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An insulin-like peptide regulates size and adult stem cells in planarians

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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 explored the role of insulin signaling in *S. mediterranea*. We disrupted insulin signaling via RNA interference and showed that animals, despite eating, degrew similarly to starved animals. Utilizing *in situ* hybridization and immunofluorescence, we assessed cellular changes in proliferative populations including the planarian adult stem cell population (neoblasts) and the germline. Both impaired insulin signaling and nutritional deprivation correlated with decreased neoblast proliferation. Additionally, insulin signaling played a role in supporting spermatogenesis that was distinct from the effects of starvation. In sum, we have demonstrated that insulin signaling is responsible for regulation of adult animal size and tissue homeostasis in an organism with plastic adult size. Importantly, insulin signaling continued to affect stem cell and germline populations in a mature organism. Furthermore, we have shown that adult organisms can differentially regulate specific cell populations as a result of environmental challenges.

KEY WORDS: *insulin, planarians, adult stem cell, germ cell, spermatogenesis*

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; Ueishi *et al.*, 2009). 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 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.*

Abbreviations used in this paper: dsRNA, double-stranded RNA; IGF, insulin growth factor; ILP, insulin-like peptide; INR, insulin receptor; INSL, insulin-like factor; RNAi, RNA interference; Tral, Trailer-hitch.

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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, 2000; 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 and Romero, 1981; 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.*, 2007; Wang *et al.*, 2010) 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

