabolism generate the majority of cellular energy via the TCA cycle and oxidative phosphorylation (OXPHOS). Disruption of the ETC activity leads to an imbalance in the NAD+/NADH ratio, which reduces SIRT1/PGC1α pathway activity and reduces mitochondrial biogenesis and membrane potential. Decreased INDY transport is associated with altered lipid and glucose metabolism, which is presumed to be a result of decreased intermediate substrate availability. Moreover, reduced levels of *InR* and *dilp3* accompanied by elevated *dPGC-1* and ETC complex gene expression in

the *Indy* mutant midgut suggests that *Indy* mutation decreases IIS. Furthermore, these data highlight the potential for INDY as a therapeutic target to prevent age-related insulin resistance or decreased insulin sensitivity during metabolic disease.

The recent development of a mammalian *Indy* mutant model highlights the translational potential for INDY as a therapeutic target to delay age-related pathologies. *mINDY* is a high-capacity plasma membrane transporter for citrate that is most abundant in the liver (Birkenfeld et al., 2011). Similar to *Indy* mutant flies and worms, deletion of *mIndy* in mice led to phenotypes strikingly similar to CR. *mIndy*$^{-/-}$ show increased energy expenditure, which led to reduction of whole-body fat content, skeletal muscle lipid storage, and increased insulin sensitivity (Birkenfeld et al., 2011). Moreover, analysis of *mIndy*$^{-/-}$ liver tissue shows activation of AMPK with subsequent induction of PGC-1α-mediated mitochondrial biogenesis, which is observed in the *Indy* mutant fly midgut and considered a hallmark of CR-mediated healthy aging (Birkenfeld et al., 2011). While *Indy* expression patterns differ between flies and mice, the beneficial changes in intermediary metabolism and global effects on aging suggest that improved regulation of INDY activity during aging may reduce the likelihood of detrimental age-related pathologies.

Genetic interventions that conserve mitochondrial energetic capacity by way of altering nutrient sensing have been shown to maintain a favorable redox state and regenerative tissue homeostasis (Biteau *et al*., 2008; Biteau *et al*., 2011; Rera *et al*., 2011; Hochmuth *et al*., 2011). This is particularly beneficial in the fly midgut, which facilitates nutrient uptake, waste removal and response to bacterial infection. ISCs maintain the midgut epithelia by proliferating in response to changes in intracellular ROS and the availability of oxidized TCA intermediates. Thus, decreased INDY transport likely influences ISC regenerative capacity by augmenting the intestinal redox state and decreasing intermediate availability. Although INDY overexpression in

esg-positive cells did not achieve significant changes in fly longevity, the observed changes in tissue architecture and ISC proliferation suggest that regulated tissue homoestais may be one of the contributing factors to mutant longevity extension.

These studies suggest that INDY may function as a physiological regulator of mitochondrial function and related metabolic pathways, modulating metabolite flux in response to nutrient availability. The ability to induce a metabolic state akin to CR coupled with the finding that *Indy* levels decrease in response to CR highlight the potential for INDY as a therapeutic target to mediate healthy aging. Reduced *Indy* expression causes similar physiological changes in flies, worms and mice, indicating its regulatory role would be conserved. Recent studies investigating phenotypic changes in the knockout mouse model of the mammalian *Indy (mIndy),* show many shared phenotypes with respect to mitochondrial activity and insulin signaling in the liver to those described in fly midguts. Specifically, deletion of *mIndy* protects mice from HFD-induced and age-associated insulin resistance, as a result of altered PGC-1α-mediated mitochondrial biogenesis**,** which is mechanistically similar to the effect of *Indy* mutation on midgut physiology. Together, these studies suggest that *Indy* is a physiological regulator of intermediary metabolism that may be an attractive therapeutic target to prevent the onset of age-related decline, obesity, and type 2 Diabetes.

