

1 **A nuclear hormone receptor and lipid metabolism axis are required for the**
2 **maintenance and regeneration of reproductive organs**

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31

32 **ABSTRACT**

33

34 Understanding how stem cells and their progeny maintain and regenerate reproductive organs
35 is of fundamental importance. The freshwater planarian *Schmidtea mediterranea* provides an
36 attractive system to study these processes because its hermaphroditic reproductive system
37 (RS) arises post-embryonically and when lost can be fully and functionally regenerated from
38 the proliferation and regulation of experimentally accessible stem and progenitor cells. By
39 controlling the function of a nuclear hormone receptor gene (*nhr-1*), we established conditions
40 in which to study the formation, maintenance and regeneration of both germline and somatic
41 tissues of the planarian RS. We found that *nhr-1(RNAi)* not only resulted in the gradual
42 degeneration and complete loss of the adult hermaphroditic RS, but also in the significant
43 downregulation of a large cohort of genes associated with lipid metabolism. One of these,
44 *Smed-acs-1*, a homologue of Acyl-CoA synthetase, was indispensable for the development,
45 maintenance and regeneration of the RS, but not for the homeostasis or regeneration of other
46 somatic tissues. Remarkably, supplementing *nhr-1(RNAi)* animals with either bacterial Acyl-
47 CoA synthetase or the lipid metabolite Acetyl-CoA rescued the phenotype restoring the
48 maintenance and function of the hermaphroditic RS. Our findings uncovered a likely
49 evolutionarily conserved role for nuclear hormone receptors and lipid metabolism in the
50 regulation of stem and progenitor cells required for the long-term maintenance and
51 regeneration of animal reproductive organs, tissues and cells.

52

53 INTRODUCTION

54

55 The adult organs of most organisms are actively maintained by a complex interplay of cellular
56 and metabolic homeostatic processes that include hormonal regulation, the maintenance of
57 stem cell pools, removal of degenerated cells and the generation and functional integration of
58 new cells through proliferation and differentiation (Guo and Cantley, 2010; Knapp and Tanaka,
59 2012; Rock and Hogan, 2011; Tu et al., 2016). This plasticity is observed in organs such as
60 lung, muscle, skin, heart, and liver, but it is most dramatically manifested in the reproductive
61 system (RS) of many animals. In humans, many organs of the RS are highly plastic in
62 adulthood and require cyclical regeneration and extensive remodeling (Nair and Taylor, 2010).
63 For instance, the human endometrium undergoes growth, differentiation and shedding during
64 every menstrual cycle, a homeostatic process that requires the coordination of proliferation
65 and differentiation of epithelial progenitor and mesenchymal/stromal stem cells with estrogen
66 and progesterone fluctuations during the estrus cycle (Gargett et al., 2012).

67

68 Although it is well-established that nuclear hormone receptors (NHRs) for androgen
69 (Yeh et al., 2002), progesterone (Chappell et al., 1997) and estrogen (Walker and Korach,
70 2004) are essential for RS development and function, precisely how the endocrine system
71 affects the stem cell populations responsible for the maintenance and cyclical regeneration of
72 adult RS organs remains incompletely understood. For example, the precise location and
73 types of both myometrial and endometrial stem cells chiefly responsible for the 500- to 1,000-
74 fold increase in volume and 24-fold increase in weight of the human uterus have yet to be fully
75 determined (Kørbling and Estrov, 2003; Ono et al., 2008; Ramsey, 1994; Shynlova et al.,

76 2006). However, the deep evolutionary conservation of endocrine regulation of the RS has
77 allowed research organisms such as fruitflies and nematodes to shed light on some of the
78 roles lipophilic hormones play in the regulation of germ cells, and the embryonic development
79 of reproductive organs (Allen and Spradling, 2008; Asahina et al., 2000; Gissendanner et al.,
80 2004; Gissendanner et al., 2008; Sun and Spradling, 2013).

81

82 Lipid metabolism also plays an evolutionarily conserved role during RS embryonic
83 development and fertility (Barton et al., 2016; Sano et al., 2005). Cholesterol and β -oxidation of
84 fatty acids are essential for meiosis, embryo development, and uterus function in both mice
85 and humans (Downs et al., 2009; Dunning et al., 2010; Mouzat et al., 2013; Seli et al., 2014).
86 In *D. melanogaster*, female feeding behavior, lipid accumulation in the oocyte, and diet nutrient
87 coordination, lipid metabolism and oocyte development are controlled by the Ecdysone
88 receptor (EcR) (Sieber and Spradling, 2015). And in *Caenorhabditis elegans*, fatty acids and
89 their derivatives not only influence reproductive growth and fertilization (Tang and Han, 2017;
90 Wang et al., 2015; Zhu and Han, 2014), but also regulate germ cell fate via Acyl CoA
91 synthetase and its downstream product Myristoyl-CoA (Tang and Han, 2017).

92

93 The fundamental importance of lipophilic hormones and their receptors on sexual
94 reproduction is underscored by the effect metabolism has on endocrine functions. When
95 systemic metabolism fails due to either disease or aging (Conboy and Rando, 2005; Lopez-
96 Otin et al., 2016), both germ and somatic cells in the RS gradually degenerate in both men and
97 women (Makabe et al., 1998; Motta et al., 2002; Paniagua et al., 1991). Changes in fat and
98 energy storage in the body have been shown to affect the reproductive activity of many

99 vertebrates (Ballinger, 1977; Eliassen and Vahl, 1982; Reznick and Braun, 1987). Similarly,
100 the adult females of *D. melanogaster* can remodel their midgut to adjust the energy balance
101 between the body and the RS after mating (Reiff et al., 2015) and in *C. elegans* dietary
102 restriction has been shown to delay aging related degeneration of the egg-laying apparatus
103 (Pickett and Kornfeld, 2013). Conversely, age-related and disease-induced changes in RS
104 metabolism also feed back to the metabolic homeostasis of the body. For example,
105 menopause alters lipid metabolism in adipose tissue causing atopic lipid accumulation in liver,
106 macrophages, and the cardiovascular system (Della Torre et al., 2014); and diseases of the
107 RS, such as polycystic ovary syndrome, are often associated with metabolic ailments such as
108 nonalcoholic fatty liver disease (Della Torre et al., 2014).

109

110 While the evolutionarily conserved roles for NHR and lipid metabolism in RS embryonic
111 development and germ stem cell functions are well established, their precise role in regulating
112 somatic stem and progenitor cell functions during adult RS maintenance and regeneration
113 remains an open question. The recent discovery that the nuclear hormone receptor *nhr-1* is
114 required for the post-embryonic development of the hermaphroditic RS in the freshwater
115 planarian *Schmidtea mediterranea* (Tharp et al., 2014), has made this organism an attractive
116 system to study the roles that NHRs and lipid metabolism may play in the plasticity of the RS.
117 Unlike other invertebrate research organisms (*i.e.*, fruitflies and nematodes), planarians are
118 devoid of both gonads and attendant somatic organs and tissues of the RS upon birth. As
119 animals grow, the entire RS, including the germline, arises from the proliferation and
120 differentiation of adult stem cells known as neoblasts (Newmark et al., 2008). Once established,
121 the RS may be subjected to changes related to either injury and/or nutritional intake. If an

122 animal is amputated, the resulting fragments resorb their RS as they regenerate the missing
123 body parts. A similar resorption of the RS is also observed when the animals are subjected to
124 starvation. In both cases, once food becomes available, the animals regenerate a fully
125 functional RS (Guo et al., 2016; Newmark and Sanchez Alvarado, 2002).

126

127 Numerous evolutionarily conserved genes and pathways have also been shown to
128 regulate RS development, homeostasis and regeneration in *S. mediterranea* (Chong et al.,
129 2013; Guo et al., 2016; Iyer et al., 2016a; Newmark and Sanchez Alvarado, 2002; Newmark et
130 al., 2008). For instance, *Dmd1*, one the most conserved genes in male sex determination
131 across bilaterians, is essential for the maintenance of the planarian male RS, including germ
132 stem cell specification, and differentiation (Chong et al., 2013). *nanos*, the nuclear factor Y-B
133 (*NF-YB*), *Smed-boule2* and several azoospermia (DAZ) family members are also required for
134 germ stem cell maintenance (Iyer et al., 2016a; Iyer et al., 2016b; Wang et al., 2010; Wang et
135 al., 2007). And *Smed-boule1*, homologs of vertebrate DAZ-associated proteins, synaptonemal
136 complex protein 1 (SYCP1), *Smed-CPEB*, *Smed-eIF4E-like* and t-complex proteins have all
137 been shown to be essential for meiosis in testis (Iyer et al., 2016b; Rouhana et al., 2017; Xiang
138 et al., 2014). Moreover, the size of testes and sperm maturation are determined by the
139 neuropeptide, NPY-8, and its upstream prohormone convertase, *PC2* (Collins et al., 2010),
140 and depletion of insulin-like peptide or insulin receptor have been shown to affect
141 spermatogenesis (Miller and Newmark, 2012). Therefore, *S. mediterranea*, an animal in which
142 the molecular and cellular interactions underpinning the maintenance and regeneration of the
143 RS can be mechanistically dissected, provides unique opportunities to advance our
144 understanding of reproductive biology.

145

146 Here, we exploit the biology, evolutionary conservation of regulatory networks and the
147 functional genomic tools available in *S. mediterranea* to uncover molecular mechanisms
148 driving the maintenance and regeneration of the RS. We carried out a discrete RNA mediated
149 genetic interference (RNAi) screen of genes found to be preferentially expressed in sexually
150 mature animals, and found new specific functions in the maintenance and regeneration of the
151 planarian hermaphroditic RS for the previously identified NHR *nhr-1* (Tharp et al., 2014). We
152 report that *nhr-1(RNAi)* not only resulted in the gradual degeneration and complete loss of the
153 hermaphroditic RS, but also in the significant downregulation of a cohort of novel reproductive
154 accessory gland markers and a large set of genes associated with lipid metabolism. We
155 discovered that the planarian gene *Smed-accs-1*, a homologue of Acyl-CoA synthetase (ACS),
156 is indispensable for the development, maintenance and regeneration of RS organs and
157 tissues, but not for the homeostasis or regeneration of other somatic tissues. Remarkably,
158 supplementing *nhr-1(RNAi)* animals with either bacterial ACS or the lipid metabolite Acetyl-
159 CoA rescued the phenotype restoring the maintenance and function of the hermaphroditic RS.
160 Our findings not only demonstrate that the planarian RS depends on an NHR/lipid metabolism
161 axis for its maintenance and regeneration, but also may have uncovered a conserved
162 molecular mechanism regulating reproductive capacity in long-lived organisms.

163

164 **RESULTS**

165

166 ***nhr-1* is required for the homeostatic maintenance of adult sexual reproductive organs**

167

168 We sought to identify genes important for the maintenance and regeneration of the RS in *S.*
169 *mediterranea* by comparing the transcriptomes of decapitated sexually mature animals to
170 immature juveniles. 7,154 transcripts (7.89% Figure 1A) were found to be highly expressed in
171 sexually mature animals. We prioritized for study genes with coiled-coil domains or zinc finger
172 domains (n=34), because proteins with these domains are among the most commonly
173 associated with biological functions in meiosis, hormonal response, and RS development in
174 eukaryotic organisms (Lupas and Bassler, 2017; Razin et al., 2012; Truebestein and Leonard,
175 2016). Whole mount *in situ* hybridizations confirmed that 30 genes were expressed in the
176 gonads (88.2%, Figure S1A and S1B), and 10 genes (29.4%, Figure S1A and S1B) were
177 expressed in the accessory reproductive organs. Only 2 genes (5.9% Fig S1A and S1B) did
178 not have detectable expression in the RS. The high levels of expression of genes coding for
179 proteins with coiled-coil or zinc finger domains in RS suggested that these proteins may be
180 important for RS functions.

