AN INSULIN-LIKE PEPTIDE REGULATES SIZE AND ADULT STEM CELLS IN PLANARIANS

BY

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DISSERTATION

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ABSTRACT

Animal growth depends on nutritional intake during development. In many animals, nutritional status is uncoupled from moderation of adult stature after adult size is achieved. However, some long-lived animals continue to regulate adult size and fertility in a nutrition-dependent manner. For example, the regenerating flatworm *Schmidtea mediterranea* becomes smaller, or degrows, during periods of starvation. These animals provide an opportunity to readily observe adult stem cell population dynamics in response to nutritional cues. We explore the role of insulin signaling in *S. mediterranea*. We disrupt insulin signaling via RNA interference and show that animals, despite eating, degrow similarly to starved animals. Utilizing in situ hybridization and immunofluorescence, we assess cellular changes in proliferative populations including the planarian adult stem cell population (neoblasts) and the germline. Both impaired insulin signaling and nutritional deprivation correlate with decreased neoblast proliferation. Additionally, insulin signaling plays a role in supporting spermatogenesis that is distinct from the effects of starvation. In sum, we demonstrate that insulin signaling is responsible for regulation of adult animal size and tissue homeostasis in an organism with plastic adult size. Importantly, insulin signaling continues to affect stem cell and germline populations in a mature organism. Furthermore, we show that adult organisms can differentially regulate specific cell populations as a result of environmental challenges.

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Chapter 1

Introduction

1.1 Significance

Insulin/insulin-like growth factor family signaling misregulation is closely tied to several diseases that affect a large portion of the population. The prevalence of Diabetes Mellitus in the United States is estimated to be as high as 7.8%; of this group, the majority (up to 90%) have Diabetes Mellitus Type 2, which is frequently characterized by adult onset (NIDDK, 2008). Additionally, downstream components involved in insulin/insulin-like growth factor signaling are up-regulated in many cancers (Brader and Eccles, 2004, Maki, 2010, Samani *et al.*, 2007). The incidence of cancer increases with age (NCI, 2011). Thus, studies of the cellular effects of insulin/insulin-like growth factor misregulation in a post-development context have broad implications for understanding and attenuating disease states that affect a large portion of the adult population.

The insulin peptide superfamily, including insulin-like growth factors (IGFs) and insulin-like peptides (ILPs), plays an evolutionarily conserved role in cellular maintenance and proliferation (Chan and Steiner, 2000). Insulin family signaling, in part through communicating an organism's nutritional status, affects growth and development (Böhni *et al.*, 1999, Kimura *et al.*, 1997), puberty and fertility (Arantes-Oliveira *et al.*, 2002, Böhni *et al.*, 1999, Drummond-Barbosa and Spradling, 2001, Michaelson *et al.*, 2010, Schlueter *et al.*, 2007, Tissenbaum and Ruvkun, 1998),

lifespan (Barbieri *et al.*, 2003, Bonafè *et al.*, 2003, Kimura *et al.*, 1997, Piper *et al.*, 2008, Russell and Kahn, 2007, Tatar *et al.*, 2003), and tissue regeneration (Desbois-Mouthon *et al.*, 2006). The overall effects of insulin/IGF signaling are apparent via animal models. Ames and other dwarf mice breeds, in comparison to wild type, have decreased levels of circulating insulin and insulin-like growth factor 1 (IGF-1) accompanied by small stature and long lifespan reminiscent of a calorically restricted state (reviewed in (Bartke and Brown-Borg, 2004)).

Disruption of insulin family signaling carries similar consequences in several invertebrate model systems. *daf2* (insulin receptor) mutations in *C. elegans* inhibit insulin signaling and are associated with extended lifespan (Kenyon *et al.*, 1993, Kimura *et al.*, 1997). *chico* (insulin receptor binding substrate) mutations in *D. melanogaster* result in smaller organism size and extended lifespan (Böhni *et al.*, 1999). These animal models display the organism-wide influence of insulin/IGF signaling over the course of growth, development, and adulthood. However, because of the prevalence of adult onset insulin/IGF misregulation diseases, models that isolate the disruption of insulin/IGF signaling to animals in a state of maturity could prove ultimately useful in identifying cellular mechanisms of disease and disease prevention.

I utilize the regenerating planarian *Schmidtea mediterranea* to study the role of insulin in adult stem cell function and spermatogenesis. *S. mediterranea* is essentially immortal when properly cared for in the laboratory. The worm's longevity and great regenerative capacity are due to a pool of adult pluripotent stem cells

called neoblasts, which allow the animal to stay in a state of slow but constant tissue turnover (Baguñá *et al.*, 1989, Brøndsted, 1969, Newmark and Sánchez Alvarado, 2000, Wagner *et al.*, 2011). Adult size, because of the rate of tissue turnover, is directly dependent on nutritional intake. Fed worms grow or maintain their size, while those that go without food long enough begin to shrink. While shrinking, the worms maintain their tissue proportionality and the organized process is called degrowth (Baguñá and Romero, 1981, Newmark and Sánchez Alvarado, 2002, Oviedo *et al.*, 2003, Wenemoser and Reddien, 2010). Because of these traits, *S. mediterranea* is an ideal organism for studies of the effects of insulin/IGF signaling on adult stem cells.

1.2 Specific Aims

The primary goals of the following work are: (1) To identify and characterize components of insulin signaling in *S. mediterranea.* (2) To establish a phenotype for disrupted insulin signaling in *S. mediterranea*. (3) To characterize the phenotype of disrupted insulin signaling.

To accomplish these goals, first, a candidate insulin-like peptide and putative insulin receptor tyrosine kinase are identified with bioinformatics and molecular techniques. The expression patterns for these genes are characterized with in situ hybridization. Additionally, the insulin-like peptide is identified in a mass spectrometry screen for neuropeptides. Then, gene knockdown is performed via RNA interference and animals are observed for phenotypic changes. Animal size is measured over time and testis lobe size is assessed after gene knockdown. Finally, cellular and molecular techniques including in situ hybridization, immunohistochemistry, and real-time quantitative PCR are used to analyze the observed phenotypes. Functions of specific cell populations, including neoblasts and the process of spermatogenesis, are analyzed. The phenotype resulting from impaired Insulin signaling is also discussed in the context of how it relates to ethanol exposure.

Chapter 2

Literature Review

2.1 Why study insulin in *S. mediterranea*?

Insulin/insulin-like growth factor (IGF) signaling regulates the intersections of important physiological processes including metabolism, growth, fertility, and lifespan (Barbieri *et al.*, 2003, De Meyts, 2004). The signaling pathway regulates cell and tissue dynamics during development and also after organism maturity is achieved (Shabanpoor *et al.*, 2009). The insulin peptide aspect of insulin/IGF signaling is associated with the pancreas in vertebrates and is primarily involved in relaying an organism's nutritional status to its tissues to regulate energy utilization and immediate growth. The growth factor (IGF-1 and IGF-2) aspect controls growth and maturation over the course of development. IGFs are not necessary for survival, but complete loss of pancreatic insulin, which occurs in Diabetes Mellitus Type 1 (DMT1), is lethal without treatment. On the other hand, excessive insulin/IGF activity is harmful and can be indirectly lethal. High circulating levels of insulin (i.e. Diabetes Mellitus Type 2: DMT2) or IGF can place one at increased risk for cancer development (Gallagher and LeRoith, 2010). Tumors themselves frequently have increased local levels of insulin/IGF activity, and insulin/IGF upregulation appears to be necessary but not sufficient for the transition into metastasis (Maki, 2010, Samani *et al.*, 2007). Thus, as insulin/IGF signaling is intimate with many aspects of life, including disease states, it is a signaling system of both scientific and medical

significance. Specifically, knowledge of the pathway can contribute to understanding stem cells and the micro-environmental niche they reside in, and can promote the ability to manipulate stem cells in vivo. Not surprisingly, the pathway is already a target of potential cancer therapies (Maki, 2010).

The subtle yet powerful effects of insulin/IGF signaling on adult stem cells are a challenge to study, first because of the difficulty of isolating pathway perturbations to adulthood, and second because the pathway regulates so many aspects of an animal's physiology, which then feed back upon each other. The planarian *S. mediterranea* is known (and envied) for its spectacular regenerative capacity. The worm's unique attributes, including a pluripotent adult stem cell population and a disconnection between animal size and age (adult animals proportionally shrink if food is unavailable and can degrow back to a state of reproductive immaturity), offer an exciting opportunity to examine the effects of insulin/IGF signaling on adult stem cells in vivo.

Planarians caught the interest of T.H. Morgan and other scientists around the turn of the 19th century because of their regenerative prowess (reviewed in (Newmark and Sánchez Alvarado, 2002)). However, for many years, scientific techniques lacked the sophistication to dissect the molecular mechanisms underlying regeneration. Recently, the advent of modern molecular methods has caused a resurgence of interest in and ability to study planarian regeneration (Newmark and Sánchez Alvarado, 2002). One planarian species, *S. mediterranea*, is especially tenable as a model organism and is currently studied in laboratories

around the world. *S. mediterranea* has a stable diploid genome (2n=8, ~800Mb) that was sequenced to 11.6x coverage and assembled at the Washington University Genome Sequencing Center ((Robb *et al.*, 2007); http://smedgd.neuro.utah.edu/). Cellular and molecular techniques including in situ hybridization, immunohistochemistry, and RNA inteference have been developed for this organism (Newmark *et al.*, 2003, Newmark and Sánchez Alvarado, 2000, Newmark and Sánchez Alvarado, 2002, Pearson *et al.*, 2009, Robb *et al.*, 2007, Zayas *et al.*, 2005), leading to studies that explore the animal's stem cells and regenerative capacity (Oviedo *et al.*, 2008, Reddien *et al.*, 2005a, Wagner *et al.*, 2011, Wenemoser and Reddien, 2010).

Planarians can regenerate an entire new worm from an estimated 1/279th of an organism (Morgan, 1897). Its regenerative ability depends on a population of pluripotent adult stem cells, or neoblasts (Baguñá *et al.*, 1989, Brøndsted, 1969, Newmark and Sánchez Alvarado, 2002, Wagner *et al.*, 2011). The neoblasts not only support regeneration, but maintain a state of constant tissue turnover, which means the organism holds particular promise for developing an understanding of tissue identity, maintenance, and homeostasis (Oviedo *et al.*, 2003, Pellettieri *et al.*, 2010, Pellettieri and Sánchez Alvarado, 2007). *S. mediterranea* does not appear to develop observable age-related diseases or age-related health problems in the laboratory, and is thus easy to maintain. Although it is impossible to empirically state that an animal is immortal, *S. mediterranea* can be described as a negligibly senescent animal (i.e. it does not appear to senesce or age over time); the lifespan

of an individual worm is not currently known or even predictable because there is no known phenotype of aging or age-related decline.

There are two strains of *S. mediterranea*: asexual and sexual reproducers. Asexuals reproduce by fission; sexual worms, while hermaphroditic, lay cross-fertilized eggs. By taking advantage of the species' regenerative ability, clonal lines of both strains have been established (Sánchez Alvarado *et al.*, 2002, Zayas *et al.*, 2005). The sexual strain of *S. mediterranea* develops germ cells post-embryonically; *nanos*, a primordial germ cell-associated gene, is not expressed in newly hatched worms but appears within several days in the region where testis lobes will develop (Wang *et al.*, 2007).

An intriguing aspect of both the asexual and sexual strain is that, no matter how old a worm is, its size depends on nutritional intake. In other words, fed animals grow until an approximate maximal size, but underfed or starved animals shrink. Adult size or stature in *S. mediterranea* is therefore plastic rather than fixed. The worms shrink proportionally rather than becoming thin or misshapen; all tissues become smaller and specific cell populations generally remain a constant percentage of whole cell counts (Baguñá and Romero, 1981, Newmark and Sánchez Alvarado, 2002, Oviedo *et al.*, 2003, Wenemoser and Reddien, 2010). Because the animal largely maintains its proportionality while shrinking, the physiological process is referred to as degrowth. In the sexual strain, gonad development and maintenance are dependent on animal size. Newly hatched animals grow to an approximate stature, at which point gonads (two ovaries and

multiple testes lobes) and accessory reproductive organs develop. However, if sexually mature *S. mediterranea* are starved and degrow smaller than that required stature, the gonads actually regress, although *nanos*-positive clusters of germline stem cells remain (Wang *et al.*, 2007). Subsequent feeding and growth reestablishes sexual tissues and reproductive maturity (Wang *et al.*, 2007). The plasticity of *S. mediterranea*'s size and germline is fascinating and also renders these animals an asset to studies of adult stem cells, sexual development, and fertility.

Insulin/IGF signaling is functionally conserved throughout metazoa and plays an important role during the development of many organisms (Chan and Steiner, 2000, Chen *et al.*, 1996, Christoforidis *et al.*, 2005, Tatar *et al.*, 2003). However, cellular changes in mature organisms that result from post-growth insulin/IGF signaling misregulation are difficult to appreciate. Thus, the cellular consequences of insulin/IGF signaling manipulations that are masked in other adult organisms may be more readily apparent in mature *S. mediterranea* because of the organism's adult stem cells, germline stem cells, and plastic adult size. The unique qualities of the model organism *S. mediterranea* offer an opportunity to examine adult stem cell and germline function after insulin/IGF signaling disruption.

2.2 Insulin peptide family evolution

Insulin protein, purified in 1922, was one of the first proteins crystallized and was the first protein sequenced by Sanger (Chan and Steiner, 2000, De Meyts,

2004, Rosenfeld, 2002). The early protein purification was driven by medical need, and virtually as soon as the protein was obtained, it was administered to patients with Diabetes Mellitus Type 1, saving lives (Rosenfeld, 2002).

In subsequent decades, vertebrate insulin peptide superfamily members were identified because of insulin-like activity or sequence similarity. All insulin peptide superfamily members have six canonical cysteine residues that contribute to the highly conserved tertiary structure of the peptide (Chan and Steiner, 2000, Ivell *et al.*, 2005, Pierce *et al.*, 2001). Many vertebrates have two insulin growth factors (IGFs) with mitogenic (mitosis promoting) properties that function during development (Shabanpoor *et al.*, 2009). Vertebrates also have a number of insulin-like peptides (ILPs), including members of the relaxin family. The effects of the relaxins and insulin-like peptides are subtle and pleiotropic (Shabanpoor *et al.*, 2009). The insulin-like peptide sub-family is not necessarily involved in promoting tissue expansion, although these peptides are frequently involved with reproductive or developmental functions (Ivell *et al.*, 2005, Shabanpoor *et al.*, 2009). Current understanding of insulin peptide evolution points to the vertebrate pancreatic insulin, IGF-1, and IGF-2, which separate the metabolic and growth roles of insulin signaling both by peptide and by tissue of origin, as branching from a common ancestral insulin (Chan and Steiner, 2000, De Meyts, 2004). Thus, the set of peptides is referred to as the insulin/IGF family. Invertebrates generally do not have IGF-like molecules, but invertebrate insulin signaling has similar effects to vertebrate insulin/IGF signaling.

Insulin homologs in invertebrate organisms have been relatively difficult to identify, in part because of high amino-acid sequence divergence (Chan and Steiner, 2000, Wu and Brown, 2006) and in part because none act with the precise metabolic role associated with pancreatic insulin (Chan and Steiner, 2000, De Meyts, 2004). Invertebrate insulin-like peptides still signal nutritive status to tissues, but the role is not isolated from other aspects of insulin family signaling as it is in vertebrates (Chan and Steiner, 2000, De Meyts, 2004, Kim and Rulifson, 2004). Invertebrate insulins vary not only in primary sequence, but also in quantity across invertebrates (Chan and Steiner, 2000, Wu and Brown, 2006). *Caenorhabditis elegans* and *Bombyx mori* have, respectively, 40 and 39 known insulin-like peptides (and likely still counting), most of which have only been discovered in the last two decades (Michaelson *et al.*, 2010, Wu and Brown, 2006). *Drosophila melanogaster* has 7 insulin-like peptides (Toivonen and Partridge, 2008, Wu and Brown, 2006). Although the precise number of insulin family members is not conserved across species, overall family function is, indicating that understanding of insulin family signaling gained through model systems such as the planarian will likely translate to humans.