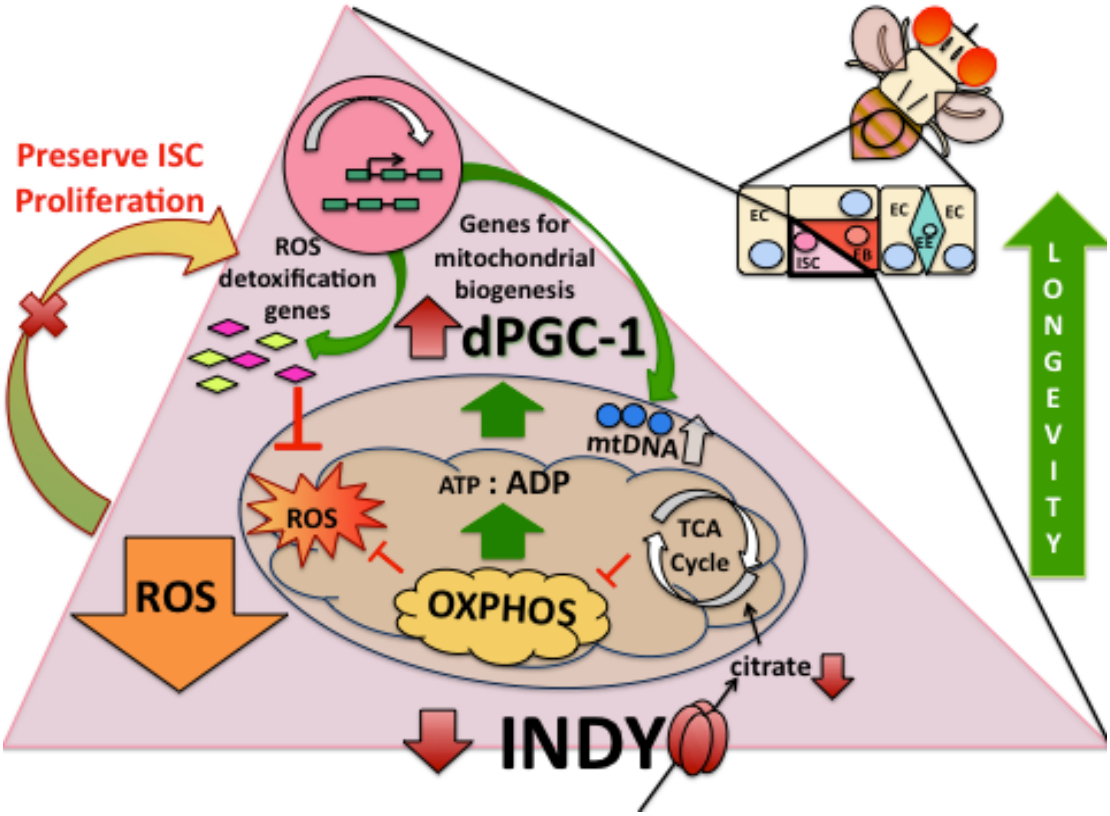

Figure 7.1 Proposed model for INDY-mediated longevity. *Indy* mutations preserve ISC homeostasis. INDY transports citrate from hemolymph into the cells, which can be transported to mitochondria and used as a substrate for the TCA cycle. Reduced INDY-mediated transport decreases citrate levels and decreases the ATP/ADP ratio. Such changes activate AMPK, promoting fat oxidation and *dPGC-1* synthesis. The increase in *dPGC-1* activity increases mitochondrial biogenesis and transcription of ROS-detoxification genes. Decreased ROS production preserves ISC homeostasis, which contributes to *Indy* mediated longevity extension.

7.2 Future Directions

Gastrointestinal disorders are a major cause of illness in the aging population and are associated with premature mortality. This dissertation focused on the way in which reduced *Indy* affects homeostatic conditions of the *Drosophila* midgut by regulating intermediary metabolism and energy balance. Given the similarity in phenotypes between *mIndy* and other *Indy* mutant model organisms, it would be of interest to investigate *mIndy*$^{-/-}$ intestinal regenerative homeostasis and examine implications for INDY as a therapeutic target for delaying aging and preventing colorectal cancer.