181

182 Next, we tested the functions of the identified genes in sexually mature animals by
183 RNAi, followed by DAPI and peanut agglutinin (PNA) staining (Figure 1B). Among the genes
184 found to be essential for RS maintenance was the recently described nuclear hormone
185 receptor *nhr-1*, which was shown to be required for the post-embryonic development of the
186 planarian RS (Tharp et al., 2014). *nhr-1* possesses two zinc finger domains of the C4 class
187 near its N terminus (PFAM Bit scores 38.0 and 92.6, respectively). We tested the function of
188 this receptor in the mature RS by subjecting animals to multiple rounds of RNAi treatment
189 (Figure 1B). Initially, 100% of testes had mature spermatids, but after 4 rounds of treatment
190 29.4% of testes (5 out of 17) had lost mature spermatids. By 8 rounds of treatment, spermatids

191 could not be detected in 100% of testes assayed (Figure 1C). The accessory reproductive
192 glands can be detected by PNA staining (Chong et al., 2011) in both dorsal and ventral sides
193 of sexually mature animals. PNA staining showed that the reproductive glands started to
194 degenerate after 4 rounds of RNAi feedings and completely disappeared after 8 rounds of
195 treatment (Figure 1D). Interestingly, while *nhr-1(RNAi)* did not lead to significant changes in
196 the expression of *nanos* in germ stem cells, even after prolonged RNAi feeding (Figure 1C),
197 *nhr-1(RNAi)* worms were sterile, and did not lay any egg capsules (Figure S2). Knockdown of
198 a meiosis specific gene (*sycp1*), or an ovary related gene (*gld*) did not affect fertility (Figure
199 S2). These results indicate that *nhr-1* is essential for the homeostatic maintenance of
200 differentiated germ cells and somatic tissues of the RS.

201

202 ***nhr-1* is required for the progression of normal meiosis in testes**

203

204 We sought to better characterize the observed defect in male meiosis caused by of *nhr-*
205 *1(RNAi)*. We quantified the percentage of male germ cells at different stages of meiosis in the
206 testes of both control and *nhr-1(RNAi)* animals using DAPI and two-photon confocal
207 microscopy. Multiple testes from the same locations of different animals were imaged from
208 dorsal surface of testes to the middle of the testes at an approximate depth of 50 μ m, which
209 was sufficient to consistently distinguish male germ cells at different meiotic stages based on
210 their nuclear morphology (Movie-S1). Knocking down *nhr-1* led to decreased germ cells at
211 pachytene stages and to a reduced number of round spermatids (Movie-S2, Figure S3A). The
212 distribution of spermatocytes at the pachytene stage was also decreased (Figure 2B). Since
213 bouquet disruption changes the distribution of prophase cells (Chretien, 2011), we also

214 checked bouquet formation in the spermatocytes (Figures 2A, 2C and S3B). During male
215 meiosis, the telomeres of all 8 chromosomes cluster at the nuclear envelope in the early
216 leptotene stage to form a bouquet, which is considered essential for the progression of meiosis
217 (Figures 2C and S3B)(Chretien, 2011; Xiang et al., 2014). The bouquets persist until the
218 pachytene stage (Figure 2C). In normal male meiosis, 50% of the spermatocytes at leptotene
219 stage had bouquets (Figure 2C). Almost 100% of the spermatocytes at zygotene or pachytene
220 stages formed bouquets (79.2% in zygotene stage and 94% in pachytene stage; Figure 2C). In
221 *nhr-1 RNAi* animals, even though chromosome condensation at each prophase stages was
222 similar to regular meiosis, the telomeres were scattered around or clustered in several
223 locations of the nuclear envelope (Figure 2C). After 6 rounds of *nhr-1(RNAi)* treatment,
224 spermatocytes at leptotene and zygotene stages had significantly fewer bouquets (33.3% in
225 leptotene stage, 52.8% in zygotene stage and 84.6% in pachytene stage. For leptotene stage,
226 $p=0.0034$; for zygotene stage $p<0.0001$; for pachytene stage, $p=0.172$. Figure 2A). These
227 results indicate that in the absence of *nhr-1* function, bouquet formation is slowed down but not
228 blocked in leptotene and zygotene stages. Altogether, the data suggest that *nhr-1* is required
229 for normal meiosis I progression through mechanisms likely involving either the regulation of
230 normal bouquet formation or the maintenance of bouquets at the nuclear envelope.

231

232 **Expression of known specific marker genes of the RS are affected by *nhr-1(RNAi)***

233

234 *nhr-1* is expressed in most organs of the adult hermaphroditic planarian RS. In order to gain a
235 better understanding of the dynamics of resorption of the RS after *nhr-1(RNAi)*, we
236 investigated the expression of known reproductive organ markers during this process. Most

237 *nhr-1*⁺ cells in both male and female gonads expressed *gh4*, a germ stem cell and
238 spermatogonial cell marker (Saber et al., 2016)(Figure S4B and S4D), but the expression of
239 *nanos* and *nhr-1* did not overlap extensively (Figure S4A and S4C). Given that *nanos* is an
240 evolutionarily conserved marker for germ stem cells (Wang et al., 2007), this result suggested
241 that *nhr-1* is expressed in more differentiated states of the germ stem cells. In fact, after 4
242 rounds of *RNAi* treatment the ovary appeared vacated of cells, including differentiated oocytes,
243 and by the 6th round of *RNAi* feeding the ovaries became undetectable (Figure 2E). Co-
244 localization of *nhr-1* with *eyesabsent (eya)*, *grn* and *tsp-1*(Figure S4E and S4F) showed that
245 *nhr-1* is expressed in oviduct, sperm duct and accessory glands, respectively (Chong et al.,
246 2013; Chong et al., 2011). After 4 rounds of *nhr-1(RNAi)*, *tsp-1* and *grn* showed markedly
247 reduced expression levels while retaining their overall spatial expression patterns, suggesting
248 that *nhr-1* may directly maintain gene expression of *tsp-1* and *grn*. After 6 rounds of *RNAi*
249 feeding, *tsp-1* and *grn* expression became undetectable (Figure 2D). Interestingly, even
250 though the ovaries became undetectable after the 6th round of *RNAi* feeding, the oviduct could
251 still be labeled by the expression of *eya* (Figure 2E). Additionally, the expression of *eya* in the
252 brain region persisted even after 11 rounds of *nhr-1 RNAi* feeding (Figure 2E), suggesting that
253 *nhr-1* likely regulates gene expression specifically in the RS. Altogether, our data indicated that
254 *nhr-1* may specifically regulate gene expression in the RS, with gene expression in different
255 RS cells and tissues responding to *nhr-1* loss with different kinetics.

256

257 ***nhr-1* is specific and essential for the regeneration of all reproductive system**
258 **components**

259

260 To determine whether *nhr-1* may play a role in RS regeneration, we characterized the timing of
261 *nhr-1* expression along with the re-establishment of the accessory reproductive organs in
262 regenerating fragments derived from adult, sexually mature planarians. We followed adult
263 head fragments produced by amputating in the pre-pharyngeal region (Figure 3A, B). These
264 fragments initially retain ovaries and some anterior reproductive organ tissues but are devoid
265 of all RS tissues found in the trunk and tail of the animal after amputation (Figure 3A, B). Head
266 fragments fully regenerate the RS 32 days post amputation (DPA) (Figure 3A). During this
267 process, *nanos*⁺ germ stem cells persist, while differentiated gonad cells disappear soon after
268 amputation (Wang et al., 2007). Consistent with data from decapitated fragments (Table S1),
269 detection levels of *nhr-1* expression became sharply reduced after amputation (Figure 3B).
270 However, its expression became noticeable once again 12 DPA, when a newly regenerated
271 pharynx could be detected by DAPI staining, and *nanos*⁺ cell had already lined up in a pattern
272 indistinguishable from primordial testes (Figure 3A and 3B). By 12 DPA, *nhr-1* expression was
273 detected in the center of the regenerating tail, where the future dorsal glands will regenerate
274 (Figure 3A and 3B). By 22 DPA, *nhr-1*⁺ cells formed a circle in this region (Figure 3A and 3B),
275 and by 28 DPA the marked *nhr-1* expression highlighted the prospective gland structure that
276 eventually became visible by 32 DPA (Figure 3A and 3B). The gland was established after the
277 gonad pore formed, which happened in about half of the tested animals by 32 DPA. It is
278 interesting to note that *nanos*⁺ cells started to differentiate 28 DPA, a time when *nhr-1*
279 expression was abundant at the gland region anlagen. These results suggested that *nhr-1*
280 expression precedes the establishment of the definitive RS tissues during regeneration.

281

282 We proceeded to determine the function of *nhr-1* in RS regeneration by first subjecting
283 sexual adult animals to RNAi for 8 times followed by amputation of heads and tails and further
284 RNAi treatments of these anterior and posterior fragments to chronically sustain *nhr-1* loss of
285 function (Figure 3C, D). Regeneration of the RS in these fragments was examined 68 DPA. In
286 the first week of regeneration, *nhr-1 RNAi* fragments formed blastemas that were
287 indistinguishable from control animals (Figure 3H). While most control animals formed
288 gonopores by 60 DPA, *nhr-1 RNAi* animals had not developed gonopores by 68 DPA (Figure
289 S5A). By 68 DPA, control head fragments had regenerated the full set of mature RS organs,
290 including the copulatory bursa, the bulbar cavity, the penis papilla and testes containing
291 mature spermatids, while the head *nhr-1(RNAi)* fragments lacked all of these structures
292 (Figure 3E and 3F). No testis-like structures could be found by DAPI staining on regenerated
293 *nhr-1(RNAi)* fragments (Figure 3G), and neither testes nor accessory glands could be detected
294 by the molecular markers *tsp-1* and *plastin* (Figure 3G). We also failed to detect sperm ducts
295 and oviducts by either DAPI or molecular markers on the ventral side (Figure S5C).
296 Nevertheless, gut and pharynx regenerated normally in these fragments with *nhr-1* expression
297 depleted, suggesting the function of this NHR is specific to the RS. Next, we examined the
298 regenerated heads from *nhr-1(RNAi)* tail fragments (Figure 3D). Consistently, the *nhr-1(RNAi)*
299 fragments failed to regenerate tissues of the RS including the ovary, oviduct and sperm duct
300 (Figure 3I), but regenerated photoreceptors (Figure 3G) as well as the cephalic ganglia of the
301 central nervous system normally (Figure S5B). We conclude from these data that *nhr-1* is
302 specific and essential for the regeneration of the RS in planarians.

303

304 **Gene expression profiling of *nhr-1(RNAi)* animals revealed both novel genes and**
305 **metabolic regulators of the RS**

306

307 To better understand how *nhr-1* maintains tissue homeostasis and regulates regeneration of
308 the RS, we profiled differential gene expression changes between intact sexual adult worms
309 subjected to *nhr-1(RNAi)* and *unc-22(RNAi)* controls (Figure 4A). As knocking down of *nhr-1*
310 expression became stronger with increased numbers of RNAi treatments (n=4, 6 and 8), more
311 genes showed reduced expression levels (Figure 4B). The numbers of genes with reduced
312 expression increased from 31 after 4 rounds of feeding to 3,604 after 8 rounds of feeding
313 (Figure 4B). Reported molecular markers of testes, ovaries, and accessory organs were
314 among the genes displaying decreased expression levels after *nhr-1(RNAi)* (n=134, Figure 4C),
315 hence validating the quality of our gene set. Expression of molecular markers of germ stem
316 cells (*i.e.*, *nanos*, *gh4*) were not affected by *nhr-1(RNAi)*, consistent with previous observation
317 that *nhr-1* is required to maintain differentiated states of germ cells and RS somatic structures
318 (Figures 1 and 2). Gene Ontology analyses of the gene sets with downregulated expression
319 after 6 and 8 rounds of RNAi feeding showed an overrepresentation of functions associated
320 with metabolic processes, especially lipid catabolic process (Figure 4D), including the nutrition
321 sensor AMPK (Chantranupong et al., 2015), Acyl-coenzyme A catabolism-related genes (Acyl-
322 coenzyme A thioesterase, Acyl-CoA-binding domain-containing protein, and Acyl-CoA
323 synthetases) (Tillander et al., 2017), the lipid droplet consuming gene (LPA
324 acyltransferase)(Leung, 2001; Pol et al., 2014), the cholesterol synthesis gene 3-hydroxy-3-
325 methylglutaryl-coenzyme A reductase (HMGCR) (Sharpe and Brown, 2013) and the β -
326 oxidation related genes Acetyl-CoA acetyltransferase, Mitochondrial carnitine/acylcarnitine

327 carrier protein, Peroxisomal carnitine O-octanoyltransferase (Indiveri et al., 2011). Taken
328 together, these results indicate that *nhr-1* likely regulates the tissue homeostasis and
329 regeneration of the RS through the activation of genes associated with discrete metabolic
330 pathways.