Many invertebrates, including *C. elegans* and *D. melanogaster*, have only one known insulin receptor, although more receptors may be present but undiscovered (Wu and Brown, 2006). Studies of disrupted insulin signaling focus on gene knockdowns involving the single insulin receptor (*daf-2* in *C. elegans*) or the insulin receptor binding substrate (*chico* in *D. melanogaster*). Interestingly, the

genes downstream of the insulin receptor and receptor binding substrate are those involved in the AKT pathway, which is also regulated by other growth factors such as epidermal growth factor (EGF). AKT activity is associated with cell growth and survival. (Maki, 2010, Samani *et al.*, 2007) Thus, the only conserved insulin pathway-specific genes are insulin-like peptides, the insulin receptor, and the insulin receptor binding substrate (in vertebrates, circulating IGF levels are also controlled by insulin binding substrates). However, in *C. elegans*, knockdown of individual insulin-like peptides can reveal phenotypes opposite to knockdown of the insulin receptor indicating a signaling complexity and nuance that is under-appreciated when only the *daf-2* or the downstream transcription factor *daf-16* phenotypes are considered (Michaelson *et al.*, 2010). Generally, vertebrates appear to have an insulin receptor for each superfamily member, although there can be cross-talk to varying degrees amongst insulin superfamily members and receptors (Denley *et al.*, 2004, Jensen and De Meyts, 2009).

In sum, the specific players in insulin signaling across species are not conserved, yet the family functions similarly across vertebrate and invertebrate species. Invertebrate models are thus useful in understanding the overall insulin superfamily function, although analogies cannot be drawn across species between single insulin-like peptides. One strength of many invertebrate models is the presence of only one insulin receptor, which means the insulin-family function is easy to assess even if all members of that family in an organism are not necessarily known.

2.3 The primary effects of insulin/IGF signaling

Insulin/IGF family signaling relays an organism's nutritional status to its tissues, promoting utilization of energy resources and growth (Chan and Steiner, 2000, Shabanpoor *et al.*, 2009). There are many physiological processes that insulin/IGF signaling influences. For instance, decreased insulin/IGF signaling in *D. melanogaster chico* mutants as well as Ames and other dwarf mice strains is associated with smaller organism size (Bartke and Brown-Borg, 2004, Böhni *et al.*, 1999, Ikeya *et al.*, 2002). Increased lifespan is observed after decreased insulin/IGF signaling in vertebrates (Bartke and Brown-Borg, 2004, Bonafè *et al.*, 2003, Tatar *et al.*, 2003) or ablated insulin/IGF signaling in invertebrates (Broughton *et al.*, 2005, Kimura *et al.*, 1997, Tissenbaum and Ruvkun, 1998). These phenotypes are similar to those resulting from nutritional deprivation. Scientifically and medically, it is important to understand if and how the many consequences of insulin signaling can be distinguished from metabolic state, and furthermore, if those functions can be individually regulated.

Although insulin frequently regulates an organism's metabolic state, it can act separately from nutritional cues. The most extreme example of how insulin/IGF can be uncoupled from nutritional state is the upregulation of insulin signaling observed in some cancers that allows tumors to grow despite inhibitory environmental factors; microarray studies indicate that misregulated insulin/IGF signaling may be one of the factors that is key in a cancer's transition to a metastatic state (Kalaany and Sabatini, 2009, Maki, 2010, Samani *et al.*, 2007). Additionally, nutritional status can

be separate and distinct from insulin signaling. Dwarf mice with decreased insulin signaling and extended life spans can, through caloric restriction, live even longer (Bartke and Brown-Borg, 2004, Bartke *et al.*, 2001). Understanding the nuances of metabolic state in contrast to insulin pathway signaling has implications pertaining to tumor growth control and tissue health in persons suffering from Diabetes Mellitus Type 2.

In vertebrates, pancreatic insulin is perceived to have a direct and immediate role in relaying nutritional status. Insulin peptide is released into circulation/bloodstream in response to a proximal cue of food (Shabanpoor *et al.*, 2009). Its activity, however, depends on insulin receptor concentrations and modifications that can differ across various tissues. For example, the course of Diabetes Mellitus Type 2 begins with differing sensitivity across tissues to circulating insulin peptide. In contrast, the vertebrate IGFs are a downstream element in the pituitary somatotropic (growth) axis; their secretion is stimulated by growth hormone and they negatively feed back onto growth hormone (GH) release (Shabanpoor *et al.*, 2009). The IGFs do not play as direct and immediate a role in nutritive status communication as pancreatic insulin peptide does. However, a state of caloric restriction results in decreased circulating levels of IGF-1 (Zhao *et al.*, 2010), and impaired organism growth. The actions of the IGFs are mediated both through insulin binding proteins that bind the majority of circulating IGFs and also, like insulin peptide, through differing receptor concentration and moderation in specific tissues (Shabanpoor *et al.*, 2009).

The difference in the actions of insulin peptide vs. the IGFs is perhaps best understood by considering their associated disease states. Pancreatic insulin signaling ablation results in Diabetes Mellitus Type 1 and misregulated blood sugar, which can be acutely fatal if not controlled on a day-to-day basis. A lack of pancreatic insulin effectively puts the body into a state of starvation. On the other hand, decreased IGF signaling leads to dwarfism, but is not fatal for an organism over the same timescale as pancreatic insulin signaling disruption. A phenotype of stunted growth during development is consistent with a lack of adequate nutrition, however, individuals with decreased levels of IGF are still able to become obese.

In vertebrates it appears that the metabolic and growth roles of insulin family signaling have been, to an extent, divided into pancreatic insulin and the IGFs (Barbieri *et al.*, 2003, Chan and Steiner, 2000). However, the respective receptors for insulin and IGFs are not exclusive; the homo-dimeric insulin receptor binds IGF-1 and vice versa (Denley *et al.*, 2004, Jensen and De Meyts, 2009). In fact, it is also possible to form an active hetero-dimeric insulin receptor containing one insulin receptor subunit and one IGF-1 receptor subunit, and furthermore a hetero-dimeric receptor can also be formed with an EGF receptor subunit (Chan and Steiner, 2000, Denley *et al.*, 2004, Jensen and De Meyts, 2009). The non-specificity between these receptors is only beginning to receive attention on a clinical level (Jensen and De Meyts, 2009), perhaps because the overt phenotypes of pancreatic insulin vs. the IGFs are distinct from each other to the point of masking other functions.

Pancreatic insulin and the two vertebrate IGFs classically activate and signal through the AKT pathway, as does EGF. In sum, the non-exclusivity of the insulin/IGF receptors, as well as the downstream AKT pathway indicate that there are poorly understood but distinct downstream events resulting from ligand-receptor binding (Jensen and De Meyts, 2009, Samani *et al.*, 2007). Indeed, current studies are beginning to focus on transcriptional changes post insulin/IGF signaling alteration as a method of examining the subtle and complex ways in which this signaling pathway can be regulating downstream components (Murphy, 2006, Murphy *et al.*, 2003).

2.4 Invertebrates and insulin/IGF signaling

Broadly, amongst invertebrates, there is only one known insulin receptor, but many insulin-like peptides acting through the one receptor (De Meyts, 2004). The confluence of signaling makes it difficult to assign specific roles or functions to particular insulin-like peptides. On the other hand, invertebrates are without pancreata, thus reducing the tissue-of-origin complexity observed in vertebrates (note that insulin-like peptides can be associated with gut tissue in invertebrates, but there is no endocrine-specific specialized organ similar to the pancreas). Invertebrate insulin-like peptides typically have a neural origin (though also are expressed in other tissues) and are attributed neuroendocrine peptide hormone status (Barbieri *et al.*, 2003, Chan and Steiner, 2000, Collins *et al.*, 2010). Thus, for

invertebrates, the metabolic and growth roles of insulin are currently not easily separable on a molecular level (Kim and Rulifson, 2004).

Although the signaling pathway for the insulin family in invertebrates appears to be convergent, distinct differences in family member properties are emerging. Recently, in *C. elegans*, two of 40 known insulin-like peptides were found to promote larval germline proliferation (Michaelson *et al.*, 2010), which alludes to a signaling complexity of this family of peptides in invertebrates that scientists are only now beginning to appreciate. Consistently, overt phenotypes resulting from insulin can be distinguished from each other experimentally, despite the signaling convergence on the *daf-2* insulin receptor. Insulin signaling in *C. elegans* moderates both reproductive status and lifespan, but does so at differing times across the worm's development such that the effects of altered insulin signaling can be specific to reproduction or lifespan (Dillin 2002).

Across vertebrates and invertebrates, the growth factor signaling aspect of insulin becomes less obvious post-growth. Its effects are clear when an organism is in a state of growth, as the growth can be inhibited. However, with many adult stem cell populations post growth, there may not be an obvious phenotype that results from a slight decrease in activity caused by altered insulin signaling. There is growing evidence that decreased insulin signaling does alter stem cell function in adults. In Drosophila, stem cell proliferation slows after either nutritional deprivation or insulin signaling inhibition; these effects are reversible with rescue by feeding or increasing insulin signaling (McLeod *et al.*, 2010). However, in both invertebrates

and vertebrates, it is important to further study insulin signaling in a post-development scenario.

2.5 Vertebrate reproduction

One difficulty in trying to understand the effects of insulin peptide family signaling in any organism is that the specific peptide functions are not conserved across organisms. The family itself, as a whole, has pleiotropic effects on many different physiological processes. The systems that the insulin peptide family affects can in turn affect and feed back upon each other. For instance, reproductive maturation and fertility clearly depend on an organism's nutritional and metabolic state (Partridge *et al.*, 2005). However, in mammals, there is a subfamily of insulin-like peptides, including the relaxins, that have very specific roles in reproduction and appear to act independently of metabolic state.

In mammals, there is a greater degree of conservation of both number and function of insulin peptide family members (Chan and Steiner, 2000, De Meyts, 2004). However, because of multiple insulin receptors as well as other insulin-like peptide family members, the effects of insulin family signaling on reproduction and fertility are multiple and complex (Shabanpoor *et al.*, 2009). Relaxin-2, an insulin-like peptide, signals through a G-protein coupled receptor and is associated with pregnancy, in particular with cervical softening prior to birth. Relaxin-2 is also found in the male reproductive tract as well as non-reproductive tissues, and its role in those tissues is not well established (Shabanpoor *et al.*, 2009). Insl3, another

insulin-like peptide, was first identified in a screen for testis-specific genes. Insl3 is expressed within the testosterone-regulating Leydig cells of mammalian testes. Insl3 is also found in female reproductive organs, including ovarian follicle and corpus luteum. Like the relaxins, Insl3 signals through a G-protein coupled receptor (Ivell *et al.*, 2005, Shabanpoor *et al.*, 2009). Mice with a homozygous mutation in Insl3 are sterile and cryptorchid (i.e. testes fail to descend; (Nef and Parada, 1999)). Mutations in either Insl3 or its receptor, LGR8/GREAT result in a sterile and cryptorchid (failure of testes to descend) phenotype (Ivell *et al.*, 2005, Shabanpoor *et al.*, 2009). Currently, there are no known complete G-protein coupled receptors for invertebrate insulins (Wu and Brown, 2006), and therefore these signaling mechanisms and their direct effects could be isolated to mammals.

The more canonical members of the insulin peptide superfamily also play roles in vertebrate reproduction. Insulin-like growth factor 1 (IGF-1) is thought to regulate spermatogenesis (Colombo and Naz, 1999, Spiteri-Grech and Nieschlag, 1993). Its primary receptor, insulin-like growth factor I receptor (IGF-IR), is expressed in spermatogonia, primary spermatocytes, and elongated spermatids (Yagci and Zik, 2006). IGF-IR is also expressed in Leydig cells, which regulate secretion of testosterone; IGF-IR mediates an anti-apoptotic effect on the Leydig cells (Colón *et al.*, 2007). Because the primary source of IGF-1 in mammals is the liver, the testicular source of IGF-1 is one example of IGF-1 signaling acting in both endocrine (hormone released into broad circulation) and paracrine (hormone released in a localized, tissue-specific) manner (Stratikopoulos *et al.*, 2008).

The role of insulin/IGF signaling in vertebrate gametogenesis is not limited to males. Insulin/IGF signaling seems to play a similar role in promoting the proliferation, survival, and maturation of ovarian follicles (Quirk *et al.*, 2004). In infertile women undergoing IVF, higher follicle fluid IGF-II levels are correlated with oocyte maturation and better fertilization rate (Wang *et al.*, 2006b). Hyperinsulinemia, (excessive insulin levels) is associated with polycystic ovary syndrome. In a normal ovary, typically one dominant oocyte develops and matures at a time; polycystic ovary syndrome is characterized by several eggs beginning the maturation process, but the eggs are never released during ovulation and form cysts (Chakrabarty *et al.*, 2006). Female mice lacking insulin receptor substrate-2 (IRS-2), which is phosphorylated by the insulin receptor, have decreased fertility, smaller ovaries, and fewer follicles (Burks *et al.*, 2000).

In sum, although some members of the mammalian insulin peptide family have clear (albeit subtle) roles in reproduction and fertility, the insulin/IGF members, which appear to be more functionally conserved with invertebrate species, also have explicit metabolic roles. Thus, given the number of adult disease states with misregulated insulin/IGF signaling, it is important to know if and to what extent the metabolic and reproductive roles of insulin/IGF can be separated.

2.6 Invertebrate reproduction

Studies in invertebrate model organisms are uncovering important functions of the insulin/IGF pathway related to reproductive development and fertility. In the late 1990s, insulin signaling was first identified as a modulator of lifespan in *C. elegans* (Kimura *et al.*, 1997). Worms lacking insulin signaling via mutations in *daf-2* (insulin receptor) or *age-1* (catalytic subunit of PI3 kinase, downstream of *daf-2*) were not only found to have an extended post-dauer lifespan but also to have decreased fertility (Hsin and Kenyon, 1999, Kimura *et al.*, 1997, Tissenbaum and Ruvkun, 1998). The decreased fertility is due at least in part to decreased germline proliferation during the larval expansion phase (Michaelson *et al.*, 2010). Altered germline proliferation is significant not only because it is associated with impaired adult fertility, but also because it is one example of how decreased insulin/IGF signaling affects a proliferative cell population in a near-adult state. It also is an excellent example of the difficulty in distinguishing if a phenotype occurs primarily because the insulin/IGF pathway is acting as a communicator of nutritional status or as a growth factor, or both simultaneously.

An additional difficulty in studying the effects of insulin/IGF signaling on reproduction is the need to distinguish reproductive effects from other effects of the signaling pathway. For instance, reduced insulin/IGF signaling results in both decreased germline proliferation and extended lifespan, but ablation of germline precursors or reduction of germline proliferation independent of any insulin/IGF pathway manipulations also results in increased lifespan (Arantes-Oliveira *et al.*,

2002, Hsin and Kenyon, 1999). In a developmental model for which effects are measured as an adult, it is impossible to distinguish between these possible mechanisms without further experimental manipulation. One possible approach to avoiding these complications is to temporally isolate insulin/IGF pathway manipulations. For example, to affect fertility, insulin/IGF signaling must be disrupted during *C. elegans* development. But lifespan effects can be observed by a post-development disruption of insulin/IGF signaling (Dillin *et al.*, 2002). Thus, exploring each of these manipulations separately furthers the understanding of both fertility and lifespan effects.