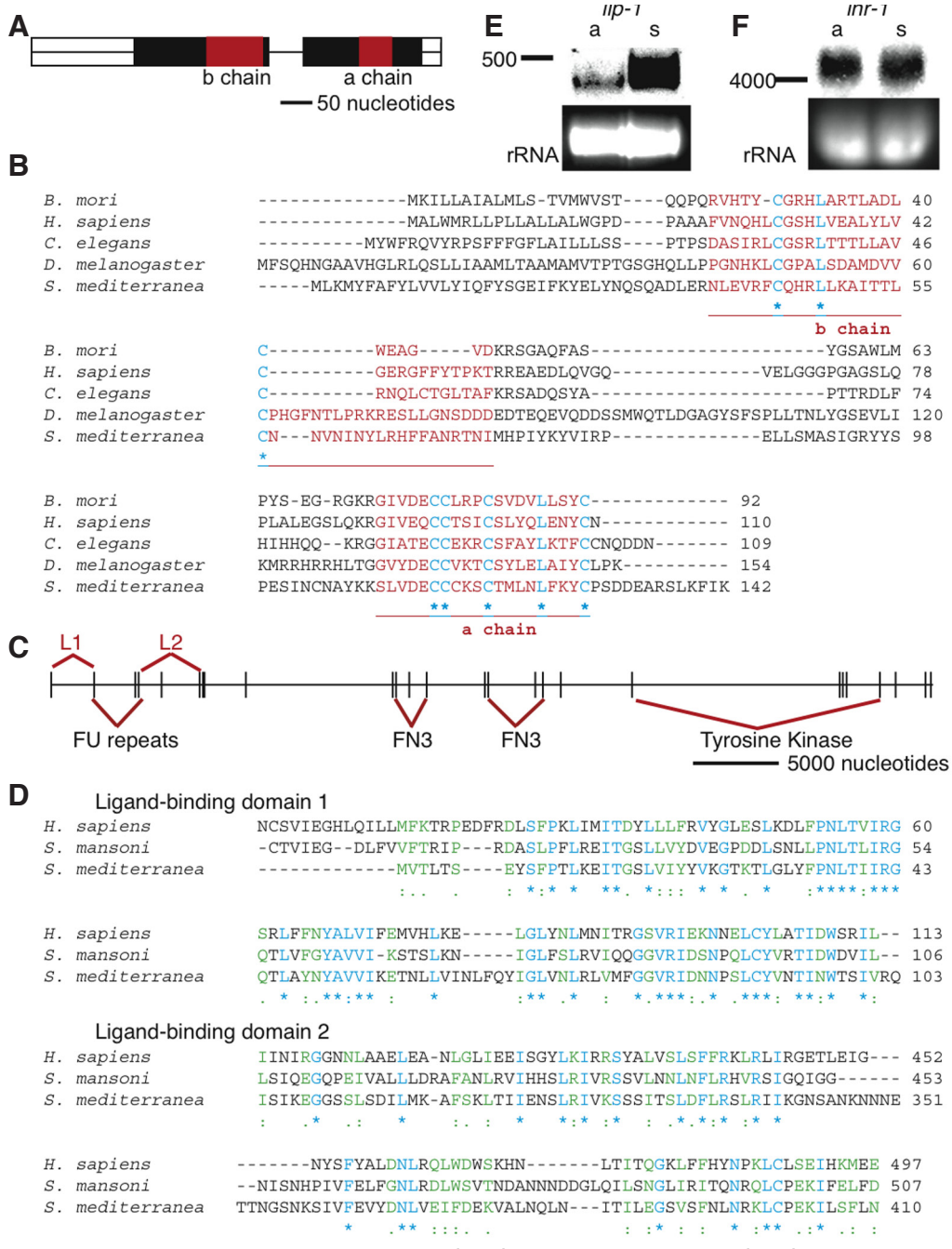

Fig. 1. Genes encoding an insulin-like peptide *(ilp-1)* and an insulin receptor *(inr-1)* are present in *S. mediterranea*. **(A)** *Map of the* ilp-1 *gene sequence including untranslated regions (hollow boxes), an intron (line) and translated regions (shaded boxes) with the conserved b and a chain 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.*

neoblast or germline function have been identified (Collins *et al.*, 2010; Guo *et al.*, 2006; Reddien *et al.*, 2005a,b; Rouhana *et al.*, 2010; Salvetti *et al.*, 2005; Wang *et al.*, 2007; Wang *et al.*, 2010), 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.

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. 1E), while *inr-1* mRNA is approximately 4000 nucleotides (Fig. 1F). 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. 1E). 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. 1F) 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 testes lobes (Fig. 2A); the abundance of signal in more luminal regions

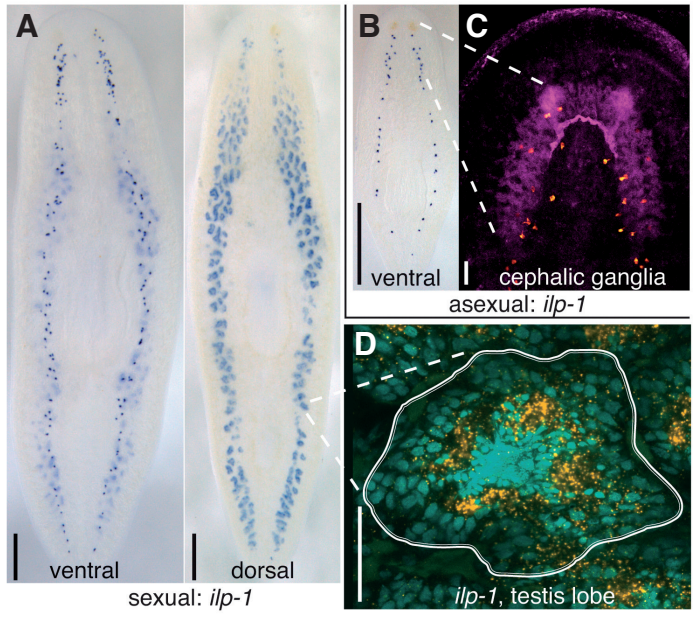

Fig. 2. Expression patterns of *ilp-1 and* inr-1 *visualized by whole-mount* in situ *hybridization.* (A) *Ventral view of a sexual planarian shows* ilp-1 *expression in the cephalic ganglia and nerve cords; dorsal view reveals* ilp-1 *expression in testis lobes.* **(B)** ilp-1 *expression in an asexual 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: 0.5 mm (black; A,B) or 50 μm (white; C,D).*

(Fig. 2D) 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. *inr-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. 3A). 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 labeled specifically with anti-phosphohistone H3 (Newmark and Sánchez Alvarado, 2000). 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. 3C; $p<0.01$). 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). We did not detect a significant difference in *smedwi-1* or *smedwi-2* relative expression levels (normalized to ubiquitously-expressed *b-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. 3C). 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 proliferation 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. 3C; $p<0.01$). *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. 3C; $p<0.05$).