331

332 ***nhr-1(RNAi)* uncovered novel and specific accessory reproductive organ markers**

333

334 By profiling gene expression changes in *nhr-1(RNAi)* at different times after treatment, we
335 reasoned that genes reduced their expression levels at the earliest time point (*i.e.*, 4 times of
336 feeding) could be upstream regulators of different tissues in the RS, or represent tissues that
337 were affected earliest upon loss of *nhr-1* expression. Interestingly, only 31 genes reduced their
338 expression levels after 4 rounds of *nhr-1 RNAi* feedings (Figure 4B), a time when visible
339 changes in the RS were observed in the testes and reproductive glands (Figure 1 and 2). No
340 gene increased at this time point. Among those decreased genes, the expression patterns in
341 the RS were only known for two genes, *tetraspanin 66e* and *PL04022B1F07*(Rouhana et al.,
342 2017). 23 genes were planarian specific, *i.e.*, devoid of obvious homologs in other species. 6
343 genes were homologous to metabolism-related genes, such as Sodium/potassium-transporting
344 ATPase subunit beta-1, Ectonucleoside triphosphate diphosphohydrolase 5 and ACS
345 (Grevengoed et al., 2014; Lingrel et al., 2003; Read et al., 2009). Importantly, all 31 genes
346 have higher expression levels in sexual adult animals, compared to juvenile animals or the
347 asexual line, CIW4. Notably, expression of 30 genes decreased in the first week after
348 amputation (Figure S6C and Table S2). Thus, we hypothesize that this cohort of 31 genes are
349 likely to include specific regulators of different tissue types in the RS.

350

351 We successfully cloned the 30 genes identified and examined their expression patterns
352 by whole mount *in situ* hybridization (WISH) (Figure S6A). Consistent with previous
353 observation with PNA staining that the reproductive glands were affected after 4 rounds of *nhr-*
354 *1* RNAi (Figure 1), we found 23 genes expressed in the posterior reproductive glands.
355 Additionally, 7 genes were expressed in the testes, 4 genes were expressed in the ovaries, 2
356 genes were expressed in the yolk glands and 1 gene was expressed in the oviduct (Figure
357 S6A). Hence, testes, ovaries, reproductive glands and yolk glands were among those tissues
358 in the RS that were among the earliest affected by loss of *nhr-1* expression. These results
359 reinforce the function that the NHR encoded by *nhr-1* may play a role in activating downstream
360 genes in a cell-autonomous way.

361

362 **Novel markers help refine the anatomical description of posterior accessory** 363 **reproductive glands**

364

365 Next, we took advantage of the expanded cohort of markers for the reproductive glands
366 uncovered by the transcriptome analyses (n=19) to study the cell type complexity and fine
367 spatial organization of the posterior reproductive glands. We used fluorescent whole mount *in*
368 *situ* hybridization (FISH) to dissect the expression patterns of the markers and their relative
369 spatial relationships. From single-gene and multi-gene FISH experiments, we found that gland
370 cells have different distribution patterns on dorsal and ventral sides of the tails. Most gland
371 cells are distributed as circles surrounding the copulatory apparatus. Different markers labeled
372 different gland cells which showed distinct distribution domains. We found the expression of

373 the novel gland markers in both dorsal and ventral posterior regions. Based on their
374 expression patterns, we defined dorsal and ventral gland areas and subdivided each of these
375 into 5 spatially-defined regions (Figure 5A and K).

376

377 The 19 gland markers subdivided the dorsal gland area into 5 discrete regions which
378 allowed us to name the novel genes after their detected spatial location. Region 1 was defined
379 by the *dorsal inner gland 1 and 2* genes (*dig-1* and *dig-2*) and *tetraspanin-66e*, which were
380 expressed in the dorsal regions lateral to the bursa canal (Figure 5B and Figure S7A). *dig-1*
381 co-localized with *tetraspanin-66e* in the exterior part of the *tetraspanin-66e*⁺ domain (Figure
382 5F), while *dig-2* co-localized with *tetraspanin-66e* in the anterior part of the *tetraspanin-66e*⁺
383 domain (Figure S7A). Region 2 was defined by the *dorsal middle gland* genes 1 through 10
384 (*dmg-1* to *dmg-10*) and *PL04022B1F07*, all of which were expressed in more exterior circles
385 relative to *dig-1* and *-2* and *tetraspanin-66e*. Gland cells in Region 2 marked by these genes
386 were distributed around the testes, dorsal and ventral to the bursa canal (Figure 5A). *dmg-1*
387 and *PL04022B1F07* were co-expressed in the same gland cells, while *dmg-2* was expressed
388 in a broader domain (Figure 5D and 5H). *dmg-3* was expressed in a smaller, distinct domain
389 that does not overlap with *PL04022B1F07* expression (Figure 5E and 5I). *dmg-4* and *dmg-5*
390 were expressed in the same cells, which were different from those cells expressing *dmg-6*
391 (Figure S7B and S7F). *dmg-7* and *dmg-8* were co-expressed in the same cells (Figure S7C and
392 S7G). Region 3 was defined by the *dorsal posterior gland 1* gene (*dpg-1*), which was
393 expressed in a small number of gland cells posterior to the bursa canal (Figure 5A, 5C, 5G and
394 Figure S6A). Region 4 was determined by the *interstitial gland* genes (*igg-1*, *-2* and *-3*), which
395 were robustly expressed in a very broad domain wrapping around the posterior testes (Figure

396 5A). *igg-1* showed partial colocalization with *dmg-3*, but not *PL04022B1F07* (Figure 5E and 5I),
397 while *igg-2* labeled the more exterior part of the *igg-3* domain (Figure S7D and S7H). Region 5
398 was specified by one gene which we named *tsp-1-like* (i.e., *tsp-1-l*) as it is expressed in similar
399 domains as *tsp-1* (Figure S6A). Interestingly, neither *dpg-1*, nor *igg-1* overlapped with *tsp-1*,
400 which was widely expressed in the dorsal reproductive gland (Figure 5C and 5G, Figure S7E).

401

402 The anatomical distribution of the reproductive glands in the ventral side of the worm
403 could likewise be divided into 5 regions according to the spatial expression pattern of the
404 above described genes. Ventral Region 1 was defined by *dig-1*, *tetraspanin-66e* and *dmg-3*
405 which were expressed in the gland next to the penis papilla, including 1-2 layers of cells
406 located in front of this gland (Figure 5K, L and S7K), with *dig-1* expressed in the exterior part of
407 *tetraspanin-66e*⁺ cells (Figure 5P). Ventral Region 2 was characterized by the expression
408 patterns of *dmg-1*, *PL04022B1F07*, *dmg-2* and *igg-2* in the gland close to the end of the
409 seminal vesicles (Figure 5N and S7I). Similar to their expression in the dorsal side, *dmg-1* and
410 *PL04022B1F07* were expressed in the same gland cells, while *dmg-2* was expressed in more
411 peripheral cells (Figure 5N and 5R). Ventral Region 3 was defined by 7 transcripts expressed
412 around the copulatory apparatus (Figure 5K, O and S7J). Unlike the dorsal side, *dmg-4* and
413 *dmg-6* were likely expressed in the same gland cells, while *dmg-5* was expressed in different
414 cells (Figure 5O, S). *dmg-7* and *dmg-8* were likely expressed in the same gland cells (S7J, M).
415 The fourth ventral or cement gland region was defined by *dig-2*, *igg-1* and *tsp-1-l* expression
416 (Figure 5K and 5M). We found that the majority of the *dig-2*⁺ cells did not express *igg-1* (Figure
417 5Q), and that *igg-1* was not expressed in *tsp-1*⁺ cells (Figure S7L). *igg-3* and *dpg-1* were not
418 detected in the ventral region (Figure S7I for *igg-3*).

419

420 Consistent with the transcriptome analyses (Figure 4B), expression in adult animals of
421 these 30 transcripts decreased after four *nhr-1(RNAi)* treatments (Fig 5J, T, U and S8). The
422 expression of *dig-1* in the gland region decreased, while its expression on the testes did not
423 show appreciable changes (Fig 5J, T). This suggested that *dig-1* expression in the gland is
424 likely to be specifically regulated by *nhr-1*. *dig-11*, *dig-2*, *tetraspanin-66e*, *dmg-6*, *dmg-8*, *igg-2*
425 and *igg-3* also changed their expression patterns after *nhr-1(RNAi)* (Figures 5J, T, U, and S8A,
426 E, F and C). In the dorsal regions, expression of both *tetraspanin-66e* and *dig-2* extended to
427 the dorsal middle region (Fig S8A), while *dmg-8*, *igg-2* and *igg-3* expanded their expression to
428 the inner region (Figure 5J and S8C). In the ventral side, *dig-1* and *tetraspanin-66e* extend to
429 the region around the end of seminal vesicles (Fig 5T), while the expression of *dmg-6* and
430 *dmg-8* were now detected in the central region (Figure 5U and S8F). These data suggest that
431 *nhr-1* is not only essential for the development and regeneration of the RS, but also for
432 regulating anatomical expression domains of the accessory reproductive organs.

433

434 ***Smed-acs-1* is essential for development, maintenance and regeneration of the RS**

435

436 To determine the functions of the 30 genes responding earliest to loss of *nhr-1* expression, we
437 carried out RNAi screening in sexual adults and examined for defects in the RS. Of these, we
438 focused on the planarian homolog of Acyl-CoA synthetase *Smed-acs-1*, as we were struck to
439 find out that its silencing by RNAi phenocopied the defects observed for *nhr-1(RNAi)*. PNA
440 staining showed that both dorsal and ventral glands degenerated after 6 rounds of *acs-1(RNAi)*
441 treatments. After 9 rounds of RNAi, the glands became undetectable (Figure 6A).

442 Degeneration or loss of other tissues in the RS, including the penis papilla, testes, ovaries and
443 oviduct were also evident (Figure 6B). Moreover, the treated worms stopped laying egg
444 capsules after *acs-1(RNAi)* treatments.

445

446 *acs-1* also shared similar expression dynamics and functions with *nhr-1* during
447 development and regeneration of the RS. *acs-1* expression increased during sexual maturation
448 and decreased after amputation (Figure S9A). Remarkably, body growth and somatic tissue
449 regeneration were not influenced by *Smed-acs-1(RNAi)* in hatchlings and regeneration
450 fragments; however, the reproductive accessory glands were not detectable in both conditions
451 (Figure 6C). The penis papilla and gonopore neither developed, nor regenerated in the *Smed-*
452 *acs-1(RNAi)* animals (Figure 6C). Similarly, neither the ovaries nor the oviduct developed or
453 regenerated (Figure 6D). Interestingly, the testes both developed and were regenerated after
454 amputation, but failed to produce mature spermatozoa (Figure 6C and 6D). We conclude from
455 these results that *acs-1*, like *nhr-1*, is essential for both RS development and regeneration.