Studies in *D. melanogaster* also support the idea that reduced insulin/IGF signaling decreases germline proliferation. Female flies with insulin receptor binding substrate (*chico*) mutations are small and sterile (Böhni *et al.*, 1999). Sterility occurs because of a lack of follicle cell proliferation and the inability to progress through vitellogenesis (Drummond-Barbosa and Spradling, 2001). Nutritional deprivation also impacts the female reproductive system, but its effects are not as severe as observed in the *chico* mutants. Nutritional status governs ovary size and oogenesis, due to altered stem cell (both germ and somatic) proliferation rate rather than a change in cell size or number (Drummond-Barbosa and Spradling, 2001). Thus, insulin/IGF signaling mediates nutritive signals to the ovaries (LaFever and Drummond-Barbosa, 2005) and promotes germline stem cell maintenance and proliferation (Drummond-Barbosa and Spradling, 2001, Hsu and Drummond-Barbosa, 2009, LaFever and Drummond-Barbosa, 2005). Similarly, insulin signaling

maintains the male *D. melanogaster* germline (McLeod *et al.*, 2010, Ueishi *et al.*, 2009).

Broadly, amongst both vertebrates and invertebrates, the insulin peptide family has several distinct roles in regulation of reproductive development. The first is in actual development of reproductive structures, such that lack of insulin signaling results in small gonads and fewer follicles. The second involves mediation of nutritional control of fertility, such that an organism can reduce maintenance costs associated with reproductive ability at times of nutritional inadequacy, and restore that ability as energy resources become more abundant. Insulin/IGF signaling could be directly involved in moderating that relationship: it could control those pathways separately, or it could control one pathway thereby affecting the other (Drummond-Barbosa and Spradling, 2001, Michaelson *et al.*, 2010, Partridge *et al.*, 2005).

For many years, fertility and lifespan have been assumed to have an inverted relationship, i.e. maintenance of reproductive ability is a stressor that decreases lifespan (Partridge *et al.*, 2005). Interestingly, in *C. elegans*, the lifespan effect of decreased insulin/IGF signaling is independent of the fertility effect, i.e. reduced fertility in this case is not the cause of the extended lifespan (Tissenbaum and Ruvkun, 1998). In fact, insulin signaling affects reproduction and lifespan during different timeframes of development (Dillin *et al.*, 2002).

2.7 Metabolism, reproduction, lifespan

Metabolism and lifespan are often theorized to be at odds with each other. Metabolism results in both survival and growth, but simultaneously causes cellular damage (i.e. via reactive oxygen species). Over time, cellular damage accumulates resulting in an "aging" phenotype in which the organism progresses through its lifespan. There is a concurrent increase in the incidence of age-associated diseases. Thus, theoretically, decreasing one's metabolic rate per unit time prolongs life (Martin *et al.*, 2006). The idea of prolonging life appeals to many people as is evidenced by the general public's consumption of antioxidants and the interest in diets that tout the health benefits and increased lifespan resulting from reduced caloric intake or "minimal nourishment" (as opposed to malnourishment). Likewise, fascination with longer life spans is part of what drives the great interest in aging research at present.

Insulin/IGF signaling serves as an indicator of an animal's nutritional status and regulates metabolism. Caloric restriction is associated with decreased insulin/IGF signaling (Martin *et al.*, 2006, Tatar *et al.*, 2003) and increased sensitivity to changes in circulating insulin levels. The effects of caloric restriction on lifespan are likely due to both direct reduction of damaging metabolic by-products and indirect effects via regulation of metabolism-associated signaling pathways (Finch and Sapolsky, 1999), such as insulin/IGF activity. For instance, long-lived animals such as Ames mice (characterized by lower circulating levels of insulin and IGF-1) that are subjected to caloric restriction have a greater degree of lifespan

extension than their normally fed Ames mice compatriots (Bartke and Brown-Borg, 2004, Bartke *et al.*, 2001, Tatar *et al.*, 2003). Therefore, lifespan extension effects are mediated by mechanisms including, but not limited to, the role of insulin/IGF signaling in relaying nutritional status.

Insulin was first identified as a regulator and communicator of metabolic status (Rosenfeld, 2002); however, in the late 1990s, insulin/IGF signaling was identified both as a modulator of lifespan and reproduction/fertility in *C. elegans* (Kimura *et al.*, 1997, Tissenbaum and Ruvkun, 1998). Just as metabolism and lifespan are generally conceived of as inversely related, reproductive ability/fertility and lifespan are also thought to draw from the same "energy resource" and thus detract from each other. Insulin/IGF signaling is therefore central in a signaling network that controls many distinct physiological axes (metabolic status, lifespan, reproduction) but these are also interrelated through complex feedback mechanisms.

Given that insulin/IGF signaling is also involved in many adult disease states, and that the pathway is receiving attention as a therapeutic target, understanding the extent to which one particular effect of insulin/IGF signaling can realistically be isolated from the others is important. There are numerous examples suggesting that metabolism can, to an extent, be uncoupled from fertility and lifespan (and similarly that fertility and lifespan are not necessarily inversely related). One example of the separation of metabolism from lifespan is that mice with a fat-specific insulin receptor knockdown are longer lived than wild type. Though the

knockdown mice have decreased adiposity, they actually have increased rates of metabolism based upon food intake and body mass (Blüher *et al.*, 2003). A general example indicating that metabolism is not necessarily linked to fertility or lifespan is that different organisms have different aging and lifespan characteristics. In other words, not all species have a similar progression through their lifespan: some are fertile for only set periods of time, while others are fertile for the duration of their lives; some animals live with high energy and metabolism but suffer no ill effects until the very end of their lifespan, while others undergo an age-related decline (Finch, 1990).

Caleb Finch created three categories for describing the progression of an organism's lifespan (Guerin, 2004): rapid, gradual, and negligible senescence. Style of senescence is not necessarily tied to length of life. Rapid senescence is often characterized by a catastrophic loss of nutrition, energy, or organ function coincident with seasonal change or reproductive maturity. Gradually senescent organisms include humans and many mammals; these animals have an increased rate of mortality as they age due to compounding physiological damage, dysfunction, or disorder. Negligibly senescent organisms are those that show no age-related increased mortality rates; in other words, the physiological systems of these animals break down at a very slow rate over time, if at all, and they frequently die of external causes rather than internal disease/failure (Finch, 1990, Finch and Ruvkun, 2001). Some organisms that are thought to be negligibly senescent are rockfish, tortoises, lobsters, some mollusks, and some parasitic nematodes (Finch

and Ruvkun, 2001). Additionally, negligibly senescent organisms do not appear to have an age-associated decrease in fertility, showing that reproduction and lifespan can also be uncoupled from each other. The various and distinct types of lifespans that organisms display show that the relationship between metabolism and lifespan is not simply inversely proportional, but rather is complex and poorly understood.

Chapter 3

Insulin in *S. mediterranea*[1]

3.1 Introduction

Animal growth requires the coordination of nutrient resource availability with stem cell dynamics. The insulin/IGF family is a conserved regulator of metabolism and moderates overall animal growth and development (Chan and Steiner, 2000, De Meyts, 2004). Insulin-like peptide signaling continues to control germline stem cell dynamics in adulthood (LaFever and Drummond-Barbosa, 2005, McLeod *et al.*, 2010, Michaelson *et al.*, 2010). Given that insulin/IGF signaling plays a significant role in cancer (Maki, 2010, Samani *et al.*, 2007), the regulatory effects of this pathway on adult stem cell dynamics merit further exploration.

Though the roles of insulin/IGF signaling during development appear to be conserved across species, the actual number of insulin-like peptides and the specific functions of those peptides are highly divergent, particularly amongst invertebrates (Chan and Steiner, 2000). Invertebrate insulin-like peptides are commonly expressed within the nervous system and regulate growth and reproduction (Wu and Brown, 2006). In mammals, insulin growth factors (IGFs) play a role in the somatotropic axis of the pituitary gland and are expressed within the brain as well as other tissues (Daftary and Gore, 2005). Disrupted neuroendocrine

[1] This chapter presents data that has been accepted for publication in the *International Journal of Developmental Biology, Planarian Edition*: Miller, CM and Newmark, PA. An insulin-like peptide regulates size and adult stem cells in planarians. (In Press).

insulin/IGF signaling during development results in decreased adult size in *Drosophila melanogaster* (Chen *et al.*, 1996, Ikeya *et al.*, 2002) and several strains of dwarf mice (Tatar *et al.*, 2003). Although the role of insulin/IGF signaling during embryonic and post-embryonic growth is clear, once adult size is attained, the continuing cellular effects of the growth-regulating aspect of the pathway are poorly understood.

In both *Caenorhabditis elegans* and *D. melanogaster*, insulin-like signaling acts upon adult germline stem cells to regulate proliferation. In *C. elegans*, two of 40 known insulin-like peptides support the L3/L4 stage germline expansion by signaling through the single insulin receptor (DAF-2) (Michaelson *et al.*, 2010). In *D. melanogaster*, insulin-like signaling mediates adult gametogenesis (LaFever and Drummond-Barbosa, 2005, Ueishi *et al.*, 2009) as well as the ovarian proliferative reponse to nutrition (Drummond-Barbosa and Spradling, 2001, Hsu and Drummond-Barbosa, 2009, LaFever and Drummond-Barbosa, 2005). Furthermore, increased insulin-like signaling can partially rescue decreased germline proliferation due to poor nutrition (McLeod *et al.*, 2010). Together, these findings indicate that insulin signaling may play a conserved role in the regulation of adult fertility and germline stem cell populations, in part through communication of nutritional state. However, further studies in other adult stem cell populations, including those in a non-reproductive context, are warranted.

The flatworm *Schmidtea mediterranea* is renowned for its regenerative capacity, which is dependent on a population of totipotent adult stem cells called

neoblasts (Baguñá *et al.*, 1989, Brøndsted, 1969, Newmark and Sánchez Alvarado, 2002, Wagner *et al.*, 2011). This model organism is also intriguing because its adult size is effectively dissociated from age; size depends on nutritional status, such that fed animals grow, while starved animals shrink yet maintain their proportionality (degrow) (Baguñá *et al.*, 1989, Newmark and Sánchez Alvarado, 2002, Oviedo *et al.*, 2003, Wenemoser and Reddien, 2010). There are two strains of *S. mediterranea*: a sexually reproducing, hermaphroditic strain and an asexual, fissioning strain. These strains can be used to assess gene function during gametogenesis as well as in a context devoid of reproductive system development. The experimental ease with which adult stem cell and germline population dynamics can be observed (Eisenhoffer *et al.*, 2008, Guo *et al.*, 2006, Reddien *et al.*, 2005b, Wang *et al.*, 2010, Wang *et al.*, 2007) make *S. mediterranea* an asset to studies of developmental and regenerative biology.

In the sexual strain of *S. mediterranea*, sexual development and gonad maintenance also appear to depend on metabolic state rather than age, as animals post-embryonically develop reproductive organs only once they achieve an approximate size (Newmark and Sánchez Alvarado, 2002). Should the animal degrow past a certain size due to lack of nutritional intake, then its gonads regress. Gonads redevelop when adequate nutrition is once again available (Morgan, 1902, Wang *et al.*, 2007). Though several genes required for proper neoblast or germline function have been identified (Collins *et al.*, 2010, Guo *et al.*, 2006, Reddien *et al.*, 2005a, Reddien *et al.*, 2005b, Rouhana *et al.*, 2010, Salvetti *et al.*, 2005, Wang *et*

al., 2010, Wang *et al.*, 2007), signals that stimulate growth or degrowth of *S. mediterranea*, and how those gross changes are coordinated with stem cell activity remain unknown (Pellettieri and Sánchez Alvarado, 2007).

In this study, we examine *ilp-1*, a neuroendocrine insulin-like peptide (Collins *et al.*, 2010) and *inr-1*, a putative insulin-like peptide receptor tyrosine kinase in *S. mediterranea*. We outline a regulatory role for the insulin/IGF signaling pathway in controlling adult size homeostasis and describe correlative changes in proliferating cell populations. Our data suggest that insulin/IGF signaling differentially regulates adult stem cell populations based on nutrient availability.

3.2 Results

An insulin-like peptide is expressed in the nervous system and testis lobes

To determine how an organism that can grow and degrow as an adult (Pellettieri and Sánchez Alvarado, 2007) regulates its size, we explored insulin/IGF signaling as a candidate pathway in *S. mediterranea*. In a genome-wide analysis of *S. mediterranea* neuropeptides, Collins *et al.* (2010) reported a putative insulin-like peptide, *smed-ilp-1* (hereafter referred to as *ilp-1*). We examined the sequence of *ilp-1* and verified the presence of six conserved cysteine residues, which are known to contribute to the tertiary structure of the peptide (Fig. 1 A, B; (Chan and Steiner, 2000, Smit *et al.*, 1998). The relatively low degree of conservation shows that *ilp-1* is diverged from its invertebrate counterparts, which is consistent with the current perspective that insulin-like peptide sequences are highly diverged across

invertebrate species (Chan and Steiner, 2000, De Meyts, 2004). Additionally, we identified a single predicted protein with similarity to an insulin/IGF receptor tyrosine kinase, *smed-inr-1* (hereafter referred to as *inr-1*; Fig. 1 C, D).

According to northern blot analyses, the full-length mRNA sequence encoded by *ilp-1* is approximately 500 nucleotides (Fig. 1 E), while *inr-1* mRNA is approximately 4000 nucleotides (Fig. 1 F). To obtain full-length sequence for both *ilp-1* and *inr-1* mRNAs, we performed 5' and 3' RACE. For *ilp-1*, we obtained a full-length cDNA sequence of 627 nucleotides, which includes a 143-codon open reading frame. For *inr-1*, we obtained a full-length cDNA sequence of 4648 nucleotides that includes a 1391-codon open reading frame.

Northern blot analyses also revealed that *ilp-1* was expressed at higher levels in the sexually reproducing strain (Fig. 1 E). We validated this observation by performing reverse-transcriptase quantitative PCR (qPCR) and found a 3.2-fold difference in *ilp-1* expression between asexual and sexual animals ($p<0.05$). No significant difference was observed in expression for *inr-1* between the strains by either northern blot (Fig. 1 F) or qPCR.

The increased expression of *ilp-1* in the sexual strain suggested that insulin signaling might play a role in sexual reproduction for *S. mediterranea*. To examine which tissues are associated with insulin expression, we performed in situ hybridization on both the sexual and asexual strains of the animal. *ilp-1* expression was detected in cells within the central nervous system (cephalic ganglia and nerve cords) in both sexual and asexual animals (Fig. 2 A-C). In the sexual strain, *ilp-1*

expression was also detected in the testis lobes (Fig. 2 A); the abundance of signal in more luminal regions (Fig. 2 D) suggests that *ilp-1* is expressed in spermatocytes and spermatids (Wang *et al.,* 2010). The expression of *ilp-1* in developing and mature testes implies that, in *S. mediterranea*, insulin may play a paracrine or autocrine role distinct from neural regulation in supporting testis growth and spermatogenesis. i*nr-1* appears to be expressed at low levels throughout the animal, making it difficult to determine definitively by in situ hybridization which cell types express this gene.

Neurally expressed insulin maintains animal size

Nutritional intake and insulin-like peptides moderate growth in developing organisms(Ikeya *et al.*, 2002, Tatar *et al.*, 2003). However, little is known about how these factors act on adult stem cell populations in vivo. The ability of adult *S. mediterranea* to grow and degrow allows a gross assessment of adult stem cell function after post-development disruption of insulin/IGF signaling.