While the intestinal architecture is structurally different between mammals and flies, the cellular components are functionally conserved between species (Figure 2.1). For this reason, it is likely that the benefits conferred by *mIndy* and *Indy* mutation yield similar effects on midgut physiology. Some of the most prominent benefits associated with *mIndy*$^{-/-}$ and *Indy* mutants include increased *dPGC-1*-mediated mitochondrial functional efficiency and enhanced insulin sensitivity. Both of these phenotypes are related to augmented nutrient sensing via AMPK activation and shown to beneficially regulate ISC proliferation patterns. AMPK activation is also associated with regulating TOR pathway activity, which has an important regulatory role in both vertebrate and invertebrate stem cell lineages (Kapuria et al., 2012). Further studies investigating AMPK and the resulting effects on downstream nutrient sensing pathways would provide further insight into the molecular mechanisms mediating *Indy* mutant longevity in flies and mammals.

In adult organisms, tissue homeostasis is maintained by efficiently regulated signaling between adult stem cells and the surrounding environment (Casali and Batlle, 2009). The molecular mechanisms that control homeostatic self-renewal and those that underlie colorectal cancer are highly similar in that both are influenced by intricate signaling events (Radtke and

Clevers, 2005). While cancer is most often linked to mutational changes in generic oncogenes or tumor suppressors, the shift toward malignant transformation of the intestinal epithelial cells appears to occur as a result of impaired physiological regulator activity and subsequent changes in normal transcription (Radtke and Clevers, 2005).

The data provided in this dissertation suggest that INDY functions as a physiological regulator in the midgut, which regulates nutrient sensing pathway activity and mitochondrial functional efficiency. Consistently, studies using the *mIndy*$^{-/-}$ mice show preserved energy balance and signs of healthy aging, suggesting that mutations in *mIndy* may beneficially delay colorectal cancer progression (Birkenfeld et al., 2011). Further work should investigate the impact of INDY reduction on pathways that are involved in regulation of mammalian ISC proliferation and differentiation such as Notch, Wnt and JNK in order to further characterize the way in which INDY reduction modulates stem cell proliferation (Beebe et al., 2010; Biteau et al., 2008; Cordero and Sansom, 2012; Jiang et al., 2009; Kapuria et al., 2012; Lin et al., 2008; Liu et al., 2010; Ohlstein and Spradling, 2007; Radtke and Clevers, 2005).

Aging is a cumulative and progressive process; therefore it is important to investigate age-related changes in all tissues in order to fully treat pathological side effects. While INDY is robustly expressed in the midgut, it would be of interest to investigate the interplay between tissue-specific *Indy* mutation. In doing so, the molecular mechanisms, which underlie *Indy* mutant longevity will be more clear and provide insight for anti-aging therapies. In that likeness, the development of pharmaceutical treatments or characterization of natural supplements that regulate INDY expression could surface as attractive therapeutic targets to delay the onset of age-related pathologies.

Bibliography

Apidianakis, Y., and Rahme, L.G. (2011). Drosophila melanogaster as a model for human intestinal infection and pathology. Disease models & mechanisms *4*, 21-30.

Arslan-Ergul, A.T.O., Michelle M Adams (2013). Aging, Neurogenesis, and Caloric Restriction in Different Model Organisms. Aging and Disease *4*, 221-232.

Barja, G. (2013). Updating the Mitochondrial Free Radical Theory of Aging: An Integrated View, Key Aspects and Confounding Concepts. Antioxidants & redox signaling.

Beebe, K., Lee, W.C., and Micchelli, C.A. (2010). JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the Drosophila intestinal stem cell lineage. Developmental biology *338*, 28-37.

Birkenfeld, A.L., Lee, H.Y., Guebre-Egziabher, F., Alves, T.C., Jurczak, M.J., Jornayvaz, F.R., Zhang, D., Hsiao, J.J., Martin-Montalvo, A., Fischer-Rosinsky, A., et al. (2011). Deletion of the mammalian INDY homolog mimics aspects of dietary restriction and protects against adiposity and insulin resistance in mice. Cell metabolism *14*, 184-195.

Biteau, B., Hochmuth, C.E., and Jasper, H. (2008). JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut. Cell stem cell *3*, 442-455.

Biteau, B., Hochmuth, C.E., and Jasper, H. (2011a). Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell stem cell *9*, 402-411.

Biteau, B., and Jasper, H. (2011). EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. Development *138*, 1045-1055.