456

457 The identified planarian *acs-1* is a homolog of ACS, which is known to be important in
458 fatty acid metabolism (Ellis et al., 2010). *acs-1* codes for a protein containing an AMP-
459 forming/AMP-acid ligase II domain at its N-terminus and phosphopantetheine attachment sites
460 at its C-terminus. The deduced *Smed-ACS-1* protein sequence in sexual and asexual
461 planarian share 99.0% identity, yet when compared to sexual animals, the expression of
462 *Smed-acs-1* is higher in sexual than in asexual planarians (Figure S9A). Unlike *nhr-1*, *acs-1*
463 expression is restricted to comparatively smaller RS domains. *acs-1* is expressed around the
464 testes region of the planarian dorsal side (Figure S9B and S9C) and in the ovary and yolk

465 gland cells on the ventral side (Figure S9B and S9D). These data indicate that *acs-1* function
466 has been restricted to sexual organs and suggest that *acs-1* may regulate RS functions non-
467 cell autonomously.

468

469 ***nhr-1* regulates reproductive system maintenance through lipid metabolism**

470

471 Given that genes regulating lipid metabolism decreased their expression after *nhr-1(RNAi)*,
472 and that ACS is important for fatty acid uptake, we tested whether *nhr-1* or *acs-1* regulated the
473 RS by modulating lipid metabolism. We first assayed dietary lipid accumulation in sexually
474 mature planarians using Oil Red O staining. Lipid droplets were quantified 7 days after feeding
475 animals with liver (Material and Methods). Well-fed wild type and *unc-22(RNAi)* animals rarely,
476 if at all, accumulated detectable amounts of lipids (Figure 7A, B), suggesting that under normal
477 conditions planarians efficiently uptake and metabolize dietary lipids. In contrast, animals
478 treated with either repeated rounds of *nhr-1(RNAi)* or *acs-1(RNAi)* displayed readily detectable
479 lipid accumulation in the form of lipid droplets (Figure 7A, C), with the most notable
480 accumulation being observed in the areas around the intestine and testes (Figure 7B, D).
481 Interestingly, neither *nhr-1(RNAi)*, nor *acs-1(RNAi)*, had measurable effects on lipid
482 accumulation in asexual worms (Figure S10A). Thus, we conclude from these data that both
483 *nhr-1* and *Smed-acs-1* are essential for lipid accumulation specifically in sexual adult
484 planarians.

485

486 Because Acyl-CoA Synthetase is a critical enzyme for fatty acid activation (Ellis et al.,
487 2010), we attempted to rescue the RS by restoring Acyl-CoA Synthetase or lipid metabolites in

488 the sexual adults fed with *nhr-1(RNAi)* food. Fatty acid can be converted by the Acyl-CoA
489 Synthetase *in vitro* (Knoll et al., 1994). Planarian RNAi food is a homogenate of beef liver and
490 bacteria, which together with free fatty acids from planarian tissues may offer the substrates for
491 fatty acid conversion. After feeding adult worms with *nhr-1(RNAi)* food for 4 weeks, the Acyl-
492 CoA Synthetase from *Pseudomonas* was added to the RNAi feeding schedule for the
493 remaining 6 weeks of the experiment. After 10 weeks of *nhr-1(RNAi)* treatment without Acyl-
494 CoA Synthetase supplementation, adult animals lost their testes and accessory reproductive
495 glands as before (7 out of 8) (Figure 7E and S10B). Remarkably, the animals supplemented
496 with Acyl-CoA Synthetase retained their testes (Figure S10B) and gland cells (Figure S10D)
497 indicating that addition of this enzyme was sufficient to rescue the loss of the RS in *nhr-*
498 *1(RNAi)*-treated animals (n=7 out of 7). Additionally, after long-term *nhr-1(RNAi)* feedings,
499 clusters of germ stem cells, spermatogonia and other meiotic cells were not detectable by
500 either *gh4* or *plastin* staining (Figure 7F and 7G). However, clusters of *gh4*⁺ and *plastin*⁺ cells
501 were restored after *nhr-1(RNAi)* was provided (Figure 7F and 7G), which suggested that Acyl-
502 CoA Synthetase rescued both the proliferation and differentiation of germ stem cells in the
503 absence of *nhr-1* function. To further evaluate this discovery, we asked if the *nhr-1(RNAi)* RS
504 phenotypes could be rescued by the exogenous addition of the metabolite Acetyl-CoA, which
505 is made by Acyl-CoA Synthetase and has been shown to be taken up from the extracellular
506 space by transporters (Pietrocola et al., 2015). We fed or injected Acetyl-CoA to *nhr-1(RNAi)*
507 animals and the results were consistent with Acyl-CoA Synthetase feeding, with 5 out of 8
508 animals displaying rescue of the phenotype as illustrated by the recovery of *tsp-1 expression* in
509 dorsal gland cells and of *plastin* and *gh4* cells in the dorsal testes region (Figure 7E, S10B and
510 S10C). Taken together, increasing an enzyme or exogenously providing a metabolite essential

511 for lipid metabolism was sufficient in both cases to rescue the RS defects caused by *nhr-*
512 *1(RNAi)* in planarians. These results demonstrated that *nhr-1* likely regulates RS maintenance
513 and regeneration via an Acyl-CoA Synthetase-activated lipid uptake process.

514

515 **DISCUSSION**

516

517 We have shown that both male and female RS components of the planarian *S. mediterranea*
518 are actively maintained by the nuclear hormone receptor *nhr-1* and its downstream target *acs-*
519 *1* through lipid metabolic pathways (Figure 7H). In many organisms, including humans, the
520 energetic costs associated with reproduction are considerable and rely in great measure on fat
521 metabolism, the major source of energy in animals (Bronson, 1989; Valencak et al., 2009;
522 Wang et al., 2008). In planarians, *nhr-1* activates lipid metabolic genes in sexually mature
523 animals (Figure 4D), including a gene homologous to Acyl-CoA Synthetase (*Smed-acs-1*).
524 During the reproduction of many animals, fat reserves are mobilized, but reducing or
525 abolishing reproduction increases lipid storage in many species (Corona et al., 2009; Judd et
526 al., 2011). Dietary lipids are converted to Acyl-CoAs by Acyl-CoA Synthetase, a role likely
527 played by *Smed-acs-1* in the RS of planarians, where their β -oxidation yields Acetyl-CoA to
528 fuel both reproduction and the lifelong maintenance of the RS. Without *nhr-1*, the expression of
529 *acs-1* is inhibited. Free lipids fail to be taken up by the RS and accumulate in the body,
530 followed by dramatic hypogonadism and the general resorption of all accessory reproductive
531 glands in planarians (Figure 7H). Our study, therefore, has uncovered a reproductive-
532 endocrine signaling axis causally linked to dietary lipid metabolism.

533

534 **Lipid metabolism plays a critical role in RS maintenance and regeneration**

535

536 The insulin and neuroendocrine pathways have been previously shown to be required for
537 sexual maturation and germ cell differentiation in planarians. In *S. mediterranea*, the insulin
538 pathway is activated by insulin like pheromone (*ilp-1*) through its receptor (*inr-1*), and
539 influences both body size and reproductive organs (Miller and Newmark, 2012). The
540 neuroendocrine pathway involves the neuropeptide NPY-8 and its cognate G protein-coupled
541 receptor NPYR-1, and their knockdown results in the loss of differentiated germ cells and
542 sexual maturity (Saber et al., 2016). Although the insulin and lipid metabolism pathways are
543 known to crosstalk in other species, the lipid metabolic processes regulated by *nhr-1* are likely
544 independent of the insulin pathway. Even though *ilp-1* expression decreased after 6 rounds of
545 *nhr-1(RNAi)* treatment, neither *nhr-1*, nor *Smed-acs-1*, influenced body size even after 12
546 feedings. Similarly, *nhr-1* does not respond to NPY8, suggesting that *nhr-1* is likely not part of
547 the neuropeptide pathway, an observation supported by the fact that NPYR-1 is the receptor of
548 NPY-8. Moreover, neither the insulin, nor the neuropeptide pathway-related genes altered their
549 expression after *nhr-1(RNAi)*. These data suggest that the lipid metabolism regulated by *nhr-1*
550 is a third and novel pathway that plays a critical role in the reproductive system of *S.*
551 *mediterranea*.

552

553 *nhr-1*, its putative target *acs-1* and the downstream lipid metabolic genes and
554 metabolites may also have conserved roles in other species of the Lophotrochozoa. In
555 planarian *Dugesia ryukyuensis*, yolk gland regeneration is inhibited by excess 17 β -estradiol
556 (Miyashita et al., 2011), which is a lipid catabolite (Wollam and Antebi, 2011) and a key

557 regulator of lipid metabolism (Sieber and Spradling, 2015). Also, the hydrophobic fraction of
558 tissue homogenates from the planarian *Polycelis nigra* is known to induce the sexual state in
559 the asexual *Dugesia gonocephala sensu lato* (Grasso et al., 1975), which suggests that lipid
560 and its metabolites are important for the sexual maturation and reproduction. In *Schistosoma*
561 *mansoni*, fatty acid oxidation and acyl-CoA synthetase are required for egg production (Huang
562 et al., 2012). Both *nhr-1* and *Smed-acs-1* have conserved homologs in other free-living and
563 parasitic platyhelminthes. Our work suggests that antagonists to *nhr-1* and/or *Smed-acs-1* may
564 repress sexual reproduction in the Lophotrochozoa by desexualization and thus may be useful
565 drug targets for parasitic control.

566

567 ***nhr-1* is required for lipid metabolism in the planarian reproductive organs**

568

569 *nhr-1* likely directs the expression of multiple downstream genes. The protein encoded
570 by *nhr-1* has two conserved nuclear hormone receptor DNA binding domains in the N terminal
571 and one ligand binding domain in the C terminal. After *nhr-1(RNAi)*, the majority of affected
572 genes showed significantly downregulated expression suggesting that *nhr-1* mostly activates
573 gene transcription. In fact, the expression of only 31 genes appeared to be significantly
574 affected when defects were first observed during the early stages of *nhr-1(RNAi)* treatment.
575 Most of these genes were predominantly expressed in the dorsal and ventral glands around
576 the copulatory organs (Figure 5). *nhr-1* is a lophotrochozoan-specific nuclear receptor (Tharp
577 et al., 2014), but a homologue to the retinoid-related orphan receptor β (ROR β). Members of
578 the ROR family of receptors such as ROR γ t can be activated by oxysterols, which are
579 cholesterol derivatives produced during lipid metabolism (Soroosh et al., 2014). Given the low

580 levels of *nhr-1* and *acs-1* expression in the asexually reproducing planarians, and that glands
581 are important organs for sterol synthesis (Kurzchalia and Ward, 2003; Niwa and Niwa, 2016;
582 Wollam and Antebi, 2011), we speculate that a likely source of the unknown ligand for *nhr-1*
583 may originate from the accessory gland organs of the RS. Because *nhr-1* expression is
584 dramatically decreased after amputation and reactivated during RS regeneration a future
585 comparison of the metabolome between these two stages may help identify the ligands for *nhr-*
586 *1*.

587

588 **A reproductive-endocrine signaling axis is required for the maintenance and** 589 **regeneration of the RS**

590

591 Our data indicate that *nhr-1* triggers conserved lipid metabolism pathways to maintain
592 the planarian RS. Depletion of *nhr-1* expression during homeostasis decreased the expression
593 levels of lipopolysaccharide and other downstream putative target genes, such as tumour
594 necrosis factor (TNF) α and interleukin (IL), which are known to initiate liver regeneration after
595 partial hepatectomy(Taub, 2004). Additionally, our studies revealed a downregulation of
596 *caveolin* and *HMGCR* gene expression after *nhr-1(RNAi)*, both of which are also involved in
597 lipid metabolism and are essential for liver regeneration (Delgado-Coello et al., 2011;
598 Fernandez et al., 2006; Gazit et al., 2010; Trentalance et al., 1984). Additionally, recent work
599 has showed that intestine regeneration is also be boosted by dietary lipid(Beyaz et al., 2016).
600 Thus, the molecular pathway of RS regeneration, which is triggered by *nhr-1* and regulated by
601 *Smed-acs-1*, may function in different organs. Altogether, we take these data to suggest that
602 the NHR-dependent regeneration of the RS may not be a unique feature of this system and

603 that other organ-endocrine axes may exist that are mediated by other not yet characterized
604 NHRs encoded by the *S. mediterranea* genome.