To test if insulin/IGF signaling in *S. mediterranea* might be responsible for regulating adult size homeostasis, we created dsRNA feeding vectors for *ilp-1* and *inr-1*. Then, according to established RNA interference (RNAi) protocols (Newmark *et al.*, 2003), we fed bacteria expressing dsRNA to large, sexually reproducing *S. mediterranea* on a weekly basis. A schedule of weekly feeding promotes growth or maintenance of animal size. Over the course of these feedings, *ilp-1(RNAi)* and *inr-1(RNAi)* animals degrew to 90% and 70% respectively of the control cohort mean

size, despite eating normally (Fig. 3 A, B; $p<0.01$). Animals with both genes knocked down simultaneously had a phenotype indistinguishable from the *inr-1(RNAi)* cohort, which is consistent with *ilp-1* and *inr-1* acting through a similar mechanism to control animal size. To further assess the possibility that *ilp-1* and *inr-1* act through a similar mechanism, we performed qPCR on animals after RNAi. After four weeks, *inr-1(RNAi)* animals had an approximately 10-fold increase in *ilp-1* expression ($p<0.01$), which is consistent with a feedback mechanism between these two members of the insulin pathway. No change in *inr-1* expression was detected in *ilp-1(RNAi)* animals.

In *S. mediterranea*, animals starved for longer than a few weeks noticeably shrink (Newmark and Sánchez Alvarado, 2002). To examine how disruption of insulin signaling compared to lack of nutritional intake, we compared insulin-disrupted animals to starved animals. After eight weeks of RNAi, the phenotypes resulting from disrupted insulin signaling are, on a gross level, similar to those of nutritional deprivation (Fig. 3 A, B). However, in the sexual strain of *S. mediterranea*, there are two distinct populations of cells producing insulin-like peptides: neuroendocrine cells and gonadal cells. To examine whether the neuroendocrine *ilp-1* signaling is necessary for maintaining adult tissue homeostasis and size, we repeated the degrowth experiments with a cohort of asexual animals. Over the course of 4 weeks, we observed that animals with disrupted insulin/IGF signaling were 5 to 10% smaller than control animals (*inr-1(RNAi)* = $p<0.05$; data not shown), consistent with the response of sexual animals

at this time point (Fig. 3 A). Therefore, we demonstrate that regulation of adult size homeostasis in *S. mediterranea* is dependent not only on nutritional status, but also on neuroendocrine *ilp-1* signaling. We also show that, on a gross level, the phenotypes of disrupted insulin signaling and nutritional deprivation are similar.

Disruption of insulin signaling affects stem cell proliferation

When *S. mediterranea* degrow after lack of nutritional intake, there is a correlative increase in apoptotic activity (Pellettieri *et al.*, 2010) but no significant change in individual cell size (Pellettieri and Sánchez Alvarado, 2007, Romero and Baguñá, 1991). However, changes in overall animal size could reflect not only changes in overall cell survival, but also altered stem cell dynamics. In asexual *S. mediterranea*, neoblasts are the only mitotically active somatic cells, and mitotic neoblasts can be specifically labeled with anti-phosphohistone H3. Therefore, to explore whether or not adult stem cell activity changes are correlative with changes in adult size, we examined mitotic activity in asexual animals after nutritional deprivation or disruption of insulin signaling.

Animals with no nutritional intake over four weeks showed approximately 30% fewer mitotic cells in comparison to controls (Fig. 3 C; $p<0.01$). This finding correlates an overall decrease of proliferative neoblasts with a decrease in animal size. The decrease in total proliferative neoblasts could arise from a decrease in total neoblasts with a constant rate of proliferation and/or a decrease in rate of proliferation across a constant population of neoblasts. Earlier work supports a

scenario in which the percentage of neoblasts within the total cell population remains roughly constant during growth and degrowth due to lack of nutrition (Baguñá and Romero, 1981). To distinguish between these two possible scenarios, we performed qPCR on two *piwi* homologues preferentially expressed in neoblasts: *smedwi-1* and *smedwi-2* (Guo *et al.*, 2006, Reddien *et al.*, 2005b et a). We did not detect a significant difference in *smedwi-1* or *smedwi-2* relative expression levels (normalized to ubiquitously-expressed *β-tubulin*) between fed or starved animals (data not shown). These data are consistent with the maintenance of a roughly proportional population of neoblasts as observed by Baguñá and Romero (1981). Though subtle changes in the total neoblast population may still be present, they are not equivalent to the approximately 30% decrease of mitotic cells (Fig. 3 C). Therefore, the observed decrease in mitotic cells likely results in part from a decrease in cell proliferation rate.

With fed and starved animals as baselines, we examined the proliferation rate in animals with disrupted insulin signaling. We found that *ilp-1(RNAi)* or *inr-1(RNAi)* animals, like starved animals, had approximately 30% and 50% respectively decreased proportions of mitotic cells compared to fed animals (Fig. 3 C; $p<0.01$). *ilp-1(RNAi)* animals were similar in size to starved animals, and the proportion of proliferative cells was indistinguishable across these two conditions. *inr-1(RNAi)* animals, which after eight weeks were smaller compared to starved animals, also had a smaller proportion of mitotically active cells (Fig. 3 C; $p<0.05$).

As with the unfed cohort, there was no difference in relative expression of either *smedwi-1* or *smedwi-2* in the *ilp-1(RNAi)* or *inr-1(RNAi)* conditions via qPCR. Thus, it appears that animal size, in conditions of nutritional deprivation or disrupted insulin signaling, is regulated at least in part by decreasing the rate of proliferation across a population of neoblasts. Furthermore, the mechanism by which starved animals and insulin-disrupted animals degrow appears to be similar in terms of changes in adult stem cell proliferation.

Disruption of insulin signaling impairs testis growth and spermatogenesis

As sexually mature *S. mediterranea* degrow past a certain point, their gonads regress (Newmark and Sánchez Alvarado, 2002). Thus, it follows that size and nutritional status are factors in gonad development and maintenance. Nutritional deprivation and disruption of insulin signaling both result in decreased size and correlatively decreased neoblast proliferation (Fig. 3 A-C). If insulin signaling affects the neoblast population in *S. mediterranea*, it may also moderate germline stem cell proliferation. Consistent with this hypothesis, *ilp-1* is expressed within testis lobes (Fig. 2 A, D, E). To explore whether insulin signaling influences germline dynamics, we examined testis lobe size and morphology after nutritional deprivation or disruption of insulin signaling.

In sexually mature *S. mediterranea* that were starved for four weeks, we found that, in the size range of animals observed, there was actually no proportional decrease in lobe size compared to control animals (Fig. 4 A, B), although the

starved population was smaller than control animals (Fig. 4 C; $p<0.05$). Correlating with the overall lobe size decrease in the smaller starved population, there were fewer spermatids, as visualized by the nuclear stain, DAPI (spermatids were observed in: 4/12 starved animals; 11/12 control animals). We therefore performed in situ hybridization to detect *germinal histone H4* RNA, which is expressed in neoblasts as well as in spermatogonia and spermatocytes (Wang *et al.*, 2007). We co-labeled with anti-Trailer-hitch antibodies that recognize RAP55 protein expressed in spermatocytes and spermatids (Fig. 4 C; see Wang *et al.*, 2010). We detected RAP55 protein in the luminal regions of testis lobes when spermatids were visible in both fed and starved animals.

After establishing that testis lobe size in mature animals appears to be similarly proportional to animal size across normally fed and starved animals, we examined lobe sizes in animals after *ilp-1(RNAi)* or *inr-1(RNAi)* treatment. In these conditions, testis lobes were proportionally smaller by approximately 30% and 35% respectively in comparison to the control cohort (Fig. 4 A-C; $p<0.05$). Correlated with the decreased lobe size, we also observed a decrease in maturing spermatids (5/12 *ilp-1(RNAi)* animals; 4/12 *inr-1(RNAi)* animals). We observed testis lobes with luminal cavities, as if there were room for maturing spermatids, but some of these hollows lacked the condensed nuclei/RAP55-positive indicators of cells progressing through spermatogenesis; the *inr-1(RNAi)* animals generally appeared to have a more severe phenotype (Fig. 4 C). Thus, insulin signaling is not necessary for the maintenance, per se, of spermatogonia in testis lobes, because *gH4*-positive cells

are present in both *ilp-1(RNAi)* and *inr-1(RNAi)* animals. Rather, insulin-like signaling appears to be involved in the subsequent amplification divisions and/or differentiation of these cells.

3.3 Discussion

In *S. mediterranea*, neuroendocrine *ilp-1* and *inr-1* are involved in regulation of growth and tissue homeostasis, consistent with the conserved developmental functions of insulin signaling in other species (Chan and Steiner, 2000, De Meyts, 2004). *S. mediterranea* has a plastic adult size and its adult stem cell population is readily observable by modern molecular techniques (Newmark and Sánchez Alvarado, 2002). Thus, the planarian provides a unique perspective on how insulin signaling regulates proliferating stem and germline cells in an adult organism.

We observed that after disrupting insulin signaling via RNAi, animals degrew but otherwise remained healthy on a gross level. In contrast, RNAi knockdown of genes that are required for proper neoblast function as well as lethal irradiation (which causes loss of the neoblast population), result in a distinct phenotype: animals without neoblasts fail to regenerate, curl up ventrally, and die on the order of days to weeks (Eisenhoffer *et al.*, 2008, Guo *et al.*, 2006, Hayashi *et al.*, 2006, Reddien *et al.*, 2005a, Reddien *et al.*, 2005b, Rouhana *et al.*, 2010, Salvetti *et al.*, 2005). Thus, under conditions of nutritional deprivation or disrupted insulin signaling, a basal rate of stem cell proliferation is maintained, allowing the organism to survive in the chance that more nutrition will become available. Insulin signaling

in *S. mediterranea* is not necessary for immediate survival/health of the organism or stem cells, but acts on a longer time scale to coordinate nutrition, adult tissue homeostasis, and stem cell dynamics.

The effects of *inr-1(RNAi)* on animal size, mitotic index, and spermatogenesis are consistently more severe than those of *ilp-1(RNAi)* alone. These data suggest that there is complexity in *S. mediterranea* insulin signaling that is unaccounted for by considering only these two genes. Given the degree of primary sequence divergence in insulin-like peptides (Chan and Steiner, 2000) and the fact that there are many organisms with more than one insulin-like peptide (Daftary and Gore, 2005; Wu and Brown, 2006), it seems likely that there are other currently unidentified insulin-like peptides in *S. mediterranea*. Additional members in the insulin signaling pathway would contribute to greater complexity and control of the pathway in response to distinct environmental cues.

We show that insulin signaling not only moderates neoblast proliferation, but also testis lobe maintenance. In *D. melanogaster* and *C. elegans*, nutritional deprivation or insulin signaling disruption leads to a decrease in germline stem cell proliferation in gonads (Drummond-Barbosa and Spradling, 2001, McLeod *et al.*, 2010, Michaelson *et al.*, 2010). Ovarian germ cell proliferation is regulated specifically by neurally produced insulin-like peptides (LaFever and Drummond-Barbosa, 2005). In *S. mediterranea*, *ilp-1* is expressed in developed testis lobes. Similarly, leydig cells in mammalian testes express an insulin-like factor (Insl3), however the effects of Insl3 on germ cell maintenance and spermatogenesis are yet

to be understood (Ivell *et al.*, 2005). *S. mediterranea* provides an opportunity to study another organism with an insulin-like peptide of testicular origin, which has implications for understanding spermatogenesis and causes of infertility in mammals.

In *S. mediterranea*, we show that insulin signaling and nutritional deprivation regulate both adult stem cells and germline cells. However, disruption of insulin signaling has a more severe effect on the germline than starvation does (Fig. 4 A-C). These data imply that insulin signaling is not only a possible mechanism for the communication of nutritional status, but also has a separate role as a growth factor. Furthermore, these data demonstrate the complexity and variety of tissue responses to a single environmental cue. During times of starvation, animals may initially favor maintenance of the germline and potential fertility, selectively suppressing proliferation of other adult stem cell populations to compensate for the lack of nutritional intake. Exploration of the transcriptional changes in neoblasts and germline cells after starvation or insulin signaling disruption would yield clues as to how these cell populations respond differently to an environmental challenge.

Our observations contribute to a greater understanding of the roles of insulin family signaling and nutrition in stem cell dynamics. We show that insulin signaling moderates both adult stem cell and germline cell populations in vivo. Our findings suggest that specific stem cell populations are differentially regulated depending upon an organism's perceived resources. Intriguingly, insulin family signaling is also disrupted in many cancers (Maki, 2010, Samani *et al.*, 2007). Consistently, in *S.*

mediterranea, we show that decreased insulin/IGF signaling decreases neoblast proliferation while Oviedo *et al.* (2008) induced cancer-like growths by knockdown of PTEN, an AKT pathway (insulin-signaling) inhibitor. A better understanding of adult stem cell dynamics after alterations in insulin signaling may influence treatments for both infertility and cancer. Further analysis of the roles of insulin signaling and nutrition in the adult stem cell and germline populations of *S. mediterranea* may offer insight into organismal controls of cell proliferation and differentiation.

3.4 Figures

Fig. 1.

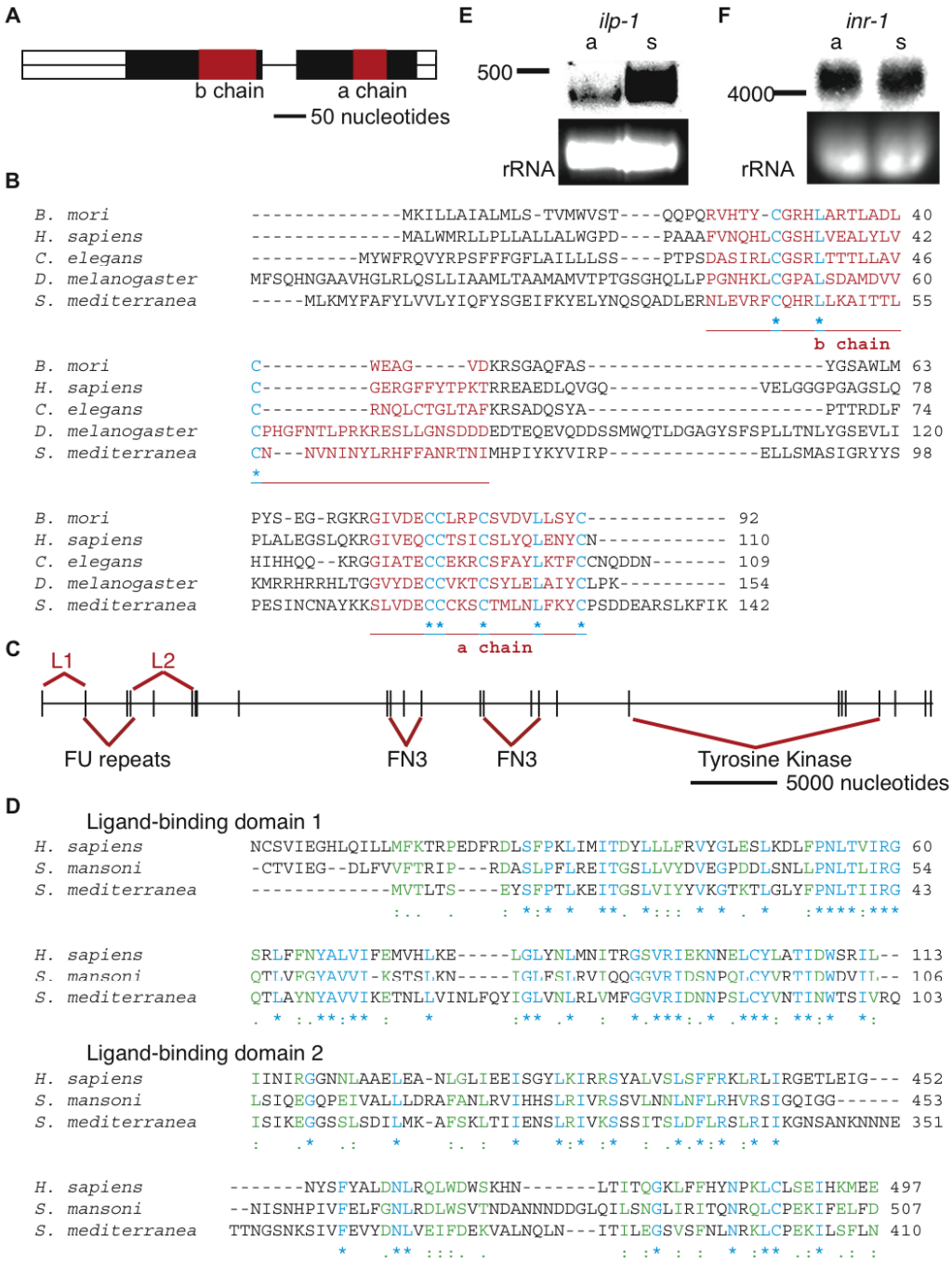

Fig. 1. Genes encoding an insulin-like peptide (*ilp-1*) and an insulin receptor (*inr-1*) are present in *S. mediterranea*. **(A)** Map of the *ilp-1* gene sequence including untranslated regions (hollow boxes), an intron (line) and translated regions (shaded boxes) with the conserved b and a chain sequences in red. **(B)** Alignment of selected insulin family member sequences with b and a chains indicated in red, and highly conserved residues, including six canonical cysteines, in blue. **(C)** Map of the *inr-1* gene spanning ~30,000 basepairs including: 26 exons (vertical lines) and 25 introns (horizontal lines); conserved domains marked in red. **(D)** Alignment of selected insulin receptor ligand binding domains with conserved residues in blue and partially conserved residues in green. **(E, F)** Northern blots of *ilp-1* and *inr-1* probe on asexual (a) and sexual (s) planarians; ribosomal RNA (rRNA) serves as a loading control.