Biteau, B., Karpac, J., Hwangbo, D., and Jasper, H. (2011b). Regulation of Drosophila lifespan by JNK signaling. Experimental gerontology *46*, 349-354.

Biteau, B., Karpac, J., Supoyo, S., Degennaro, M., Lehmann, R., and Jasper, H. (2010). Lifespan extension by preserving proliferative homeostasis in Drosophila. PLoS genetics *6*, e1001159.

Casali, A., and Batlle, E. (2009). Intestinal stem cells in mammals and Drosophila. Cell stem cell *4*, 124-127.

Chan, D.C. (2006). Mitochondria: dynamic organelles in disease, aging, and development. Cell *125*, 1241-1252.

Cheng, Z., Tseng, Y., and White, M.F. (2010). Insulin signaling meets mitochondria in metabolism. Trends in endocrinology and metabolism: TEM *21*, 589-598.

Cho, J., Hur, J.H., and Walker, D.W. (2011). The role of mitochondria in Drosophila aging. Experimental gerontology *46*, 331-334.

Choi, N., Kim, JG., Yang, DJ., Kim, YS., Yoo, MA. (2008). Age-related changes in Drosophila midgut are associated with PVF2, a PDGF/VEGF-like growth factor. Aging Cell *7*, 318-334.

Copeland, J.M., Cho, J., Lo, T. J., Hur, J.H., Sepehr B., Arabyan,T., Rabie, J., Soh, J., Walker, D.W. (2009). Extension of Drosophila Life Span by RNAi of the Mitochondrial Respiratory Chain. Current Biology *19*, 1591-1598.

Cordero, J.B., and Sansom, O.J. (2012). Wnt signalling and its role in stem cell-driven intestinal regeneration and hyperplasia. Acta physiologica *204*, 137-143.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151-156.

Dilova, I., Easlon, E., and Lin, S.J. (2007). Calorie restriction and the nutrient sensing signaling pathways. Cellular and molecular life sciences : CMLS *64*, 752-767.

Fabienne, H.a.D., S. (2001). Histone H3 phosphorylation and cell division. Oncogene *20*, 3021-3027.

Fei, J., Liu, J.C., Inoue, K., Zhuang, L., Miyake, K., Miyauchi, S., Ganapathy, V. (2004). Relevance of NAC-2 and NAD+-coupled citrate transporter to lifespan, body size and fat content in Caenorhabditis elegans. Jounral of Biochemistry *379*, 191-198.

Fei, Y.J., Inoue, K., and Ganapathy, V. (2003). Structural and functional characteristics of two sodium-coupled dicarboxylate transporters (ceNaDC1 and ceNaDC2) from Caenorhabditis elegans and their relevance to life span. The Journal of biological chemistry *278*, 6136-6144.

Feng, J., Bussiere, F., and Siegfried, H. (2002). Mitochondrial Electron Transport Is a Key Determinant of Lifespan in Caenorhabditis elegans. Developmental Cell *1*, 11.

Folmes, C.D., Dzeja, P.P., Nelson, T.J., and Terzic, A. (2012). Metabolic plasticity in stem cell homeostasis and differentiation. Cell stem cell *11*, 596-606.

Frankel, S., and Rogina, B. (2012). Indy mutants: live long and prosper. Front Genet *3*, 13.

Guarente, L. (2006). Sirtuins as potential targets for metabolic syndrome. Nature *444*, 868-874.

Guarente, L. (2008). Mitochondria--a nexus for aging, calorie restriction, and sirtuins? Cell *132*, 171-176.

Gutierrez, E., Wiggins, D., Fielding, B., and Gould, A.P. (2007). Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. Nature *445*, 275-280.

Hochmuth, C.E., Biteau, B., Bohmann, D., and Jasper, H. (2011). Redox regulation by Keap1 and Nrf2 controls intestinal stem cell proliferation in Drosophila. Cell stem cell *8*, 188-199.

Hwang, A.B., Jeong, D.E., and Lee, S.J. (2012). Mitochondria and Organismal Longevity. Current Genomics *13*, 519-532.
Ingram, D.K., Zhu, M.M., J.,, Zou, S., Lane, M.A., Roth, G.S.,, and and deCabo, R. (2006). Calorie restriction mimetics: an emerging research field. Aging Cell *5*, 97-108.