605
606 ***Smed-acs-1* specifically regulates lipid metabolism in RS**

607
608 ACS can convert fatty acids to different products, which may have different metabolic
609 fates in different tissues (Grevengoed et al., 2014). In vertebrates, long ACS isoform 4
610 (ACSL4), fatty acid transport protein 3 (FATP3), ACS bubblegum 1 (ACSBg1) and ACS
611 bubblegum 2 (ACSBg2) are enriched in the RS, producing different types of Acyl-CoAs
612 (Grevengoed et al., 2014). Though defects in brain were observed in ACSL4 and ACSBg
613 mutants, defects in the RS were not reported in animals lacking a single ACS (Grevengoed et
614 al., 2014), suggesting that different organs may depend on specific metabolites for their normal
615 function. Given the very specific expression patterns of *acs-1* in the adult planarian, it is likely
616 that *acs-1* may be non-cell autonomously regulating the maintenance and regeneration of the
617 planarian RS. Based on gene expression, the most likely tissues endowed with high ACS-1
618 activity are the ends of gut branches, the exterior of the lobes of testes, yolk glands and glands
619 surrounding the ovaries. These cell types may convert dietary lipids from the intestine and
620 transport the Acyl-CoAs to other tissues of the RS. That such transport of metabolites likely
621 exists in planarians is supported by the rescue experiments in which we fed either bacterial
622 ACS or Acetyl-CoA to *nhr-1(RNAi)*-treated animals (Figure 7). In fact, the rescue of the defects
623 in the RS in *nhr-1(RNAi)* planarians by exogenously administered bacterial ACS, suggests that
624 planarian RS may not necessarily rely on a single kind of Acyl-CoAs.

625

626 Because ACS protein and Acetyl-CoA could rescue defects in the RS after *nhr-1(RNAi)*,
627 we suspect that the AMPK and Acetyl-CoA synthesis pathways in both mitochondria and
628 cytosol may be controlled by *acs-1* in the planarian RS. After fatty acid is converted to Acyl-
629 CoAs by ACS in the cytosol, Acyl-CoAs can be transported by mitochondrial
630 carnitine/acylcarnitine carrier protein (SLC25A20) to synthesize Acetyl-CoA to be used by the
631 TCA cycle to generate ATP. Alternatively, Acyl-CoAs can be used directly in the cytosol to
632 produce Acetyl-CoA, which is further used to generate sterols by HMGCR (Pietrocola et al.,
633 2015). After 6 to 8 feedings of *nhr-1(RNAi)*, the expression of key molecules involved in Acetyl-
634 CoA generation and consumption in both mitochondria and cytosol decreased significantly
635 (Figure 4). AMPK expression also decreased after 6 rounds of *nhr-1(RNAi)*. AMPK can be
636 activated by fatty acid and modulate meiosis in the gonads of *Drosophila*, *C. elegans* and mice
637 (Bertoldo et al., 2015; Ellis et al., 2010). Given that *acs-1(RNAi)* phenocopies the RS
638 defects of *nhr-1(RNAi)*, it is likely that AMPK may be a downstream target of *acs-1* to regulate
639 planarian gonad functions.

640 641 **Implications for reproduction, fat metabolism and lifespan**

642
643 In many organisms, increased life span is associated with reduced reproduction, but drastically
644 increased lipid storage and generally improved survival under starvation conditions (Rion and
645 Kawecki, 2007; Tatar et al., 2001). As such, it has been postulated that lipids set aside for
646 reproduction are unavailable to support the maintenance and survival of other somatic tissues,
647 ultimately diminishing the life-span of the organism (Shanley and Kirkwood, 2000). This
648 tradeoff between reproduction and the homeostasis of other somatic functions has led to the

649 notion that individuals with curtailed reproduction survive better and live longer than those with
650 higher reproductive output and vice versa (Partridge et al., 2005; Reznick et al., 2000). Yet,
651 how reproduction, fat metabolism, and life span may or may not be mechanistically intertwined
652 remains poorly understood (Hansen et al., 2013). The findings reported here indicate that
653 planarians may provide a novel biological context in which to unravel this issue.

654

655 Unlike the most actively researched organisms used to study reproduction, lipid metabolism
656 and aging (*i.e.*, flies, nematodes and vertebrates), planarians are negligibly senescent and can
657 lose and regenerate their entire RS seemingly endlessly. When either starved or amputated,
658 the hermaphroditic sexual organs are resorbed likely due to loss of *nhr-1* signaling, only to be
659 restored once food is available, the animals reach an appropriate size (Newmark and Sanchez
660 Alvarado, 2002), and lipid metabolism is once again activated to maintain the RS. In both men
661 and women, the reduction of hormone synthesis and gametes are usually the earliest signs of
662 aging (Gunes et al., 2016; Makabe et al., 1998), with other organs such as the liver and brain
663 decreasing in volume in the elderly (Hedman et al., 2012; Makabe et al., 1998; Motta et al.,
664 2002; Paniagua et al., 1991; Tajiri and Shimizu, 2013). Moreover, the ectopic lipid
665 accumulation observed in normal aged people and premature menopause patients (Knauff et
666 al., 2008; Toth and Tchernof, 2000), is only observed in planarians after the loss of either *nhr-1*
667 or *acs-1*. Thus, decline of fat oxidation and lipid synthesis may be a general cause of aging
668 and resorption of organs. The activation of *nhr-1* orthologs, ACS, or other downstream
669 associated genes that may rebalance lipid metabolism may result in the rejuvenation of
670 organs. As such, planarians present a unique opportunity to both determine whether steroid

671 hormones play a critical role in the interconnection between reproduction, lifespan and fat
672 metabolism, and to help establish how these three processes may be causally connected.

673

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683

684 **Accession Numbers**

685 All RNA-seq datasets have been deposited in GEO: Accession number for *nhr-1(RNAi)* RNA-
686 seq is GSE107756. Accession number for sexual fragment regeneration data is [GSE107869](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107869).

687

688

689

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947 Figure Legends

948
949 **Figure 1:** Functional screen of sexual planarian enriched genes revealed *nhr-1* as an essential
950 gene for reproductive system maintenance.

- 951 (A) Comparison of the juvenile and sexual adult planarian transcriptome (n=3 replicates,
952 each containing 3 planarians. “D0” represents decapitated adult animals. 7.89%
953 transcripts were expressed significantly higher in adult animals (in green, t-test, adj.
954 p<0.05), while 2.58% of transcripts were expressed higher in juvenile animals (in blue, t-
955 test, adj. p<0.05).
- 956 (B) RNAi feeding schedule diagram. Adult planarians were given RNAi by feeding 8 times
957 as indicated and checked 7 days after the last round of feeding.
- 958 (C) *In situ* test of *nhr-1* and *nanos* expression in testes. DAPI staining showed the observed
959 testes morphology at the same anatomical location. Scale bar: 100µm
- 960 (D) Nuclear and gland staining of the *nhr-1(RNAi)* animals. DAPI staining was stronger in
961 spermatids (blue arrowheads), spermatozoa and other testes cells (blue triangles)
962 compared to somatic tissues. Penis papilla was also visible by DAPI staining (blue
963 asterisks). Glands were stained by PNA. Scale bar: 20µm

964
965 **Figure 2:** *nhr-1* is necessary for sexual reproductive system maintenance in adult planarians.

- 966 (A) Nuclear and telomere staining in one nuclear layer in testes. Scale bar:10µm
- 967 (B) Histogram of leptotene, zygotene and pachytene stage spermatocytes distribution in
968 meiosis I cells observed in half testes by whole mount DAPI staining (n=4 for each
969 sample, *p<0.05).
- 970 (C) Representative image showing normal bouquet in control animals (upper row) and failed
971 bouquet (lower row) in *nhr-1(RNAi)* animals. Scale bar: 5µm
- 972 (D) Nuclear and FISH staining for gland and sperm duct in control and *nhr-1(RNAi)* animal
973 tails. Scale bar: 100µm

974 (E) Nuclear and FISH staining for *nhr-1* expression in oviduct (red arrowheads) in control
975 and *nhr-1(RNAi)* animals. Red stars show the brain close to the ovary. Ovaries are in
976 red circles. Scale bar: 20 μ m
977

978 **Figure 3:** *nhr-1* specifically regulates reproductive system regeneration.

979 (A) Head fragment regeneration time course. Red rectangle indicates the imaging region in
980 panel B. Scale bar: 100 μ m
981 (B) DAPI and FISH staining for *nhr-1* and *nanos* in the dorsal gland region of the
982 regenerated tail. Yellow arrowhead shows *nhr-1* expression. Green arrowhead shows
983 expression of *nanos*. Scale bar: 100 μ m
984 (C) Feeding and amputation schedule for testing function of *nhr-1* during regeneration.
985 Adult animals were amputated after 8 rounds of RNAi feeding. 8 days after amputation,
986 the regenerated RNAi animals were fed with RNAi food for 15 more times.
987 (D) Hematoxylin and eosin (H&E) staining of animals regenerated from control *unc-22(RNAi)*
988 and *nhr-1(RNAi)* animals 68 days after amputation. Orange triangle shows the
989 copulatory bursa. Blue arrowhead indicates bulbar cavity. Blue asterisk marks penis
990 papilla. Blue triangle points at testis. Scale bar: 100 μ m
991 (E) Zoom in of the H&E staining in the putative testes region circled in panel D.
992 (F) DAPI and FISH staining for *plastin* and *tsp-1* in dorsal gland region of regenerated tail.
993 Scale bar: 100 μ m
994 (G) Image of regenerated head from *unc-22(RNAi)* control and *nhr-1(RNAi)* tail fragments 4
995 and 7 days post amputation. Scale bar: 400 μ m
996 (H) DAPI and FISH staining for *grn* and *eyesabsent (eya)* in the ventral site of regenerated
997 head. Ovaries are in the red circles. Oviducts are pointed by red arrowheads. Red star
998 shows the brain in close proximity to the ovary. Scale bar: 100 μ m
999

1000 **Figure 4:** *nhr-1* is necessary for reproductive system and lipid metabolism genes expression.

1001 (A) RNAi feeding schedule and sample collection for transcriptome analysis. Both the
1002 control (*unc-22 RNAi*) and test (*nhr-1 RNAi*) animals were collected 2 days after RNAi
1003 feeding 4, 6 and 8 times. 2 days after collection, mRNA was extracted from those
1004 animals and submitted for sequencing (n=4 replicates, each containing 2-4 planarians).
1005 The transcriptome was compared between *unc-22* and *nhr-1 RNAi* animals, which were
1006 withdrawn at the same time.
1007 (B) Venn diagram showing the number of transcripts decreased after *nhr-1 RNAi* feedings
1008 at different time points (t-test, adj. p<0.05).
1009 (C) The fold change of the expression of reproductive system related genes that decreased
1010 after *nhr-1 RNAi*.
1011 (D) The histogram shows the gene ontology (GO) terms with the 10 lowest Benjamini-
1012 Hochberg multiple testing correction (BH) value for the transcripts that decreased after
1013 *nhr-1 RNAi* feeding 6 times. The BH values of those ten terms for the transcripts
1014 decreased after *nhr-1 RNAi* feeding 8 times are shown in blue. The terms relating to
1015 lipid metabolism are shown in red. The terms relating to other metabolic process are
1016 shown in orange.
1017

1018 **Figure 5:** *nhr-1* is essential for novel gland genes expression.