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.

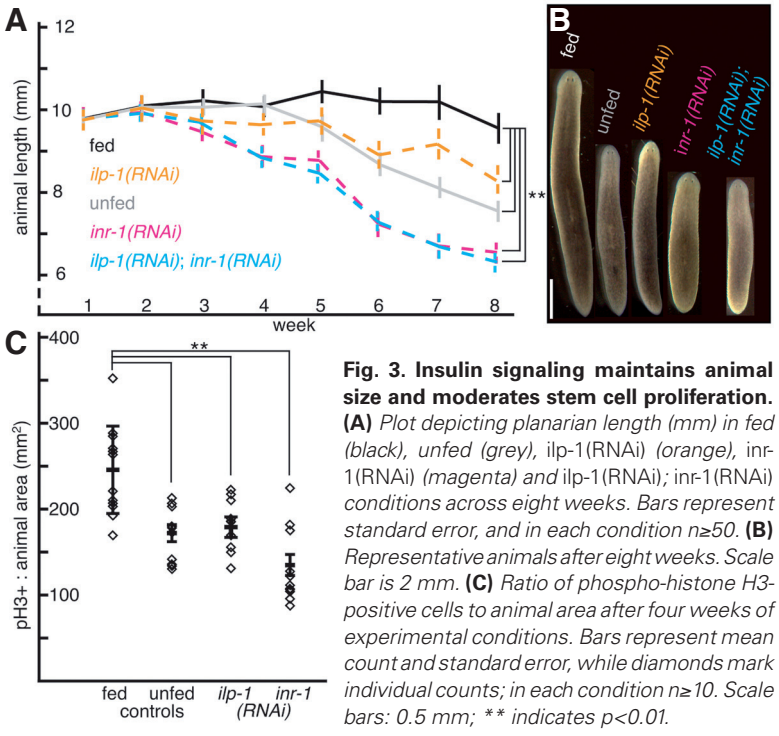

Fig. 3. Insulin signaling maintains animal size and moderates stem cell proliferation. **(A)** *Plot depicting planarian length (mm) in fed (black), unfed (grey),* ilp-1(RNAi) *(orange),* inr-1(RNAi) *(magenta) and* ilp-1(RNAi)*;* inr-1(RNAi) *conditions across eight weeks. Bars represent standard error, 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: 0.5 mm; ** indicates p<0.01.*