Jasper, H., and Jones, D.L. (2010). Metabolic regulation of stem cell behavior and implications for aging. Cell metabolism *12*, 561-565.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343-1355.

Kaeberlein, M., Powers, R.W., 3rd, Steffen, K.K., Westman, E.A., Hu, D., Dang, N., Kerr, E.O., Kirkland, K.T., Fields, S., and Kennedy, B.K. (2005). Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science *310*, 1193-1196.

Kapuria, S., Karpac, J., Biteau, B., Hwangbo, D., and Jasper, H. (2012). Notch-mediated suppression of TSC2 expression regulates cell differentiation in the Drosophila intestinal stem cell lineage. PLoS genetics *8*, e1003045.

Kennedy, H.J.a.B.K. (2012). Niche science: the Aging Stem Cell. Cell Cycle *11*, 2959-2960.

Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. Cell *120*, 449-460.

Knauf, F., Mohebbi, N., Teichert, C., Herold, D., Rogina, B., Helfand, S., Gollasch, M., Luft, F.C., and Aronson, P.S. (2006). The life-extending gene Indy encodes an exchanger for Krebs-cycle intermediates. The Biochemical journal *397*, 25-29.

Knauf, F., Rogina, B., Jiang, Z., Aronson, P.S., and Helfand, S.L. (2002). Functional characterization and immunolocalization of the transporter encoded by the life-extending gene Indy. Proceedings of the National Academy of Sciences of the United States of America *99*, 14315-14319.

Lee, S.-H., and Min, K.-J. (2013). Caloric restriction and its mimetics. BMB reports *46*, 181-187.

Liesa, M., and Shirihai, O.S. (2013). Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. Cell metabolism *17*, 491-506.

Lin, G., Xu, N., and Xi, R. (2008). Paracrine Wingless signalling controls self-renewal of Drosophila intestinal stem cells. Nature *455*, 1119-1123.

Liu, W., Singh, S.R., and Hou, S.X. (2010). JAK-STAT is restrained by Notch to control cell proliferation of the Drosophila intestinal stem cells. Journal of cellular biochemistry *109*, 992-999.

Long, Y.C., Tan, T.M., Inoue, T., and Tang, B.L. (2014). The biochemistry and cell biology of aging: metabolic regulation through mitochondrial signaling. American journal of physiology. Endocrinology and metabolism.

Lopez-Lluch, G., Hunt, N., Jones, B., Zhu, M., Jamieson, H., Hilmer, S., Cascajo, M.V., Allard, J., Ingram, D.K., Navas, P., et al. (2006). Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. Proceedings of the National Academy of Sciences of the United States of America *103*, 1768-1773.

MacNeil, L.T., Watson, E., Arda, H.E., Zhu, L.J., and Walhout, A.J. (2013). Diet-induced developmental acceleration independent of TOR and insulin in C. elegans. Cell *153*, 240-252.

Marden, J.H., Rogina, B., Montooth, K.L., and Helfand, S.L. (2003). Conditional tradeoffs between aging and organismal performance of Indy long-lived mutant flies. Proceedings of the National Academy of Sciences of the United States of America *100*, 3369-3373.

Micchelli, C.A., and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature *439*, 475-479.

Neretti, N., Wang, P.Y., Brodsky, A.S., Nyguyen, H.H., White, K.P., Rogina, B., and Helfand, S.L. (2009). Long-lived Indy induces reduced mitochondrial reactive oxygen species production and oxidative damage. Proceedings of the National Academy of Sciences of the United States of America *106*, 2277-2282.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Falcone, S., Valerio, A., Cantoni, O., Clementi, E., et al. (2005). Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science *310*, 314-317.

O'Brien, L.E., Soliman, S.S., Li, X., and Bilder, D. (2011). Altered modes of stem cell division drive adaptive intestinal growth. Cell *147*, 603-614.

Ohlstein, B., and Spradling, A. (2006). The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature *439*, 470-474.

Ohlstein, B., and Spradling, A. (2007). Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science *315*, 988-992.