- 1019 (A) The cartoon summarizes the dorsal tail expression pattern of the downstream genes,
1020 which decreased after *nhr-1* RNAi feeding 4 times. The glands around the bursa canal
1021 can be divided into 5 regions, according to those molecular markers. Examples of
1022 genes in each region are shown in panel B to E and further zoomed in single cell layer
1023 in panel F to I.
- 1024 (B) FISH for dorsal expression of *dig-1* and *tetraspanin 66e*, which express in region 1. The
1025 yellow box shows the region for further zoom in in panel F. Scale bar: 100 μ m
- 1026 (C) FISH for dorsal expression of *dpg-1*, which expresses in region 3, and *tsp-1*, which
1027 express in region 5. The yellow box shows the region for further zoom in in panel G.
1028 Scale bar: 100 μ m
- 1029 (D) FISH for dorsal expression of *dmg-1*, *dmg-2* and *PL04022B1F07*, which express in
1030 region 2. The yellow box shows the region for further zoom in in panel H. Scale bar:
1031 100 μ m
- 1032 (E) FISH for dorsal expression of *dmg-3*, *igg-1* and *PL04022B1F07*. The yellow box shows
1033 the region for further zoom in in panel I. Scale bar: 100 μ m
- 1034 (F) FISH for dorsal expression of *dig-1* and *tetraspanin 66e* in single cell layer. Scale bar:
1035 20 μ m
- 1036 (G) FISH for dorsal expression of *dpg-1* and *tsp-1* in single cell layer. Scale bar: 20 μ m
- 1037 (H) FISH for dorsal expression of *dmg-1*, *dmg-2* and *PL04022B1F07* in single cell layer.
1038 Scale bar: 20 μ m
- 1039 (I) FISH for dorsal expression of *dmg-3*, *igg-1* and *PL04022B1F07* in single cell layer.
1040 Scale bar: 20 μ m
- 1041 (J) FISH for expression of *dmg-8*, *dig-1* and *tetraspanin 66e* in dorsal tail of sexual worm
1042 after *nhr-1* RNAi feeding 4 times. Scale bar: 100 μ m
- 1043 (K) The cartoon summarizes the ventral tail expression pattern of the downstream genes,
1044 which decreased after *nhr-1* RNAi feeding 4 times. The glands around the penis papilla
1045 can be divided into 5 regions, according to those molecular markers and *tsp-1*.
1046 Examples of genes in each region are shown in panel L to O and further zoomed in
1047 single cell layer in panel P to S.
- 1048 (L) FISH for ventral expression of *dig-1* and *tetraspanin 66e*, which are in region 1. The
1049 yellow box shows the region for further zoom in in panel P. Scale bar: 100 μ m
- 1050 (M) FISH for ventral expression of *igg-1* and *dig-2*, which express in region 4 or cement
1051 gland. The yellow box shows the region for further zoom in in panel Q. Scale bar:
1052 100 μ m
- 1053 (N) FISH for ventral expression of *dmg-1*, *PL04022B1F07* and *dmg-2*, which express in
1054 region 2. The yellow box shows the region for further zoom in in panel R. Scale bar:
1055 100 μ m
- 1056 (O) FISH for ventral expression of *dmg-4*, *dmg-5* and *dmg-6*, which express in region 3. The
1057 yellow box shows the region for further zoom in in panel S. Scale bar: 100 μ m
- 1058 (P) FISH for ventral expression of *dig-1* and *tetraspanin 66e* in single cell layer. Scale bar:
1059 20 μ m
- 1060 (Q) FISH for ventral expression of *igg-1* and *dig-2* in single cell layer. Scale bar: 20 μ m
- 1061 (R) FISH for ventral expression of *dmg-1*, *PL04022B1F07* and *dmg-2* in single cell layer.
1062 Scale bar: 20 μ m

- 1063 (S) FISH for ventral expression of *dmg-4*, *dmg-5* and *dmg-6* in single cell layer. Scale bar:
1064 20 μ m
1065 (T) FISH for expression of *dig-1* and *tetraspanin 66e* in ventral tail of sexual worm after *nhr-*
1066 *1* RNAi feeding 4 times. Scale bar: 100 μ m
1067 (U) FISH for expression of *dmg-4* and *dmg-6* in ventral tail of sexual worm after *nhr-1* RNAi
1068 feeding 4 times. Scale bar: 100 μ m
1069

1070 **Figure 6:** *Smed-acs-1*, a downstream gene of *nhr-1*, is essential for reproductive system
1071 development, maintenance and regeneration.

- 1072 (A) DAPI and PNA staining of the tail region in sexual mature planarian after *Smed-acs-1*
1073 RNAi feeding 6 and 9 times. The blue triangles point to the testes without mature
1074 spermatozoa. The white triangles point to the regressing gland stained by PNA. The
1075 blue snow flake marks the penis papilla. Scale bar: 100 μ m
1076 (B) DAPI staining of the ovary and oviduct region in sexual mature planarian after *Smed-*
1077 *acs-1* RNAi feeding 2, 6 and 9 times. The red circle marks the ovary. The red arrow
1078 head points to the oviduct. Scale bar: 50 μ m
1079 (C) DAPI and PNA staining of the tail region in grow-up hatchlings (above row) and
1080 regenerated worms (below row) after *Smed-acs-1* RNAi feeding. The blue triangles
1081 point to the testes without mature spermatozoa. Scale bar: 100 μ m
1082 (D) DAPI staining of the testes and ovaries region in grow-up hatchlings (above row) and
1083 regenerated worms (below row) after *Smed-acs-1* RNAi feeding. The red circle marks
1084 the ovary. The red arrow head points to the oviduct. Scale bar: 50 μ m
1085

1086 **Figure 7:** *nhr-1* maintains planarian reproductive system through regulating lipid metabolism

- 1087 (A) Oil Red O staining of the sexual adult planarian 7 days after *unc-22*, *nhr-1* and *Smed-*
1088 *acs-1* RNAi feeding. Scale bar: 100 μ m
1089 (B) Oil Red O staining on the transverse sections of the sexual adult planarian 7 days after
1090 RNAi feeding. The left illustration shows the structure of the transverse section. Scale
1091 bar: 50 μ m
1092 (C) Histogram shows the Mean \pm SEM value of the Oil Red O staining intensity in the tails
1093 (n=3, t-test **p<0.01, ***p<0.001).
1094 (D) Oil Red O and DAPI staining around testes region. White dash line circles the testis
1095 region according to DAPI staining. Scale bar: 10 μ m
1096 (E) WISH of *tsp-1* and DAPI staining of the dorsal tail region in sexual planarian after long
1097 term *nhr-1* RNAi feeding with (right) and without (left) Acetyl-CoA rescue. Scale bar:
1098 100 μ m
1099 (F) WISH of *gh4* and DAPI staining of the testes region in sexual planarian after long term
1100 *nhr-1* RNAi feeding with (below) and without (above) Acyl-CoA synthetase (ACS)
1101 rescue. Scale bar: 20 μ m
1102 (G) WISH of *plastin* and DAPI staining of the testes region in sexual planarian after long
1103 term *nhr-1* RNAi feeding with (below) and without (above) Acyl-CoA synthetase (ACS)
1104 rescue. Scale bar: 20 μ m
1105 (H) Proposed model for reproductive system maintenance in sexual mature animals via an
1106 *nhr-1* and lipid metabolism axis.
1107

Supplementary figure legends

Figure S1, related to Figure 1

Majority of the adult worm enriched transcripts, which contain coiled-coil domain or zinc finger domain, express in the sexual reproductive system.

(F) WISH for candidate genes for screening. The color of dots represents different expression categories, which is explained in panel B. Scale bar: 750 μ m

(G) Summary of expression patterns of screened transcripts

Figure S2, related to Figure 1

Histogram showing the cocoon number from different RNAi worms

Figure S3, related to Figure 2

nhr-1 RNAi decreased pachytene stage cells and telomere formation in testis.

(A) Histogram of cell numbers in different meiosis stages in half testis (n=4, t-test, *p<0.05, **p<0.01).

(B) Representative image showing normal bouquet in *nhr-1(RNAi)* animals. Scale bar: 5 μ m

Figure S4, related to Figure 2

nhr-1 expresses in most organs in the sexual reproductive system in adult planarian.

(A) FISH for *nhr-1*, *nanos* and *eya* expression in ovary and oviduct. Scale bar: 20 μ m

(B) FISH for *nhr-1* and *gh4* expression in ovary. Scale bar: 20 μ m

(C) FISH for *nhr-1* and *nanos* in dorsal border region. *nanos* labels the germ stem cells in the testis. Scale bar: 100 μ m

(D) FISH for *nhr-1* and *gh4* in testis. Scale bar: 20 μ m

(E) FISH for *nhr-1* and *tsp-1* in dorsal tail. Scale bar: 100 μ m

(F) FISH for *nhr-1*, *tsp-1* and *grn* in ventral tail. Scale bar: 100 μ m

Figure S5, related to Figure 3

nhr-1 is specifically required for reproductive system regeneration.

(A) Percentage of worms with gonopore during regeneration.

(B) FISH for *pc2* in regenerated head and *porcupine* in regenerated tail. Scale bar: 100 μ m

(C) FISH for *grn* and *eya* in regenerated tail. Scale bar: 100 μ m

Figure S6, related to Figure 5

nhr-1 downstream genes, which decreased after *nhr-1* RNAi feeding 4 times, are enriched in the reproductive system.

(A) WISH of the 30 transcripts, which decreased after *nhr-1* RNAi feeding 4 times. Scale bar: 750 μ m

(B) Summary of the 30 transcripts expression.

(C) Expression fold changes of genes affected after 4 rounds of *nhr-1* RNAi feeding in asexual, juvenile, decapitated sexual adult fragments (D0) and 7 days post amputation (D7). Each dot shows ratio of single transcripts. Mean \pm SEM of each data group showed by lines in the figure. n=3 for juvenile, sexual adult and sexual adult fragments,

1153 each containing 3 worms. n=4 for asexual worms as previously reported (Davies et al.,
1154 2017).
1155

1156 Figure S7, related to Figure 5

1157 *nhr-1* downstream genes, which decreased after *nhr-1 RNAi* feeding 4 times, express in
1158 different regions of the glands in sexual mature planarian.

1159 Panel A to H are the in the dorsal tail region. Panel I to M are in the ventral tail region.

1160 (A) FISH for dorsal expression of *dig-2* and *tetraspanin 66e*, which express in region 1.

1161 Scale bar: 100µm

1162 (B) FISH for dorsal expression of *dmg-4*, *dmg-5* and *dmg-6*, which express in region 2. The
1163 yellow box shows the region for further zoom in in panel F. Scale bar: 100µm

1164 (C) FISH for dorsal expression of *dmg-7* and *dmg-8*, which express in region 2. The yellow
1165 box shows the region for further zoom in in panel G. Scale bar: 100µm

1166 (D) FISH for dorsal expression of *igg-2* and *igg-3*, which express in region 4. The yellow box
1167 shows the region for further zoom in in panel H. Scale bar: 100µm

1168 (E) FISH for dorsal expression of *igg-1* and *tsp-1* in single cell layer. Scale bar: 20µm

1169 (F) FISH for dorsal expression of *dmg-4*, *dmg-5* and *dmg-6* in single cell layer. Scale bar:
1170 20µm

1171 (G) FISH for dorsal expression of *dmg-7* and *dmg-8* in single cell layer. Scale bar: 20µm

1172 (H) FISH for dorsal expression of *igg-2* and *igg-3* in single cell layer. Scale bar: 20µm

1173 (I) FISH for ventral expression of *igg-2*, which expresses in region 2, and *igg-3*. Scale bar:
1174 100µm

1175 (J) FISH for ventral expression of *dmg-7* and *dmg-8*, which express in region 3. The yellow
1176 box shows the region for further zoom in in panel M. Scale bar: 100µm

1177 (K) FISH for ventral expression of *dmg-3*, which expresses in region 1. Scale bar: 100µm

1178 (L) FISH for ventral expression of *igg-1* and *tsp-1* in single cell layer. Scale bar: 20µm

1179 (M) FISH for dorsal expression of *dmg-7* and *dmg-8* in single cell layer. Scale bar: 20µm
1180

1181 Figure S8, related to Figure 5

1182 *nhr-1* is essential for the expression of novel gland genes, which decreased after *nhr-1 RNAi*
1183 feeding 4 times.