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

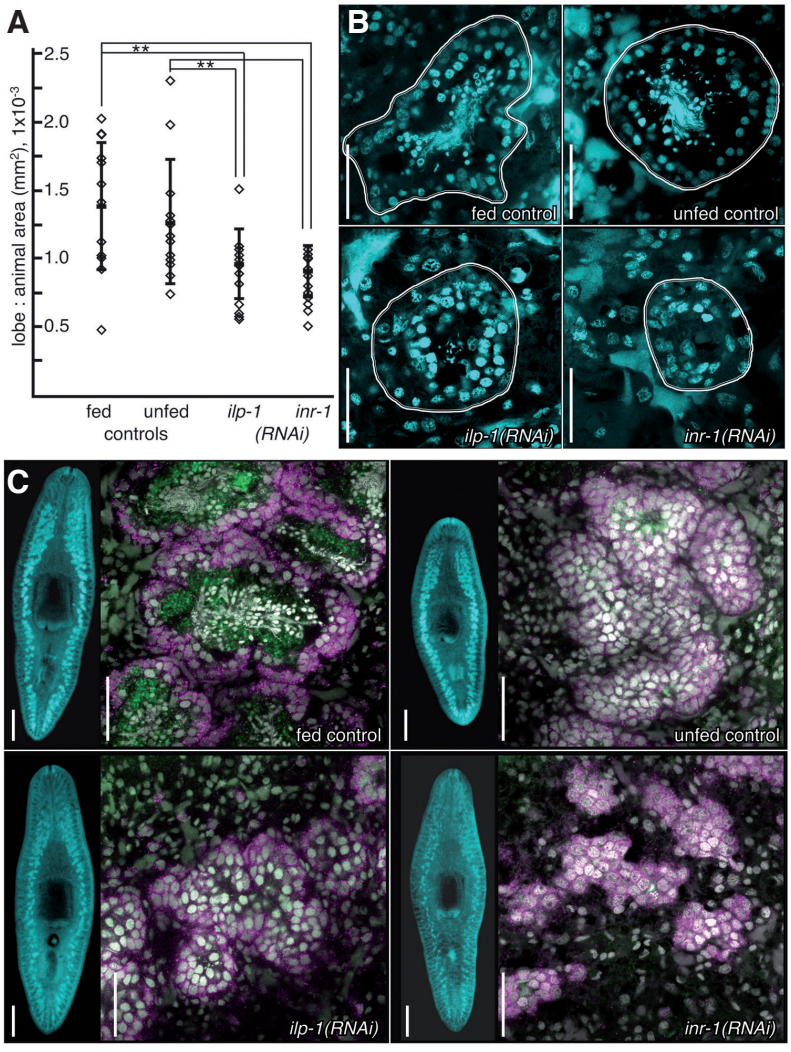

Fig. 4. Insulin signaling maintains size of testis lobes and supports spermatogenesis. (A) *Plot depicting the ratio of testis lobe area to animal area in* S. mediterranea *after RNAi for four weeks. Bars represent mean size and standard error, while diamonds mark the average area of five lobes in one 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 μm.* **(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: 50 μm for testis lobes (B,C) and 0.5 mm for whole animals (C); * indicates p<0.05.*

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. 4C; 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. 4C; 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. 5 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. 4C). Thus, insulin signaling is not necessary for the maintenance, per se, of spermatogonia in testes 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.

Discussion

In *S. mediterranea*, neuroendocrine *ilp-1* is 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,b; 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 moderates not only 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 a testis-derived insulin-like peptide, 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.

Materials and 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. The primers used were:

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 were:
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 visualization utilized digoxigenin-labeled riboprobes 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, pH 9.5; 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 2x10 minutes, 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 *b-tubulin* (PL05008A2H07) as a normalization control. Statistical analysis was performed using a Student's *t*-test on ΔCT values. The primers used were:

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

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References

BAGUÑÁ, J. and ROMERO, R. (1981). Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians Dugesia mediterranea and Dugesia tigrina *Hydrobiologia* 84: 181-194.

BAGUÑÁ, J., SALO, E. and AULADELL, C. (1989). Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. *Development* 107: 77-86.

BENDTSEN, J., NIELSON, H., VON HEIJNE, G. and BRUNAK, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340: 783-795

BRØNDSTED, H. (1969). Planarian Regeneration. *Pergamon, London.*

CEBRIÀ, F. and NEWMARK, P. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* 132: 3691-3703.

CHAN, S. and STEINER, D. (2000). Insulin through the ages: Phylogeny of a growth

promoting and metabolic regulatory hormone. *Am Zool* 40: 213-222.

CHEN, C., JACK, J. and GAROFALO, R. (1996). The *Drosophila* insulin receptor is required for normal growth. *Endocrinology* 137: 846-856.

COLLINS, J.R., HOU, X., ROMANOVA, E., LAMBRUS, B., MILLER, C., SABERI, A., SWEEDLER, J. and NEWMARK, P. (2010). Genome-wide analyses reveal a role for peptide hormones in planarian germline development. *PLoS Biol* 8: e1000509.