Fig. 2.

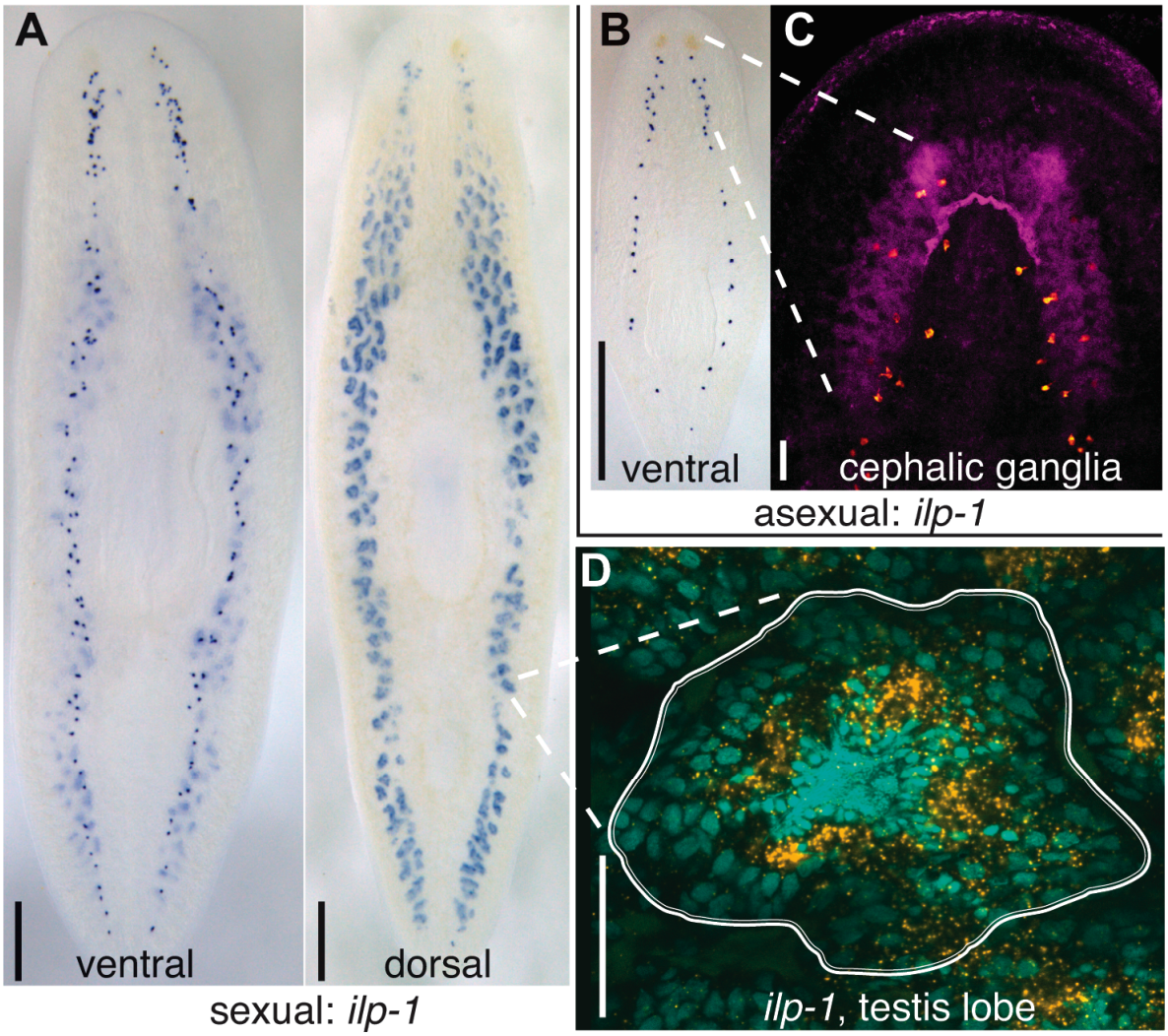
A
ventral
dorsal
sexual: *ilp-1*
B
C
ventral
cephalic ganglia
asexual: *ilp-1*
D
ilp-1, testis lobe

Fig. 2. Expression patterns of *ilp-1* and *inr-1* visualized by whole-mount in situ hybridization. (A) Ventral view of a sexual planarian shows *ilp-1* expression in the cephalic ganglia and nerve cords; dorsal view reveals *ilp-1* expression in testis lobes. **(B)** *ilp-1* expression in an asexual planarian. **(C)** *ilp-1* (orange) expression in an asexual planarian co-stained with neural markers anti-phospho-tyrosine and VC-1 (magenta). **(D)** A testis lobe with *ilp-1* (orange) expression and the nuclear stain DAPI (cyan). Scale bars are 0.5 mm (black; A, B) or 50 µm (white; C, D).

Fig. 3.

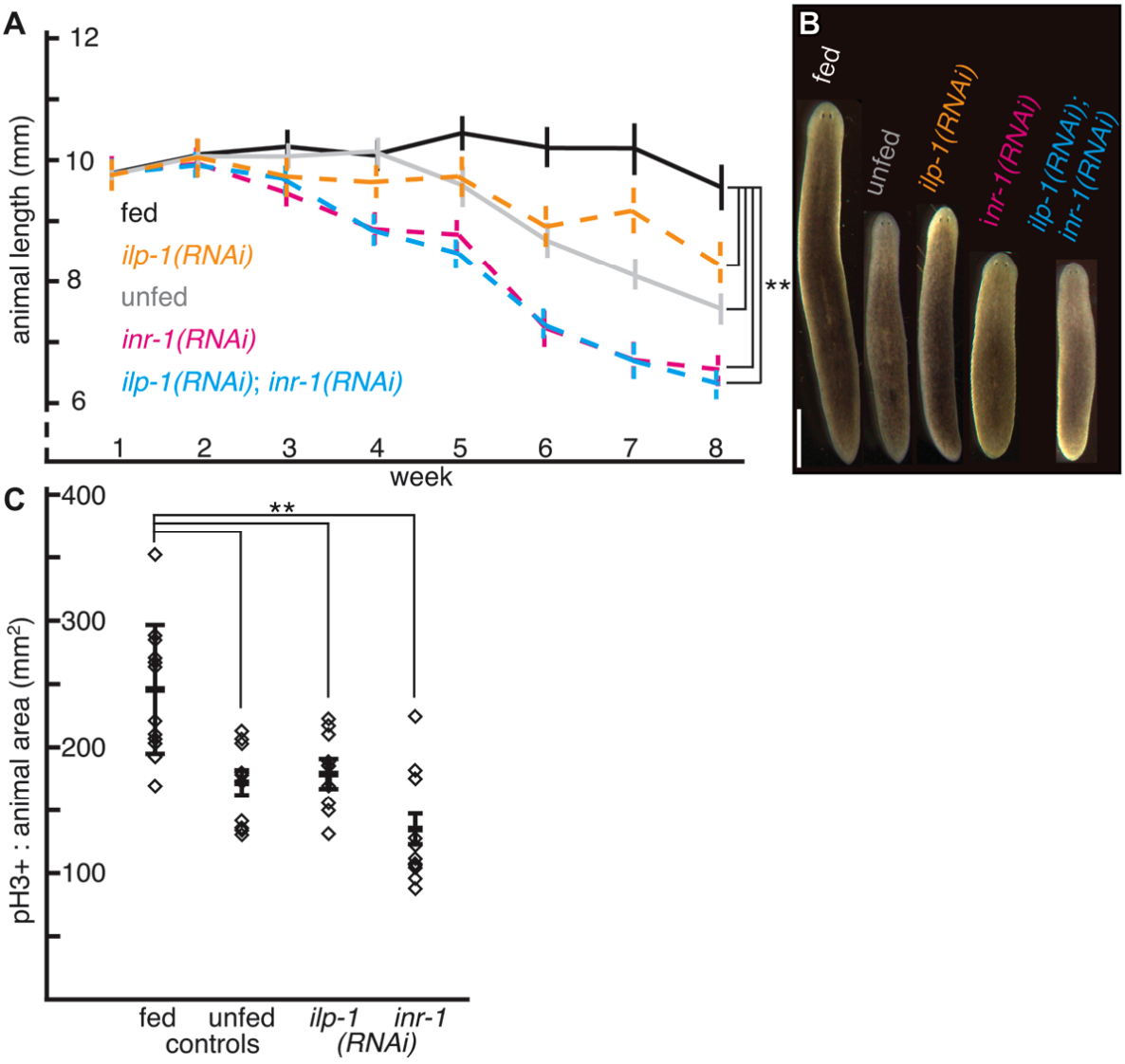
A
12
10
8
6
animal length (mm)
fed
ilp-1(RNAi)
unfed
inr-1(RNAi)
ilp-1(RNAi); inr-1(RNAi)
**
1
2
3
4
5
6
7
8
week
B
fed
unfed
ilp-1(RNAi)
inr-1(RNAi)
ilp-1(RNAi); inr-1(RNAi)
C
400
300
200
100
pH3+ : animal area (mm²)
**
fed
unfed
controls
ilp-1
(RNAi)
inr-1

Fig. 3. Insulin signaling maintains animal size and moderates stem cell proliferation. (A) Plot depicting planarian length (mm) in fed (black), unfed (grey), *ilp-1(RNAi)* (orange), *inr-1(RNAi)* (magenta) and *ilp-1(RNAi)*; *inr-1(RNAi)* conditions across eight weeks. Bars represent standard error, and in each condition n≥50. **(B)** Representative animals after eight weeks. Scale bar is 2 mm. **(C)** Ratio of phospho-histone H3-positive cells to animal area after four weeks of experimental conditions. Bars represent mean count and standard error, while diamonds mark individual counts; in each condition n≥10. Scale bars are 2 mm; ** indicates $p<0.01$.

Fig. 4.

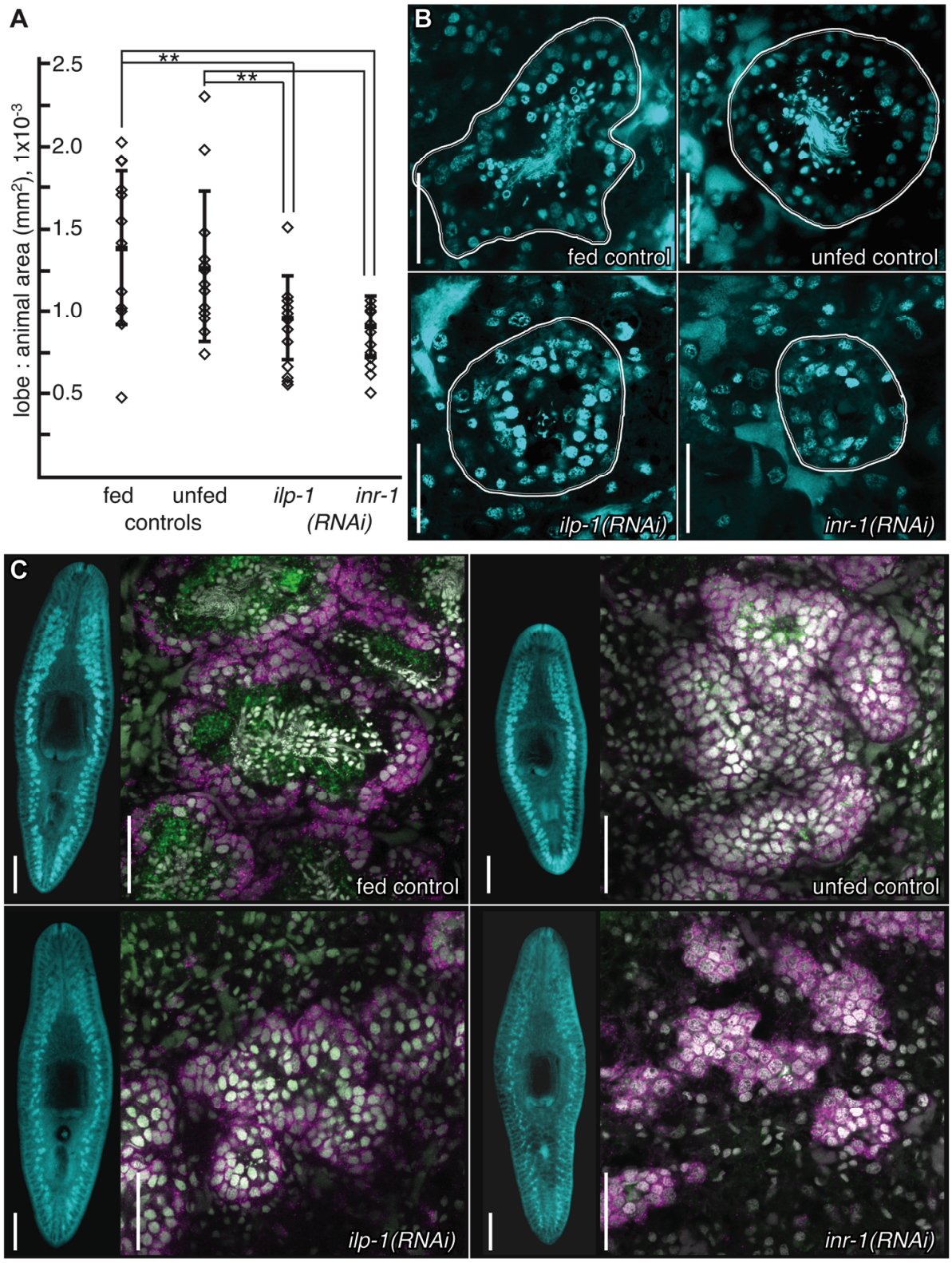
A
lobe : animal area (mm²), 1x10⁻³
2.5
2.0
1.5
1.0
0.5
**
**
fed
unfed
controls
ilp-1
inr-1
(RNAi)
B
fed control
unfed control
ilp-1(RNAi)
inr-1(RNAi)
C
fed control
unfed control
ilp-1(RNAi)
inr-1(RNAi)

Fig. 4. Insulin signaling maintains size of testis lobes and supports spermatogenesis. (A) Plot depicting the ratio of testis lobe area to animal area in *S. mediterranea* after RNAi for four weeks. Bars represent mean size and standard error, while diamonds mark the average area of five lobes in one worm; in each condition n≥10. **(B)** Representative testis lobes stained with nuclear maker DAPI (cyan), with the area outlined in white. Scale bars are 50 *μ*m. **(C)** Representative whole animals with testis lobes visualized with nuclear marker DAPI (cyan); each whole animal is next to a corresponding testis lobe after in situ hybridization to detect *germinal histone H4* (magenta) and immunostaining with anti-Tral (green). Scale bars are 50 μm for testis lobes (B, C) and 0.5 mm for whole animals (C); * indicates $p<0.05$.