1184 This figure shows the representative images of FISH for expression of novel gland genes after
1185 *nhr-1 RNAi* feeding 4 times in adult planarian. Panel A to C show the image in the dorsal
1186 region, while panel D to F show the image in the ventral region.

1187 (A) FISH for expression of *dig-2*, *igg-1* and *tetraspanin 66e* in dorsal tail region. Scale bar:
1188 100µm

1189 (B) FISH for expression of *dmg-1* and *dmg-2* in dorsal tail region. Scale bar: 100µm

1190 (C) FISH for expression of *igg-2* and *igg-3* in dorsal tail region. Scale bar: 100µm

1191 (D) FISH for expression of *dmg-1* and *dmg-2* in ventral tail region. Scale bar: 100µm

1192 (E) FISH for expression of *dig-2*, *igg-1* and *tetraspanin 66e* in ventral tail region. Scale bar:
1193 100µm

1194 (F) FISH for expression of *dmg-7* and *dmg-8* in ventral tail region. Scale bar: 100µm
1195

1196 Figure S9, related to Figure 6

1197 *Smed-acs-1* expression in sexual adult animal.

- 1198 (A) *Smed-acs-1* expression in intact asexual, juvenile, decapitated sexual adult (D0) and
1199 sexual adult fragments (7 days after amputation, D7) according to RNAseq data. Dot
1200 shows RPKM value in each replicate. Bar graph indicates the Mean±SEM value for
1201 each sample. n=3 for juvenile, sexual adult and sexual adult fragments, each containing
1202 3 worms. n=4 for asexual worms, as previously reported (Davies et al., 2017).
1203 *represents the adjusted p value for each comparison group. ****p<0.0001.
1204 (B) WISH of *Smed-acs-1* in dorsal and ventral region. The dorsal region in the white square
1205 was further zoom in in the panel B. The ventral region in the white square was further
1206 zoom in in the panel C. Scale bar: 100µm
1207 (C) Zoom in of *Smed-acs-1* dorsal expression posterior of neck region. Scale bar: 50µm
1208 (D) Zoom in of *Smed-acs-1* ventral expression in the ovary region. Scale bar: 50µm
1209

1210 Figure S10, related to Figure 7

1211 *nhr-1* maintains reproductive system through lipid metabolic pathway in sexual planarian.

- 1212 (A) Oil Red O staining of asexual animal. Scale bar: 500µm
1213 (B) DAPI staining in the dorsal tail region of the sexual mature animal after long term *nhr-1*
1214 RNAi. Blue arrowheads point to the testes after rescue, which enrich DAPI signal. Scale
1215 bar: 100µm
1216 (C) FISH of *plastin* and *gh4* in dorsal testes region after Acetyl-CoA rescue. Scale bar:
1217 50µm.
1218 (D) FISH of *tsp-1* in dorsal tail region after Acyl-CoA rescue. Scale bar: 20µm.
1219

1220 **Movie-S1**

1221 3D reconstruction of nuclei in half testis with whole-mount DAPI staining in normal sexual adult
1222 planarian.

1223 **Movie-S2**

1224 3D reconstruction of nuclei in half testis with whole-mount DAPI staining in sexual adult
1225 planarian after *nhr-1* RNAi feeding 6 times
1226

1227 **Table S1, related to Figure 1**

1228 Expression level changes of the 34 genes identified to be expressed higher in decapitated
1229 adult fragments (D0) when compared to juvenile animals. D7 shows decapitated sexual adult
1230 fragments 7 days post amputation.
1231

1232 **Table S2, related to Figure 4**

1233 Expression level changes of the 30 genes down-regulated after 4 rounds of *nhr-1*(RNAi)
1234 feeding. D0 and D7 show decapitated sexual adult fragments at 0 and 7 days post amputation.
1235
1236