DAFTARY, S. and GORE, A. (2005). IGF-1 in the brain as a regulator of reproductive neuroendocrine function. *Exp Biol Med* 230: 292-306.

DE MEYTS, P. (2004). Insulin and its receptor: structure, function and evolution. *BioEssays* 26: 1351-1362.

DRUMMOND-BARBOSA, D. and SPRADLING, A.C. (2001). Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. *Dev Biol* 231: 265-278.

EISENHOFFER, G., KANG, H. and SÁNCHEZ ALVARADO, A. (2008). Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian Schmidtea mediterranea. *Cell Stem Cell* 3: 327-339.

GUO, T., PETERS, A. and NEWMARK, P. (2006). A Bruno-like gene is required for stem cell maintenance in planarians. *Dev Cell* 11: 159-169.

HAYASHI, T., ASAMI, M., HIGUCHI, S., SHIBATA, N. and AGATA, K. (2006). Isolation of planarian X-ray-sensitive stem cells by fluorescence-activated cell sorting. *Dev Growth Differ* 48: 371-380.

HSU, H.-J. and DRUMMOND-BARBOSA, D. (2009). Insulin levels control female germline stem cell maintenance via the niche in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 106: 1117-1121.

IKEYA, T., GALIC, M., BELAWAT, P., NAIRZ, K. and HAFEN, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr Biol* 12: 1293-1300.

IVELL, R., HARTUNG, S. and ANAND-IVELL, R. (2005). Insulin-like factor 3: Where are we now? *Ann N Y Acad Sci* 1041: 486-496.

LAFEVER, L. and DRUMMOND-BARBOSA, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in *Drosophila*. *Science* 309: 1071-1073.

LETUNIC, I., DOERKS, T. and BORK, P. (2009). SMART 6: recent updates and new developments. *Nucleic Acids Res* 37: 229-232.

MAKI, R. (2010). Small is beautiful: Insulin-like growth factors and their role in growth, development, and cancer. *J Clin Oncol* 28: 4985-4995.

MCLEOD, C., WANG, L., WONG, C. and JONES, D. (2010). Stem cell dynamics in response to nutrient availability. *Curr Biol* 20: 2100-2105.

MICHAELSON, D., KORTA, D., CAPUA, Y. and HUBBARD, E. (2010). Insulin signaling promotes germline proliferation in *C. elegans*. *Development* 137: 671-680.

MORGAN, T. (1902). Growth and regeneration in *Planaria lugubris*. *Arch Ent Mech Org* 13: 179-212.

NEWMARK, P., REDDIEN, P., CEBRIÀ, F. and SÁNCHEZ ALVARADO, A. (2003). Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proc. Natl. Acad. Sci. USA* 100: 11861-11865.

NEWMARK, P. and SÁNCHEZ ALVARADO, A. (2002). Not your father's planarian: A classic model enters the era of functional genomics. *Nat Rev Genet* 3: 210-219.

OVIEDO, N., NEWMARK, P. and SÁNCHEZ ALVARADO, A. (2003). Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea Mediterranea*. *Dev Dyn* 226: 326-333.

OVIEDO, N., PEARSON, B., LEVIN, M. and SÁNCHEZ ALVARADO, A. (2008). Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *Disease Mod. Mech.* 1: 131-143.

PEARSON, B., EISENHOFFER, G., GURLEY, K., RINK, J., MILLER, D. and SÁNCHEZ ALVARADO, A. (2009). Formaldehyde-based whole-mount *in situ* hybridization method for planarians. *Dev Dyn* 238: 443-450.

PELLETTIERI, J., FITZGERALD, P., WATANABE, S., MANCUSO, J., GREEN, D. and SÁNCHEZ ALVARADO, A. (2010). Cell death and tissue remodeling in planarian regeneration *Dev Biol* 338: 76-85.

PELLETTIERI, J. and SÁNCHEZ ALVARADO, A. (2007). Cell Turnover and Adult Tissue Homeostasis: From Humans to Planarians. *Annu Rev Genet* 41: 83-105.