Chapter 4

Ethanol and *S. mediterranea*

4.1 Summary

Alcohol disrupts the maintenance and proliferation of stem and progenitor cell populations. Exposure to alcohol thus has devastating consequences during fetal development, with pleiotropic effects on multiple organ systems. In adolescence and adulthood, excessive use of alcohol can have detrimental effects on fertility as well as liver function and regeneration, which may be due, in part, to alterations of progenitor cell population function. A better understanding of the influence of alcohol on proliferating cells raises the possibility of attenuation or cure of these alcohol-associated defects.

Alcohol has an inhibitory effect on insulin signaling. The neurological defects associated with fetal alcohol syndrome are hypothesized to be in part due to alcohol's suppression of insulin signaling during development. Simiarly, liver regeneration, which is dependent on IGF-1 signaling, is also thought to be impaired after excessive alcohol use. Misregulation of insulin signaling is related to such diseases as diabetes and cancer, as well as decreased growth and fertility. Thus, interference with insulin signaling may be the source of some of the health problems associated with chronic excessive adult alcohol use. Studying the roles and interaction of alcohol and insulin in adult stem cells *in vivo* will provide insight into the magnitude of insulin's involvement in alcohol-related health defects, and

furthermore may provide the basis of future treatment to alleviate the consequences from both chronic alcohol abuse and diabetes.

4.2 Introduction

Alcohol exposure can have drastic physiological consequences on the developing fetus, resulting in a multitude of abnormalities. Fetal Alcohol Syndrome (FAS) refers to the presence of a specific collection of these symptoms: facial and central nervous system (CNS) abnormalities, as well as growth retardation. Many children are affected by alcohol during development but do not meet the criteria for FAS or are simply not diagnosed correctly (Sampson *et al.*, 1997); Fetal Alcohol Spectrum Disorder (FASD) is a more inclusive term for milder or non-FAS alcohol-related phenotypes. The prevalence of FASD is estimated to be as high as 1% of live births (Manning and Hoyme, 2007).

Detrimental effects of alcohol are not limited to the developing fetus. Just as fetal exposure to ethanol can affect many organ systems, chronic adult overuse is associated with liver damage, psychiatric conditions, muscular atrophy, and certain types of cancer (Cargiulo, 2007, Meyerhoff *et al.*, 2005, Seitz and Stickel, 2007). Alcohol has devastating pleiotropic effects on multiple organs in both fetus and adult, and understanding the mechanism through which these defects arise is important for developing preventions, treatments, and cures.

The effects of alcohol can be divided into two categories: (i) toxicity of alcohol metabolites; and (ii) long-lasting endocrine signaling changes with effects on

organism growth, sexual maturation and fertility (Ronis *et al.*, 2007). There is increasing evidence that many of the long-term detrimental effects of alcohol are due to disruptions in insulin peptide family signaling. Indeed, chronic ethanol consumption is correlated with an increased risk for adult-onset diabetes mellitus type 2 (DMII) (Kornhuber *et al.*, 1990, Ting and Lautt, 2006), characterized by insulin insensitivity rather than lack of insulin peptide. These effects are dose dependent, such that consumption of low levels of alcohol is thought to be protective against DMII because it slightly lowers overall circulating insulin levels, effectively promoting insulin sensitivity (Koppes *et al.*, 2005). Fetal exposure to ethanol can also result in long-lasting endocrine alterations; insulin resistance is observed in both neonates (Chen *et al.*, 2004, Dembele *et al.*, 2006, Soscia *et al.*, 2006) and adults (Dembele *et al.*, 2006). Puberty, mediated in part through the growth hormone/insulin-like growth factor endocrine axis (Christoforidis *et al.*, 2005), can be delayed and adult fertility reduced (Emanuele and Emanuele, 1998, Les Dees *et al.*, 1998). Chronic excess adult alcohol use results in liver disease, due not only to the primary toxic effects of ethanol, but also to impairment of the organ's regenerative potential (Diehl, 2005) that may be mediated through disruption of insulin signaling (Michalopoulos and DeFrances, 1997, Sasaki and Wands, 1994).

Insulin is best known as a regulator of energy and metabolism (Plum *et al.*, 2005), but the insulin peptide superfamily has evolutionarily conserved roles in cellular maintenance and proliferation, including growth and development (Böhni *et al.*, 1999), adult neurogenesis (Lichtenwalner *et al.*, 2001), and lifespan (Barbieri *et*

al., 2003, Bartke and Brown-Borg, 2004, Bonafè *et al.*, 2003, Kimura *et al.*, 1997, Tatar *et al.*, 2003). Additionally, there is strong evidence that insulin family signaling has an important and conserved function in reproductive system development and maintenance, observed in nematodes (Dillin *et al.*, 2002, Tissenbaum and Ruvkun, 1998), fruit flies (Böhni *et al.*, 1999, Drummond-Barbosa and Spradling, 2001, LaFever and Drummond-Barbosa, 2005), zebrafish (Schlueter *et al.*, 2007), and mammals (Burks *et al.*, 2000, Colón *et al.*, 2007, Quirk *et al.*, 2004, Wang *et al.*, 2006b, Yagci and Zik, 2006). Therefore, the consequences of insulin misregulation extend far beyond metabolic abnormalities, with implications for lifespan, health, fertility (White, 2003), and overall cellular function of stem and progenitor cells. An emergent challenge to scientists is to understand the role of insulin signaling and the consequences of disrupting that signaling, which occurs in both alcohol abuse and diabetes.

The link between alcohol use and disruption in insulin signaling extends beyond creating a state of insulin resistance that predisposes for DMII. Ethanol inhibits IGF signaling and consequently reduces proliferation of neural progenitor cells, thought to be one mechanism by which ethanol disrupts brain development in FAS (de la Monte *et al.*, 2000, Luo and Miller, 1998, Ronis *et al.*, 2007). Fetal alcohol exposure results in decreased insulin and slightly decreased IGF-I expression in the newborn cerebellum (de la Monte *et al.*, 2005, Soscia *et al.*, 2006) and brain (Singh *et al.*, 1996) and downstream factors in the insulin signaling cascade are disrupted (Hallak *et al.*, 2001, Rubin *et al.*, 2004, Xu *et al.*, 2003).

Additionally, in adult rats, chronic alcohol exposure resulted in decreased circulating IGF-1 as well as IGF-1 expression in the liver (Lang *et al.*, 1998).

Disruption of insulin signaling due to ethanol exposure also affects proliferation and differentiation of other progenitor cell populations, including hematopoietic (Meagher *et al.*, 1982, Wang *et al.*, 2006a), and bone/osteoblast progenitors (Guiliani *et al.*, 1999, Wezeman and Gong, 2004). Ethanol may impact the role of insulin signaling in adult tissue regeneration, including the liver (Ronis *et al.*, 2007, Sasaki and Wands, 1994), as well as germ cell maintenance and proliferation (Srivastava *et al.*, 2007). In neural stem cell culture, low levels of ethanol inhibited neuronal differentiation, and this effect was partially rescued by administration of IGF-1 (Tateno *et al.*, 2004); addition of insulin and IGF-1 also resulted in partial rescue from apoptosis induced by higher levels of ethanol (de la Monte *et al.*, 2000). However, the effect of ethanol on adult stem cells, how this effect relates to regeneration, and the role insulin plays *in vivo* remains largely unexplored. The societal prevalence of alcohol use and abuse, as well as increasing rates of diabetes, creates a scientific necessity to understand the roles and interactions of ethanol and insulin in adult stem cells, germ cell maintenance and proliferation, and regeneration. The planarian *S. mediterranea* is a unique model organism with an adult population of pluripotent stem cells, inductive germ cell specification, and the ability to regenerate its central nervous system. This organism is an excellent tool for the *in vivo* study of the effects of ethanol on insulin

signaling and their combined effects on adult stem cell function and tissue regeneration.

4.3 Results

Choosing a working concentration of ethanol

Records of experiments regarding the effects of ethanol exposure on planarians are available from the early 1900s. At that time, Child, by soaking planarians in a concentration of 1.5% ethanol, found that the presence of ethanol correlated with impaired regeneration of planarian fragments, as well as possible changes in animal size or animal behavior, such as extension or normal body position (Child, 1909, Child, 1911). Pursuant to these early experiments, planarians in the following experiments were placed into normal salts with a concentration of 1.5% ethanol. Ethanol was refreshed every other day, and planarians were observed to survive over the course of days to weeks, although their characteristic gliding motion was replaced by an “inch-worm” like motility. Such motility likely results, at least in part, to a disruption of the animals’ cilia (Nishimura *et al.*, 2007).

The effects of ethanol on planarian testis lobes

Six sexually mature animals were exposed to an external concentration of 1.5% ethanol for one week, after which animals were fixed and stained with the nuclear marker DAPI. In comparison to four control worms, the animals that were exposed to ethanol had smaller testis lobes (Fig 5). A decrease in testis lobe size is

consistent with ethanol's inhibitory effect on cell proliferation and exposure-associated decreases in sperm count in other organisms (Oliva *et al.*, 2006). However, the effects could be due to toxic effects on survival rather than proliferation.

The effects of ethanol on regeneration[2]

In regenerating animals exposed to 1.5% ethanol, blastemas, a population of newly formed cells at the regeneration site, were smaller than controls (Fig 6), indicating a disruption of cell proliferation or survival of newly differentiating cells (Guo *et al.*, 2006). Additionally, 14 of 49 regenerating animals developed ectopic photoreceptors as indicated with the photoreceptor neuron-specific marker VC-1 (Fig 6) (Umesono *et al.*, 1999), or seemed to have two pigment cups in one photoreceptor, indicating a disruption in normal tissue development after regeneration similar to ethanol's teratogenic effects during embryogenesis. Regenerates exposed to ethanol prior to being cut and 'rescued' into normal solution after being cut did not show any abnormal photoreceptor phenotypes, indicating that the disruptive effect is based on exposure during the cell proliferative response. In sum, these phenotypes are consistent with the effects of alcohol on progenitor cell populations, as well as the teratogenic effects observed during development in other species. The planarian is thus a promising tool for studying

[2] I want to thank Priscilla Jackson, a wonderful ISNI Summer Student whom I had the honor to mentor. She helped design and ran the experiments described in this section.

the impact of ethanol exposure on stem cells and tissue regeneration.

Similarities in the regeneration phenotypes of ethanol exposure and insulin-signaling disruption

Ethanol, like many other biologically active substances, has pleiotropic effects both on a gross level, but also on a molecular level. One of the molecular mechanisms thought to underlie some of the longer-term effects of ethanol signaling is impairment of insulin signaling. Indeed, preliminary data indicated that knockdown of insulin signaling also disrupted planarian regeneration. After knockdown, animals appeared to have slightly smaller blastemas compared to control animals during regeneration. The difference in blastema sizes was most readily observed at around 2-3 days post-regeneration; after that time, the size difference was no longer detectable by eye. The effect of disrupted insulin signaling could be described as a delay in regeneration of about 0.5 days. In order to establish a statistically significant difference in insulin-disrupted animals vs. controls, it would likely take a large number of animals and corresponding blastema measurements. Clearly, the effect of disrupted insulin signaling on regeneration was not nearly as drastic as that observed with ethanol exposure. The fact that the effects of disrupted insulin signaling on regeneration are much less obvious than those of ethanol exposure is consistent with ethanol affecting many other physiological systems as well as insulin signaling, and furthermore with ethanol and/or its metabolites acting as toxins in and of themselves.

In terms of photoreceptor phenotypes, animals with disrupted insulin signaling also appeared to have a greater number of abnormalities after regeneration in comparison to controls. Descriptively, insulin-disrupted animals typically had smaller photoreceptor pigment cups during the course of regeneration. One possible explanation for that observation is that pigment cups appeared approximately 12-24 hours after those in control animals. Furthermore, many of the insulin-disrupted animals had one pigment cup appear before the other during regeneration, and some animals developed only one or no pigment cups. Altogether, the rate of these abnormalities was around 30-40% of the animals subjected to insulin signaling disruption via knockdown of *ilp-1* or *inr-1*. In comparison, in a group of normal planarians, one might expect abnormal photoreceptor/pigment cup phenotypes in 5-10% of animals.

4.4 Discussion

One of the most exciting aspects of *S. mediterranea* as a model organism is the relative ease with which stem cell and proliferative cell behaviors can be experimentally monitored. Both ethanol and insulin signaling are involved in affecting stem cell populations; perhaps not to the extent of stem cell-associated genes like Oct, Nanog, etc. but in a more subtle way that is perhaps more relevant to understanding cell behavior across an organism's lifespan. In this spirit, accounts of preliminary data describing the effects of ethanol on proliferative populations have been presented above.

In *S. mediterranea*, ethanol appears to impair testis lobe maintenance. After only a week of ethanol exposure, lobes are smaller than those associated with control animals. Although these data do not address any possible functional changes (i.e. decrease in fertility), it is clear that the presence of ethanol impairs spermatogenesis to some degree in *S. mediterranea*.

4.5 Figures

Fig. 5.

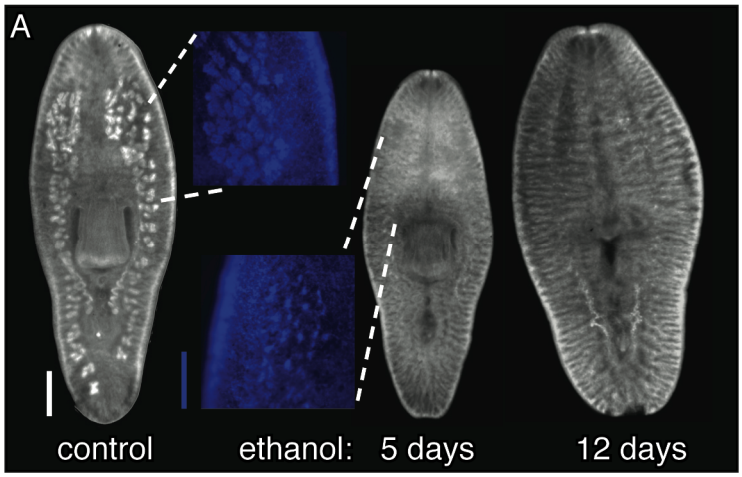

Fig. 5. Ethanol exposure results in decreased testis lobe size. (A) Dorsal views of mature sexual planarians stained with nuclear marker DAPI (white and blue) to show developed testis lobes in a control animal, but only small clusters of testis cells in planarians exposed to ethanol. White scale bar is 0.5 mm; Blue scale bar is 0.2 mm.

Fig. 6.