Osterwalder, T., Yoon, K.S., White, B.H., and Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. Proceedings of the National Academy of Sciences of the United States of America *98*, 12596-12601.

Panopoulos, A.D., Yanes, O., Ruiz, S., Kida, Y.S., Diep, D., Tautenhahn, R., Herrerias, A., Batchelder, E.M., Plongthongkum, N., Lutz, M., et al. (2012). The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. Cell research *22*, 168-177.

Poirier, L., Shane, A., Zhend, J., and Seroude, L. (2008). Characterization of the Drosophila gene-switch system in aging studies: a cautionary tale. . Aging Cell *7*, 758-770.

Puigserver, P. (2003). Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1alpha (PGC-1alpha): Transcriptional Coactivator and Metabolic Regulator. Endocrine Reviews *24*, 78-90.

Quiang, W., Weiqiang, K., Qing, Z., Pengju, Z., and Liu, Y. (2007). Aging impairs insulin-stimulated glucose uptake in rat skeletal muscle via suppressing AMPKα. Experimental and Molecular Medicine *39*, 535-543.

Radtke, F., and Clevers, H. (2005). Self-renewal and cancer of the gut: two sides of a coin. Science *307*, 1904-1909.

Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W.S., Lo, T., Jr., Jones, D.L., and Walker, D.W. (2011). Modulation of longevity and tissue homeostasis by the Drosophila PGC-1 homolog. Cell metabolism *14*, 623-634.

Rizza, W., Veronese, N., and Fontana, L. (2013). What are the roles of calorie restriction and diet quality in promoting healthy longevity? Ageing research reviews *13C*, 38-45.

Rogers, R., Rogina B. (2012). A gutsy way to extend longevity. Frontiers in Genetics *3*, 108.
Rogina, B., and Helfand, S.L. (2013). Indy mutations and Drosophila longevity. Front Genet *4*, 47.

Rogina, B., Reenan, R., Nislon, S., and Helfand, S. (2000). Extended Life-Span Conferred by Cotransporter Gene Mutations in Drosophila. Science *290*, 2137-2140.

Scarpulla, R.C. (2011). Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochimica et biophysica acta *1813*, 1269-1278.

Schlicker, C., Gertz, M., Papatheodorou, P., Kachholz, B., Becker, C.F., and Steegborn, C. (2008). Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. Journal of molecular biology *382*, 790-801.

Someya, S., Yu, W., Hallows, W.C., Xu, J., Vann, J.M., Leeuwenburgh, C., Tanokura, M., Denu, J.M., and Prolla, T.A. (2010). Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell *143*, 802-812.

St-Pierre, J., Drori, S., Uldry, M., Silvaggi, J.M., Rhee, J., Jager, S., Handschin, C., Zheng, K., Lin, J., Yang, W., et al. (2006). Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell *127*, 397-408.

Tranah, G.J. (2011). Mitochondrial-nuclear epistasis: implications for human aging and longevity. Ageing research reviews *10*, 238-252.

Verdin, E., Hirschey, M.D., Finley, L.W., and Haigis, M.C. (2010). Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. Trends in biochemical sciences *35*, 669-675.

Wallace, D.C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annual review of genetics *39*, 359-407.

Wang, A., Jeong, D., and Lee, S. (2012). Mitochondria and Organismal Longevity. Current Genomics *13*, 519-532.
Wang, P.Y., Neretti, N., Whitaker, R., Hosier, S., Chang, C., Lu, D., Rogina, B., and Helfand, S.L. (2009). Long-lived Indy and calorie restriction interact to extend life span. Proceedings of the National Academy of Sciences of the United States of America *106*, 9262-9267.

Zhang, J., Nuebel, E., Daley, G.Q., Koehler, C.M., and Teitell, M.A. (2012). Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. Cell stem cell *11*, 589-595.

Zhou, Y., Lu, T., and Xie, T. (2011). A PGC-1 tale: healthier intestinal stem cells, longer life. Cell metabolism *14*, 571-572.

Zhu, M.D.C., R.; Lane, M.A.; Ingram,D.K. (2004). Caloric Restriction Modulates Early Events in Insulin Signaling in Liver and Skeletal Muscle of Rat. Annals NY Acadamy of Science.