REDDIEN, P., BERMANGE, A., MURFITT, K., JENNINGS, J. and SÁNCHEZ ALVARADO, A. (2005a). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev Cell* 8: 635-649.

REDDIEN, P., OVIEDO, N., JENNINGS, J., JENKIN, J. and SÁNCHEZ ALVARADO, A. (2005b). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310: 1327-1330.

ROBB, S., ROSS, E. and SÁNCHEZ ALVARADO, A. (2007). SmedGD: the *Schmidtea mediterranea* genome database. *Nucleic Acids Res* 36: D599-D606.

ROMERO, R. and BAGUÑÁ, J. (1991). Quantitative cellular analysis of growth and reproduction in freshwater planarians (Turbellaria; Tricladida). I. A cellular description of the intact organism. *Invertebr Reprod Dev* 19: 157-165.

ROUHANA, L., SHIBATA, N., NISHIMURA, O. and AGATA, K. (2010). Different requirements for conserved post-transcriptional regulators in planarian regeneration and stem cell maintenance. *Dev Biol* 341: 429-43.

SALVETTI, A., ROSSI, L., LENA, A., BATISTONI, R., DERI, P., RAINALDI, G., LOCCI, M., EVANGELISTA, M. and GREMIGNI, V. (2005). DjPum, a homologue of *Drosophila* Pumilio, is essential to planarian stem cell maintenance. *Development* 132: 1863-1874.

SAMANI, A., YAKAR, S., LEROITH, D. and BRODT, P. (2007). The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* 28: 20-47.

SAMBROOK, J. and RUSSELL, D. (2001). Molecular Cloning. Molecular cloning: a laboratory manual. Irwin N, Janssen K. A, editors. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

SCHULTZ, J., MILPETZ, F., BORK, P. and PONTING, C. (1998). SMART, a simple modular architecture research tool: Identification of signaling domains *Proc. Natl. Acad. Sci. USA* 95: 5857-5864.

SMIT, A., VAN KESTEREN, R., LI, K., VAN MINNEN, J., SPIJKER, S., VAN HEERIKHUIZEN, H. and GERAERTS, W. (1998). Towards understanding the role of insulin in the brain: lessons from insulin-related signaling systems in the invertebrate brain. *Prog Neurobiol* 54: 35-54.

TATAR, M., BARTKE, A. and ANTEBI, A. (2003). The endocrine regulation of aging by insulin-like signals. *Science* 299: 1346-1351.

TIMMONS, L., COURT, D. and FIRE, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* 263: 103-112.

UEISHI, S., SHIMIZU, H. and INOUE, Y. (2009). Male germline stem cell division and spermatocyte growth require insulin signaling in *Drosophila*. *Cell Struct Funct* 34: 61-69.

WAGNER, D., WANG, I. and REDDIEN, P. (2011). Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. *Science* 332: 811-816.

WANG, Y., STARY, J., WILHELM, J. and NEWMARK, P. (2010). A functional genomic screen in planarians identifies novel regulators of germ cell development. *Genes Dev* 24: 2081-2092.

WANG, Y., ZAYAS, R., GUO, T. and NEWMARK, P. (2007). *nanos* function is essential for development and regeneration of planarian germ cells. *Proc. Natl. Acad. Sci. USA* 104: 5901-5906.

WENEMOSER, D. and REDDIEN, P. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. *Dev Biol* 344: 979-991.

WU, Q. and BROWN, M. (2006). Signaling and function of insulin-like peptides in insects. *Annu Rev Entomol* 51: 1-24.

ZAYAS, R., HERNANDEZ, A., HABERMANN, B., WANG, Y., STARY, J. and NEWMARK, P. (2005). The planarian *Schimdtea mediterranea* as a model for epigenetic germ cell specification: Analysis of ESTs from the hermaphroditic strain. *Proc. Natl. Acad. Sci. USA* 102: 18491-18496.

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