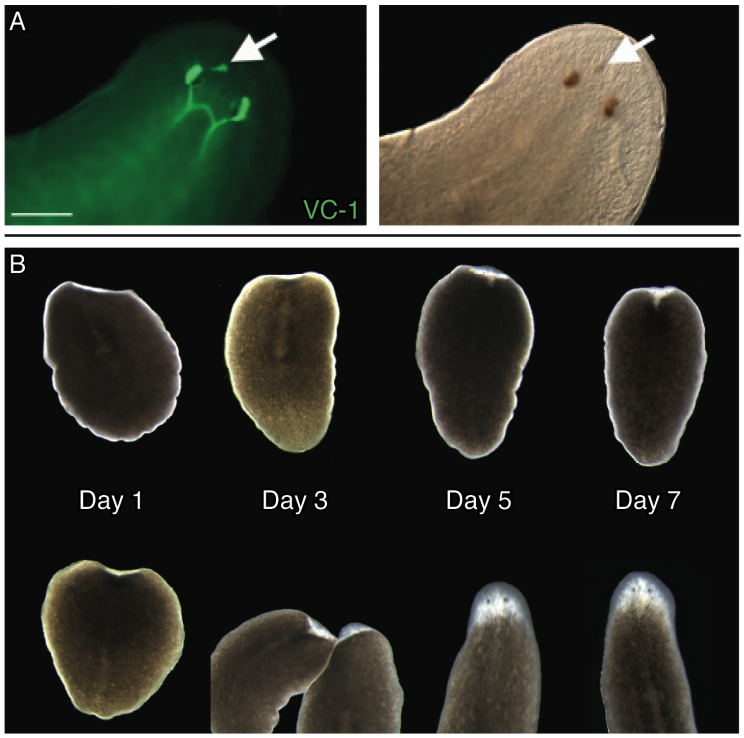

Fig. 6. Ethanol impairs regeneration and promotes abnormal photoreceptor induction. (A) An abnormal photoreceptor in a planarian that was exposed to ethanol during its regeneration, stained with the neural marker VC-1 (green), which strongly labels photoreceptor-associated neurons. **(B)** A timecourse of asexual planarian regeneration illustrates that ethanol exposure impairs the regenerative process, such that blastemas are smaller and eye-cup development is delayed in ethanol-exposed animals. Scale bar is 0.1 mm.

Chapter 5

Discussion

5.1 Conclusions

The research presented in this dissertation has demonstrated that *S. mediterranea*, a regenerating flatworm, has an insulin-like peptide (*ilp-1*) and an insulin receptor (*inr-1*) (Chapter 3, Fig 1). The *ilp-1* gene is expressed within cells associated with the animal's nervous system, which is consistent with a neuroendocrine function. Additionally, *ilp-1* is expressed within cells associated with testis lobes (Chapter 3, Fig 2). The *inr-1* gene appears to be ubiquitously expressed. RNAi knockdown of these genes results in a distinct and striking phenotype; animals degrow over time, despite eating similarly to wild type (Chapter 3, Fig 3). On a gross level, the degrowth appears comparable to that observed while an animal is in a state of starvation. The change in size is correlated with a decrease in the number of mitotic events observed in an animal (Chapter 3, Fig 3). Fewer mitotic events could result from a decrease in the overall population of neoblasts or a slower rate of mitosis in an unchanged number of neoblasts. Quantitative real-time PCR suggests that the decrease in mitotic events is due, at least in part, to an overall slower rate of mitosis. A similar alteration in mitosis is observed in a starved cohort of animals (Chapter 3, Fig 3). Finally, the germline, which is another proliferative cell population, also appears to be affected by disrupted insulin/IGF signaling. After gene knockdown, testis lobes become

disproportionately small in comparison to animal size (Chapter 3, Fig 4). In addition, there is a decreased number of lobes containing cells undergoing spermatogenesis (i.e. fewer spermatids and maturing sperm are observed; Chapter 3, Fig 4). The testis lobe phenotype resulting from decreased insulin/IGF signaling is more extreme than that resulting from starvation, which is consistent with insulin signaling playing a role that is at least in part distinct from communication of nutritional status.

The role of insulin/IGF signaling in *S. mediterranea* described in this study appears to be consistent with how the insulin/IGF family functions in other organisms (Bartke and Brown-Borg, 2004, Böhni *et al.*, 1999, Kimura *et al.*, 1997). The presented findings support the current hypothesis that insulin/IGF family function is highly conserved across species, even if particular peptides are not conserved (Chan and Steiner, 2000, De Meyts, 2004). If an animal is in a state of growth (i.e. during development), decreased insulin/IGF signaling results in decreased size (Bartke and Brown-Borg, 2004, Böhni *et al.*, 1999, Kimura *et al.*, 1997, Tatar *et al.*, 2003). *S. mediterranea*, because of its plastic mature size and ability to degrow, demonstrates that the cellular and molecular mechanisms behind growth control are not isolated to development, but persist into an organism's adulthood.

The two strains of *S. mediterranea*, asexual and sexual, provide an opportunity for examining how a gene functions in a context devoid of reproductive development. Because both strains show a similar degrowth phenotype after insulin/IGF signaling disruption, and the asexual animal only expresses *ilp-1* within

the nervous system, the growth control aspect of the pathway appears to be dependent on neuroendocrine *ilp-1*.

Similarly, in other invertebrates, insulin-like peptides frequently have a neural origin (Chan and Steiner, 2000, De Meyts, 2004, Wu and Brown, 2006); in humans, IGF-1, which controls post-embryonic growth, is primarily expressed in the liver (it is expressed to a lesser extent in the brain), but release and endocrine action of IGF-1 is controlled by the pituitary gland (Shabanpoor *et al.*, 2009).

Although the functions of insulin/IGF signaling are consistent across species, the number of insulin-like peptides across organisms is not conserved (Chan and Steiner, 2000, De Meyts, 2004). Only one insulin-like peptide was identified in *S. mediterranea*. The challenge in utilizing bioinformatic identification of insulin-like peptides is that the family has a highly divergent primary peptide sequence, both across species and within single invertebrate species. However, different methods in other invertebrates have been largely unsuccessful (Wu and Brown, 2006), indicating that bioinformatic techniques are likely the best method for identifying invertebrate insulin-like peptides. Thus, while the presence of only one insulin-like peptide in *S. mediterranea* is not surprising, it is possible that the organism has other, as-yet unidentified insulin-like peptides.

Generally speaking, although vertebrates have multiple insulin receptors, including both tyrosine kinase and G-protein coupled, invertebrate species generally appear to have only one insulin receptor gene (Wu and Brown, 2006). One insulin receptor tyrosine kinase was identified within *S. mediterranea*, though it is

impossible to rule out the presence of more insulin receptors. Indeed, the gene knockdown phenotypes for *ilp-1* and *inr-1*, when compared to each other, support the conclusion that the insulin signaling pathway in *S. mediterranea* is more complex than is accounted for by these two genes alone. The phenotype for *inr-1(RNAi)* is significantly stronger than that of *ilp-1(RNAi)* (Chapter 3, Fig 3). Possible explanations include other, unidentified insulin-like peptides or insulin receptors (through which *ilp-1* could have an inhibitory rather than excitatory signal), constitutive activity of *inr-1* in the absence of *ilp-1*, or an unknown aspect of the *ilp-1* knockdown process that makes the phenotype less representative than the *inr-1(RNAi)* phenotype (i.e. *ilp-1* protein perdurance, such that signaling is still active after the gene transcript has disappeared). Current methodology available in *S. mediterranea* makes analysis of these different possibilities very difficult.

Although the phenotypes resulting from *ilp-1(RNAi)* or *inr-1(RNAi)* are consistent with those observed in other organisms, *S. mediterranea* has unique qualities that provide an increased understanding of the roles of insulin signaling. The worm appears to have the characteristics of species that are, according to Caleb Finch's categories, negligibly senescent (Guerin, 2004). The lifespan of *S. mediterranea* is unknown, and it does not noticeably succumb to age-related disease. In contrast, model organisms in which most of the studies pertaining to insulin/IGF family signaling are performed fit into either the rapid or the gradual senescence categories.

One of the difficulties in establishing how insulin/IGF signaling functions is that it regulates many different physiological axes, including control of lifespan and "aging," and that all those physiological axes feed back onto each other. In *S. mediterranea*, insulin/IGF family signaling is active without any apparent effect on lifespan. Thus, a real advantage of the use of *S. mediterranea* is that it allows studies of insulin's effects on growth and reproductive development in a context isolated from lifespan. *S. mediterranea* is particularly useful as a model organism for the study of stem cells (Guo *et al.*, 2006, Reddien *et al.*, 2005a, Reddien *et al.*, 2005b, Wagner *et al.*, 2011, Wenemoser and Reddien, 2010). There are two distinct populations of adult proliferative cells, the neoblasts and the germline, which can be examined after experimental manipulation. Beginning with the former, the worm is full of neoblasts, which are adult totipotent stem cells (Wagner *et al.*, 2011). Neoblast regulation is of particular interest to stem cell researchers, because the cells enable a high rate of tissue turnover and are the basis of *S. mediterranea*'s regenerative capacity.

Studies have shown that when *S. mediterranea* lose their neoblast population, either by gene knockdown or lethal irradiation, the worms fail to regenerate, curl up ventrally, and die within days to a few weeks (Eisenhoffer *et al.*, 2008, Guo *et al.*, 2006, Hayashi *et al.*, 2006, Reddien *et al.*, 2005a, Reddien *et al.*, 2005b, Rouhana *et al.*, 2010, Salvetti *et al.*, 2005). In this study, I observed the effects of insulin/IGF signaling on stem cells in *S. mediterranea*. I observed that, after insulin signaling disruption, worms degrew but otherwise remained healthy.

Therefore, although insulin signaling is not necessary for neoblast survival in *S. mediterranea*, it stilli regulates neoblast function, as shown by the reduced level of mitoses under RNAi conditions (Chapter 3, Fig 3).

Insulin/IGF signaling communicates nutritional status in many organisms. Like the *ilp-1(RNAi)* and *inr-1(RNAi)* conditions, caloric reduction also results in animal degrowth, correlated with fewer mitoses (Chapter 3, Fig 3). Thus, the reduced number of mitoses observed in the gene knockdown conditions is consistent with animals scaling back their rate of metabolism in times of reduced caloric intake. Recently, in *D. melanogaster,* caloric restriction was shown to decrease adult intestinal stem cell proliferation (McLeod *et al.*, 2010). The findings presented in this dissertation support and build upon the current literature by showing that, in an adult organism, both caloric restriction and disrupted insulin signaling result in decreased adult stem cell proliferation. Insulin signaling is thus a mechanism of adaptation to an environment rather than a necessity for life.

In many organisms, germline stem cells proliferate and differentiate even during adulthood. Insulin/IGF signaling is known to control entrance into puberty, as well as post-puberty fertility. In *S. mediterranea*, testis lobe size is reduced after insulin signaling disruption (Chapter 3, Fig 4). There is a correlative decrease in spermatogenesis, but it is not possible to say if insulin signaling directly inhibits spermatogenesis or indirectly inhibits it by causing an overall decrease in testis lobe size. In *D. melanogaster* and *C. elegans*, nutritional deprivation or insulin signaling disruption leads to a decrease in germline stem cell proliferation in gonads

(Drummond-Barbosa and Spradling, 2001, Hsu and Drummond-Barbosa, 2009, LaFever and Drummond-Barbosa, 2005, McLeod *et al.*, 2010, Michaelson *et al.*, 2010). Upregulation of insulin signaling can partially rescue reduced spermatogenesis in *D. melanogaster* (McLeod *et al.*, 2010). Thus, insulin signaling appears to play a role in terms of supporting spermatogenesis across these invertebrate model organisms; further, it that insulin signaling is involved in regulating spermatogenesis in response to nutritional status.

Insulin signaling appears to support spermatogenesis in relation to caloric state in invertebrate organisms. However, in *S. mediterranea*, it also plays a role that is distinct from communication of nutritional status. In *S. mediterranea*, the phenotypes resulting from caloric restriction and insulin signaling disruption are not equivalent in terms of gametogenesis. Both conditions cause a reduction in testis lobe size and spermatogenesis. However, *S. mediterranea*, unlike most other model organisms, has a plastic adult size and degrows during times of starvation or insulin signaling disruption. When the animal degrows, its gonads eventually regress. Thus, the reduced gonad size could be due to reduction in caloric intake or insulin signaling disruption, or indirectly due to the decreased organism size, resulting from reduced caloric intake or insulin signaling disruption. The phenotype in the cohort of animals with disrupted insulin signaling is much more severe than that of the starved animals. Because the effects of insulin signaling in terms of gonad maintenance and gametogenesis are more severe than those resulting from caloric restriction, insulin signaling plays a role in planarian reproduction that is distinct

from its role as a communicator of nutritional status. Further, because *ilp-1* is expressed within testis lobes and because its actions are similar to but distinct from caloric restriction, its actions may be more similar to how IGFs and ILPs act in vertebrates than the currently understood role in other invertebrate model organisms.

Disrupted insulin/IGF signaling affects both neoblast proliferation and spermatogenesis. The comparison of the dynamics of these two populations of cells, neoblasts and the germline, is in and of itself revealing about how an adult animal can regulate tissue dynamics and homeostasis. The neoblast phenotypes that result from caloric restriction and insulin signaling reduction are broadly equivalent in terms of animal size and decreasing number of mitotic events. However, the testis lobe phenotypes differ between caloric restriction and insulin signaling disruption. As described above, the different phenotypes point to insulin having a role separate from a communicator of nutritional status. The distinct phenotypes also indicate that proliferative cell populations can be differently modulated in response to an environmental cue. In times of nutritional duress, an animal may reduce its neoblast proliferation but, for some weeks, maintain its reproductive ability until its body has degrown to the point at which maintaining reproductive status is no longer metabolically feasible. On the other hand, if insulin signaling is reduced, both proliferative populations are affected. Thus, adult organisms can differentially regulate adult stem cell dynamics in specific tissues.

5.2 Future Directions

Antibody synthesis against ilp-1 *and* inr-1 *peptides*

During the course of my graduate studies, I attempted to raise antibodies against *ilp-1* three times, all of which were unsuccessful. Thus, any interpretations of the data presented must include the caveat that manipulation of RNA transcript is not necessarily reflected by functional changes in protein activity. Furthermore, *ilp-1* is expressed within what appear to be neuroendocrine cells as well as the testis lobe lumen, but the peptide form may circulate throughout the organism; an *ilp-1* antibody would clarify how the peptide acts across the tissues of the organism. Similarly, *inr-1* appears to be expressed ubiquitously, but the protein could be relatively up- or down-regulated across tissues and an antibody would clarify where the receptor is localized, and therefore on which tissues *ilp-1* could be acting. Additionally, the success of the *ilp-1* and *inr-1* gene knockdowns could be analyzed on a protein level.

Transcriptome analysis after insulin/IGF signaling disruption

Insulin/IGF signaling drives the AKT pathway and alters cellular dynamics in large part through the foxo/*daf-16* transcription factor (Kalaany and Sabatini, 2009, Maki, 2010, Samani *et al.*, 2007). Transcriptome analysis has been done in several organisms to assess the main actions of the pathway, but the studies have not yielded any primary suspects in terms of genes up- or down-regulated by alterations

in insulin/IGF signaling (Murphy, 2006, Murphy *et al.*, 2003). However, the cohort of genes affected by insulin/IGF activity and the AKT signaling pathway are of medical significance due to the pathway's upregulation in many cancers. Therefore, it would be worthwhile to perform a transcriptome analysis on *S. mediterranea* after insulin signaling disruption in comparison to wild type. In order to isolate the analysis to a non-reproductive context, an asexual strain should be used. Transcriptome analysis could be performed on both a representative population of cells and a neoblast-enriched population of cells (which can be obtained via cell sorting; (Reddien *et al.*, 2005b, Salvetti *et al.*, 2009)). The four-way comparison that is described would yield the following useful information: transcriptional changes that result from altered insulin/IGF signaling, which could be compared to those identified in other organisms; and the transcriptional effects of insulin/IGF signaling that are specific to neoblasts.

In *S. mediterranea*, insulin/IGF signaling regulates both neoblast proliferation and spermatogenesis. Although the overt cellular phenotypes of these populations are observable, the transcriptional changes that drive alterations in cell dynamics are not. One of the intriguing findings of the work presented in this dissertation is that the phenotypes resulting from caloric restriction and disrupted insulin/IGF signaling are similar in the neoblast population, but different in terms of the male germline cells and spermatogenesis. Performing a three-way comparison of the transcriptomes of wild type animals, starved animals, and *ilp-1(RNAi)*; *inr-1(RNAi)* animals would illuminate the molecular mechanisms underlying the different

phenotypes and would aid understanding in how insulin/IGF signaling is both similar to and different from an animal's metabolic state. The transcriptome comparison would also indicate how two different cell populations, both presumably expressing *inr-1* can react differently to an environmental cue.

Regeneration

S. mediterranea has achieved recognition because of its regenerative prowess. The mammalian IGF-1 is up-regulated in the liver after hepatectomy and prior to regeneration. If the up-regulation is blocked, regeneration is impaired (Desbois-Mouthon *et al.*, 2006). IGF is also required for fin regeneration in zebrafish (Chablais and Jazwinksa, 2010). Application of insulin also increases the rate of healing after wounding or burning (Seitz *et al.*, 2010, Tuvdendorj *et al.*, 2011). Thus, it is likely that insulin/IGF signaling plays a role in planarian regeneration. Because the insulin/IGF knockdown phenotype is subtle, any analysis of its effects on regeneration would by necessity be very rigorous. Animals would undergo gene knockdown, and blastema size could be monitored at time points during regeneration. Additionally, physical markers including eyespots and pigmentation could be monitored. Animals could be fixed at a time approximately 4-5 days post-amputation and the size of the cephalic ganglia (brain lobes) could be measured. It might be necessary to perform multiple wounds in one animal, or to re-wound a regenerating animal in order to obtain a thorough understanding.

Oncogene analysis

Currently, the medical field is pursuing insulin/IGF signaling as a target for cancer therapies. One intriguing trait of *S. mediterranea* is that noticeable instances of cancer are generally not observed in the laboratory setting. Cancer-like growths have been experimentally induced by disrupting PTEN (Oviedo *et al.*, 2008), which is a known inhibitor of AKT pathway signaling. Data in this dissertation indicate that insulin signaling plays a mitogenic role in adult *S. mediterranea*, because it regulates neoblast proliferation. However, some mechanism prevents cancer formation despite the apparent conservation of a potentially oncogenic pathway (Maki, 2010, Samani *et al.*, 2007). Thus, the previously described transcriptome analysis might be revealing in terms of transcriptional changes associated with insulin/IGF signaling in *S. mediterranea*, but not in other organisms.

Given that *S. mediterranea* does not appear to generate tumors, or if it does, the tumors do not appear to be lethal, the organism may be able to contribute to general cancer research. Moving away from a focus on the insulin/IGF signaling pathway, a screen of candidate oncogenes, and tumor suppressors might identify unique roles that conserved genes are or are not playing in *S. mediterranea*. Additionally, such a screen might also uncover specialized cellular mechanisms of cancer control. In order to perform an oncogene/tumor suppressor screen, candidate genes would first need to be identified bioinformatically. Then, riboprobes could be synthesized for the candidate genes and expression patterns analyzed to

assess if the genes are associated with the neoblast population. Candidate genes could be knocked down via RNAi in a functional study. Unfortunately, in planarians, it is not yet possible to upregulate genes and all manipulations must be performed by knocking down genes. Functionally, a signaling pathway can be up-regulated by knocking down a pathway inhibitor, as is the case with the AKT pathway and its inhibitor, PTEN (Oviedo *et al.*, 2008). If knockdown of a candidate tumor suppressor results in cancer, then further analysis should focus on why those genes are not misregulated in *S. mediterranea*. If knockdown of a candidate tumor suppressor does not result in cancer, transcriptome analysis in comparison to other organisms might reveal genes that are up- or down-regulated to prevent cancer in response to loss of a tumor suppressor.

In sum, the experiments described in this thesis show that the planarian *S. mediterranea* is a useful model organism for future studies of insulin signaling.

Chapter 6

Materials & Methods

Animal Culture

Clonal asexual and sexual (hermaphroditic) strains of *Schmidtea mediterranea* were housed at 20˚C in 1.0x and 0.75x Montjuïc salts, respectively (Cebrià and Newmark, 2005). Animals were fed ground organic calf liver (Vantage USA, Lenoir, NC) once per week. Prior to fixation for imaging, animals were starved for at least one week to reduce background and increase animal integrity during processing.

For growth/degrowth experiments, we used three biological replicate groups of 15 to 20 sexual *S. mediterranea* per condition, leading to a total of at least 50 animals per condition per time point. We replicated the findings in asexual animals using one biological group of at least 20 animals per condition per time point. Live images of animals were taken with a Leica DFC420 camera mounted on a Leica M205A stereomicroscope (Leica, Wetzlar, Germany), utilizing LAS3.6 software. To accurately and consistently capture images of animal length/size, animals were accustomed to a Petri dish for a minimum of 5 minutes prior to imaging. *S. mediterranea* displays negative phototactic behavior. Thus, just prior to imaging, an LED light source was turned on, causing animals to extend as they moved away from the light. For the first time point, both animal length and area were calculated; they were found to be proportional and length was used as a proxy for area/size

thereafter. Mean animal length and standard error were calculated for each graphed experimental condition. For the final experimental timepoint, differences in length between all conditions were analyzed for significance via ANOVA, followed by Tukey's HSD post-hoc test.

Gene Identification

To identify insulin and insulin receptor homologues in *S. mediterranea*, EST (Zayas *et al.*, 2005) and genomic resources (Robb *et al.*, 2007) were searched via BLAST with query sequences from other species including *Bombyx mori*, *C. elegans*, *D. melanogaster*, *Homo sapiens* and *Schistosoma mansoni* (NCBI). Insulin identity depends on the presence of six canonical cysteine residues and a signal sequence (Chan and Steiner, 2000, Smit *et al.*, 1998). After this bioinformatic search, only one putative insulin-like peptide gene (*ilp-1*) encoded a protein with all six cysteines at the proper location in gene sequence as well as a putative signal sequence (SignalP 3.0; (Bendtsen *et al.*, 2004)). A gene encoding for a candidate insulin receptor (*inr-1*) was identified based on similarities with other insulin receptor tyrosine kinases. *inr-1* had both ligand-binding (L) and tyrosine kinase domains, as well as other conserved domains consistent with insulin receptor tyrosine kinases (SMART 6 protein domain predictor; (Letunic *et al.*, 2009, Schultz *et al.*, 1998)). ESTs with partial sequence, in the pBluescript II (SK+) vector, were available for both *ilp-1* (PL05016A1H10) and *inr-1* (PL05016A2F11) from our EST collection

(Zayas *et al.*, 2005). EST matches were verified by sequencing prior to use as template for further experiments.

Full-length sequences for *ilp-1* and *inr-1* were obtained as follows: RNA was extracted from the sexual strain of *S. mediterranea* (Trizol, Invitrogen, Carlsbad, CA), DNase digested (DNA-free RNA Kit, Zymo Research, Orange, CA), oligo (dT) purified (Poly-A Purist Kit, Ambion, Austin, TX), then used as template for cDNA synthesis (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA). Both 5' and 3' RACE (First Choice RLM RACE Kit, Ambion, Austin, TX) were performed with nested, gene-specific primers. Due to the presence of AT-rich regions in the insulin receptor that caused false 3' poly-A ends, this process was repeated until all receptor domains were identified. Accession numbers: *Smed-ilp-1*, BK007034; *Smed-inr-1,* JN049497.

Riboprobe Synthesis, Northern Blot, In Situ Hybridization

Riboprobes were synthesized for use in northern blot and in situ hybridization. In vitro transcription using T3 polymerase was performed with partial cDNA sequence template for *ilp-1* and *inr-1*. Probes were synthesized with Digoxigenin-12-UTP (Roch, Mannheim, Germany), Fluorescein-12-UTP (Roche, Mannheim, Germany) or Dinitrophenol-11-UTP (Perkin Elmer, Waltham, MA). Additionally, because the original *inr-1* partial cDNA clone corresponded to only 762 of 4654 total nucleotides, two alternate probes to unique 5' and 3' regions were transcribed from nested PCR-amplified product (Platinum Taq, Invitrogen, Carlsbad,

CA) using gene-specific primers flanked by T3 or T7 promoter sites. All three *inr-1* probes were tested side by side in northern blot and in situ hybridization to ensure that they all yielded similar results.

Primers used:

inr-1 alternate probe sequences, 5' and 3' of EST sequence:

inr-1 5' forward: 5'-CGGTGGGGAAAATTTGCAGAAA-3';

inr-1 5' reverse: 5'-CAGAACCTTCCAAAATCGTGA-3';

inr-1 3' forward: 5'-CGCTCTGGGCCATACAAATTGC-3';

inr-1 3' reverse: 5'-TGGGTTTATTCATTGACTTTCC-3'

The flanking T7 and T3 polymerase promoter sequences.:

T7: 5'-GTAATACGACTCACTATAGGG-3';

T3: 5'-CAATT AACCCTCACTAAAGGG-3'

Northern blots were performed according to standard protocol (Sambrook and Russell, 2001), utilizing digoxigenin probes (above), α-digoxigenin AP antibody (Roche, Mannheim, Germany) and chemiluminescence (CDP-STAR, Roche, Mannheim, Germany). Luminescent blots were visualized with a FluoroChem Q (Alpha Innotech, San Leandro, CA).

In situ hybridization was performed according to a standard protocol for asexual and sexual animals (Collins *et al.*, 2010, Pearson *et al.*, 2009). Chromogenic in situ utilized the digoxigenin probe and α-digoxigenin-AP antibody

previously described and was developed with NBT-BCIP (SigmaFast BCIP/NBT, Sigma, St. Louis, MO) in high salt AP buffer (100mM Tris, pH9.5; 100mM NaCl; 50mM $MgCl_2$; 0.1% tween-20) with 10% PVA. After development, animals were post-fixed (4% FA in PBS+1% Triton X-100) for 15 minutes, washed in 100% ethanol 2x10minutes, returned to PBS+1% Triton X-100, then cleared and mounted in 80% glycerol. Animals developed chromogenically were imaged over a piece of white filter paper with the Leica microscope, camera, and software described above.

Fluorescent in situ hybridization utilized all three probe types, coupled with α-digoxigenin-POD (Roche, Mannheim, Germany), α-fluorescein-POD (Roche, Mannheim, Germany) or α-dinitrophenol-HRP (Perkin Elmer, Waltham, MA). Prior to development, animals were placed in TNT buffer (100mM Tris, pH7.5; 150mM NaCl; 0.05% tween-20) for 10 minutes. FISH was developed with Cy3-, Cy5- or fluorescein-tyramide TSA-Plus kits (Perkin Elmer, Waltham, MA), and if multiple probes were utilized, then peroxidase reactions were quenched (1 hour incubation with 1% H_2O_2 in TNT buffer) prior to detection of another probe. Samples were co-stained with the nuclear marker DAPI (Sigma-Aldrich, St. Louis, MO). Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and imaged on a Zeiss LSM 710 confocal microscope (Carl Zeiss, Germany) with Zen 2008 software (Carl Zeiss, Germany) or a Zeiss SteREO Lumar.V12 microscope. Further analysis, including calculation of lobe size or manual numbering/counting of cells, was also carried out with Zen 2008 software.

Immunofluorescence

Staining with anti-phosphohistone H3 and DAPI were performed with animals killed in 5% n-acetyl cysteine for 5 minutes, rinsed once in PBS, then fixed for 20 minutes in 4% formaldehyde in PBSTx (PBS + 0.3% Triton X-100). Animals were rinsed in PBSTx for 3x 5 minutes, then were bleached overnight in approximately 6% H_2O_2 in PBSTx. Animals were rinsed again in PBSTx for 3x 5 minutes, blocked at room temperature for 4 hours in PBSTx + 0.6% BSA + 0.4% fish gelatin, and exposed to a 1:100 dilution of anti-mouse phospho-histone H3 serine 10 (05-806, Millipore, Temecula, CA) in PBSTx at 4°C overnight. After washing 6 times over a minimum of 4 hours, animals were exposed to the secondary Goat anti-Mouse POD (Pierce/Thermo Fisher Scientific, Rockford, IL) for either 4 hours at room temperature or 4°C overnight. The animals were then washed in PBSTx 6 times in 1 hour, placed into TNT buffer (above) for 10 minutes, then developed with the Perkin-Elmer TSA kit (Perkin Elmer, Waltham, MA). Staining with anti-phospho-tyrosine and VC-1 was performed as previously described (Cebrià and Newmark, 2005) after in situ hybridization. Staining with anti-Tral was performed after in situ hybridization as previously described (Wang *et al.*, 2010).

RNA interference

EST sequences for insulin and insulin receptor were cloned into the pPR244 plasmid (Reddien *et al.*, 2005a) via the Gateway BP Clonase Kit (Invitrogen, Carlsbad, CA). Plasmids were transformed into RNaseIII-deficient HT115(DE3),

which expresses T7 polymerase after induction with IPTG (Timmons *et al.*, 2001) thus producing dsRNA. Single colonies were selected for sequencing to confirm successful cloning, and cultures were grown and induced as previously described (Newmark *et al.*, 2003). Control vector was empty pPR242, transformed into HT115(DE3). For feeding, a pellet of cells from 10 ml of induced culture, stored at -80˚C, was thawed, mixed with 60 ul of liver homogenate, then administered to animals. There was an excess of food in all RNAi/feeding experiments so animals could eat to satiety. Animals undergoing RNAi were fed once per week, then fixed and processed as described above. For experiments involving pH3-positive cell counts as well as experiments involving analysis of testis lobes, a minimum of 10 animals were used in each condition. Differences between conditions were analyzed for significance via ANOVA, followed by Tukey's HSD post-hoc test.

rt-qPCR

Each reverse-transcriptase quantitative PCR (qPCR) experiment described was performed on three biological replicate samples of 2-3 worms each. RNA from each sample was extracted with Trizol (Invitrogen, Carlsbad, CA), DNase digested (DNA-free RNA Kit, Zymo Research, Orange, CA), then used as template for cDNA synthesis (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA). cDNA was diluted to 100 ng/ul prior to experimentation.

Samples were run in experimental triplicate. qPCR was performed with GoTaq Mastermix (Promega, Madison, WI) on a StepOnePlus real-time PCR

system (Applied Biosystems, Carlsbad, CA) with StepOne Software v2.1 according to the ΔΔCT protocol with *β-tubulin* (PL05008A2H07) as a normalization control. Statistical analysis was performed using a Student's *t*-test on ΔCT values.

Primers used:

β-tubulin forward: TGGCTGCTTGTGATCCAAGA

β-tubulin reverse: AAATTGCCGCAACAGTCAAATA

ilp-1 forward: CTAAGACACTTTTTCGCCAATCG

ilp-1 reverse: TTTGTAAATCGGGTGCATTATGTTA

inr-1 forward: TGGAAACCAGAACCAAGGAG

inr-1 reverse: CATGACTCCATGCACTTGTCA

smedwi-1 forward: GAAGAGCTGGGGGATGTGTA

smedwi-1 reverse: TTCACGACCACGAATCGTAA

smedwi-2 forward: GGCGATCAACCATTTGTTCT

smedwi-2 reverse: CTTTCTCGACGCATTGGTTT

Insulin Antibody Synthesis

In collaboration with the UIUC Immunological Resource Center, we attempted to raise mouse antibodies *ilp-1*. We utilized a total of four short peptide sequences to try to obtain an immunogenic response.

Short Peptide Sequences:

SLVDEC

NNVNINYL

YYSPESINC

CNAKKSLVED

After 3-4 injections across 6-8 weeks, mouse bleeds were obtained and tested on planarians. Planarians were fixed and processed in multiple ways prior to antibody exposure, including the standard HCL or N-acetyl cysteine killing protocols in combination with standard Carnoy's, Methacarne, or Formaldehyde fixation protocols. Blocking was performed with BSA or in situ blocking buffer.
Antibody reactivity was only observed in the relatively sticky cells of the oviduct and secretory system; western blot (performed according to standard protocols) was unsuccessful.

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