1237 **Table S3, related to Figure 1 and Figure 5**

1238 Primers used for gene cloning

Figure 1

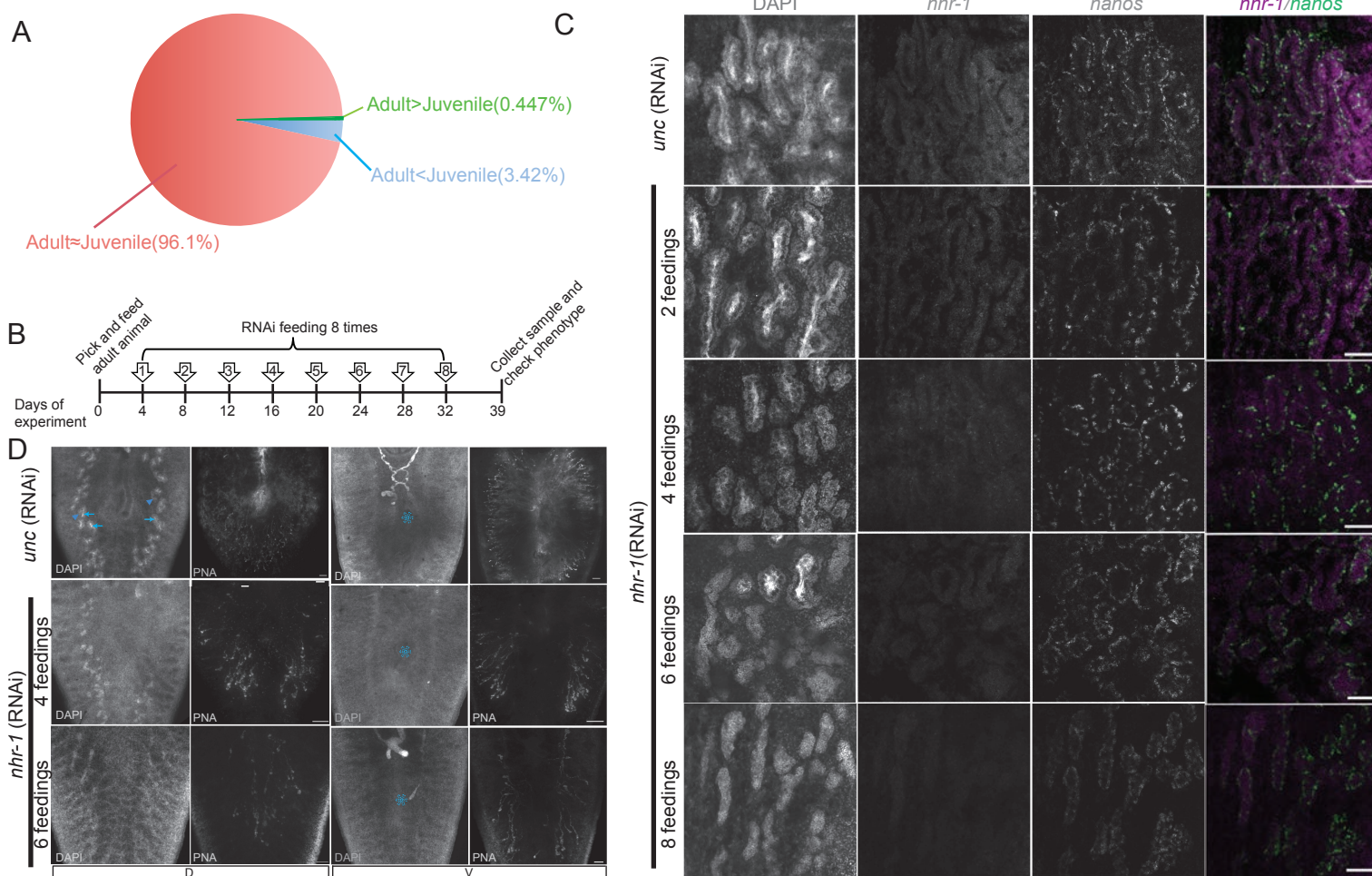


Figure 2

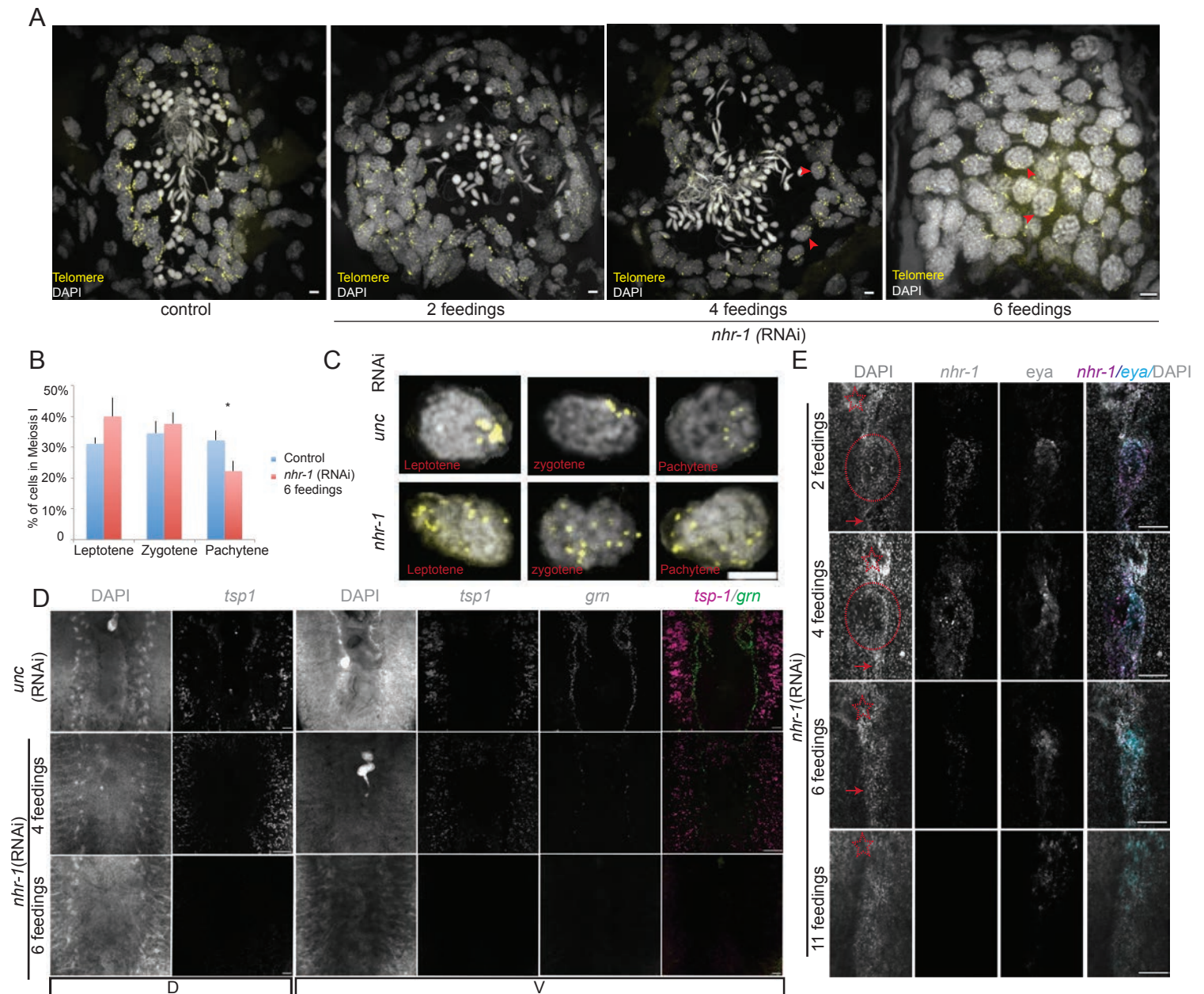


Figure 3

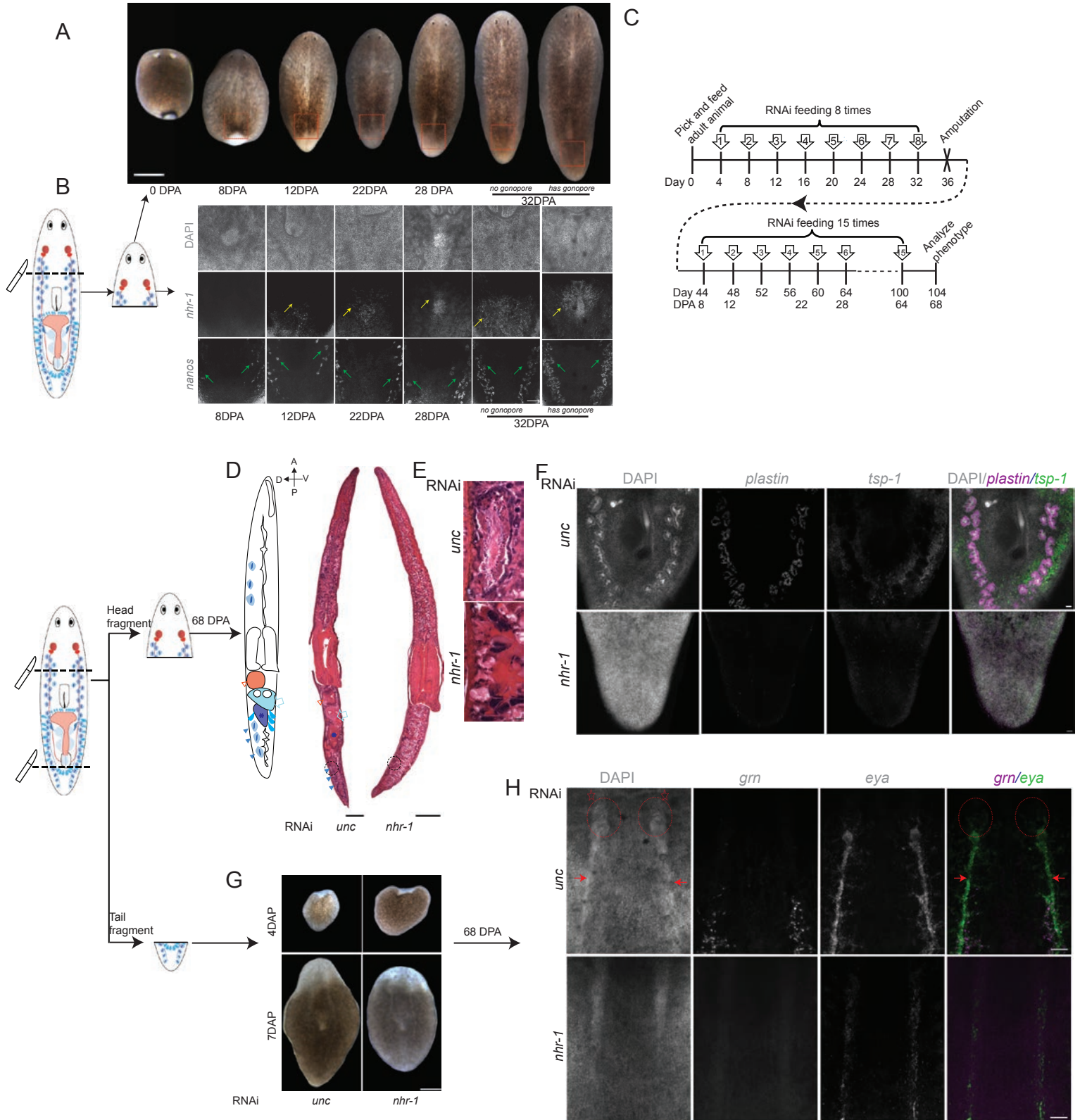


Figure 4

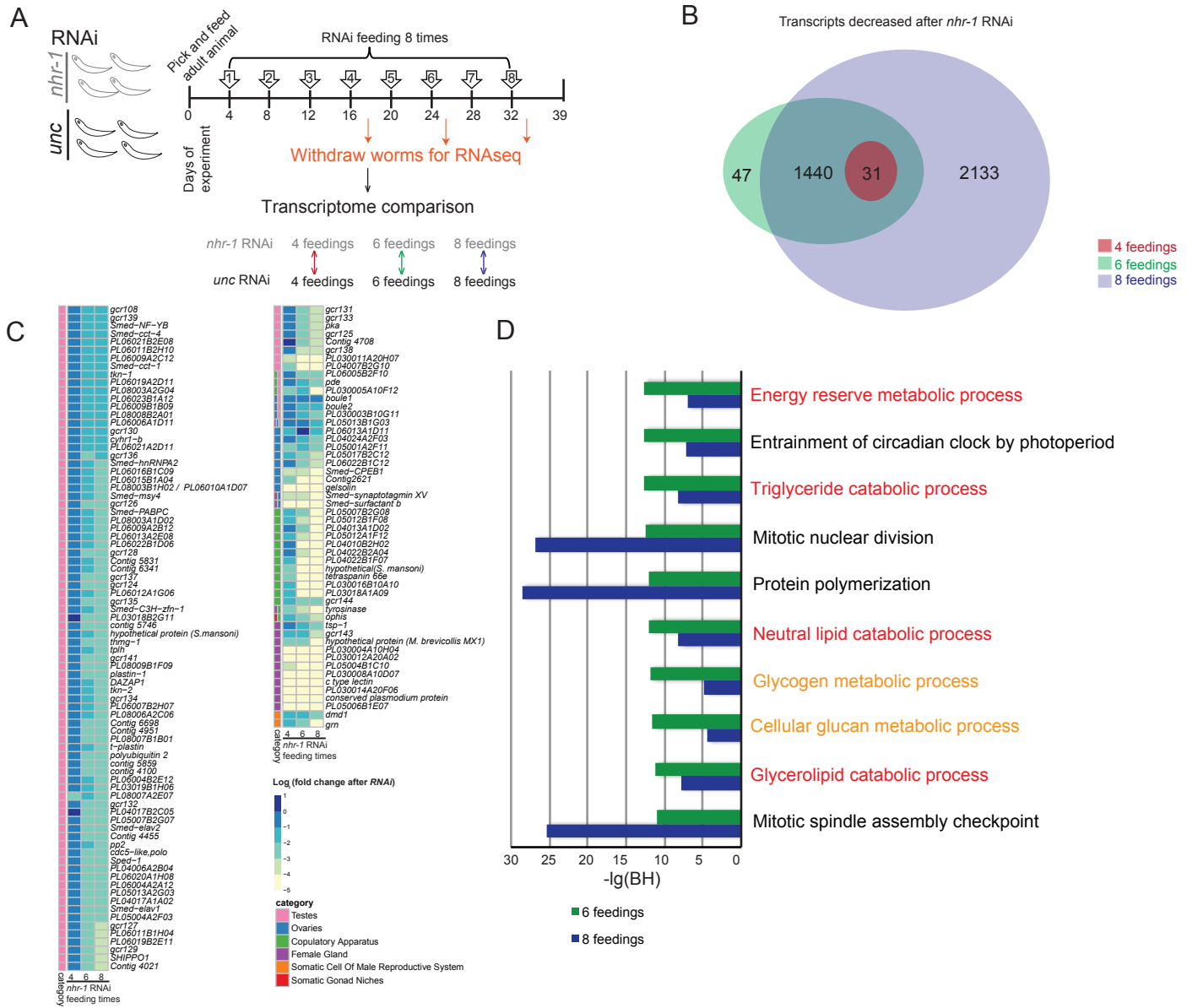


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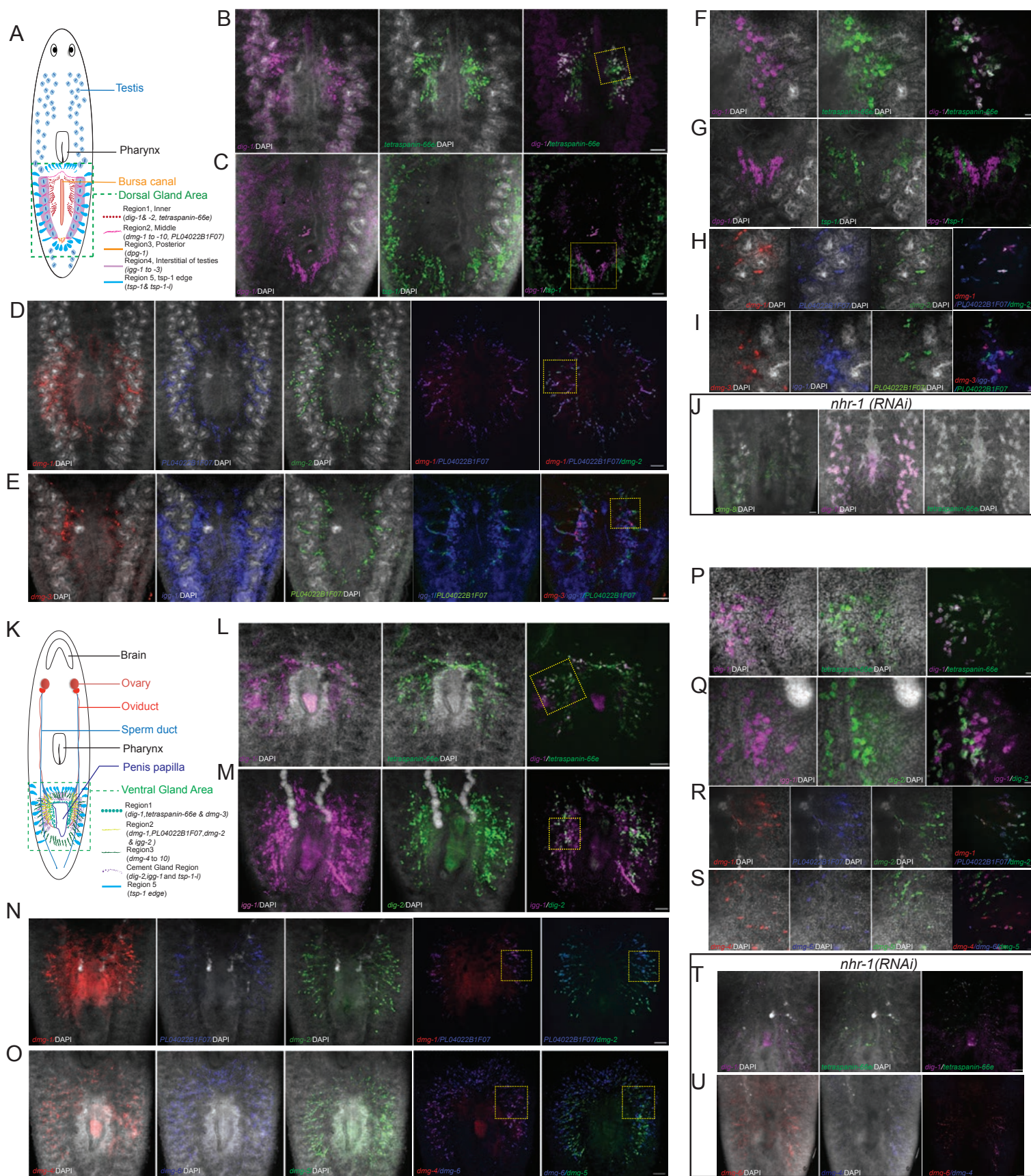


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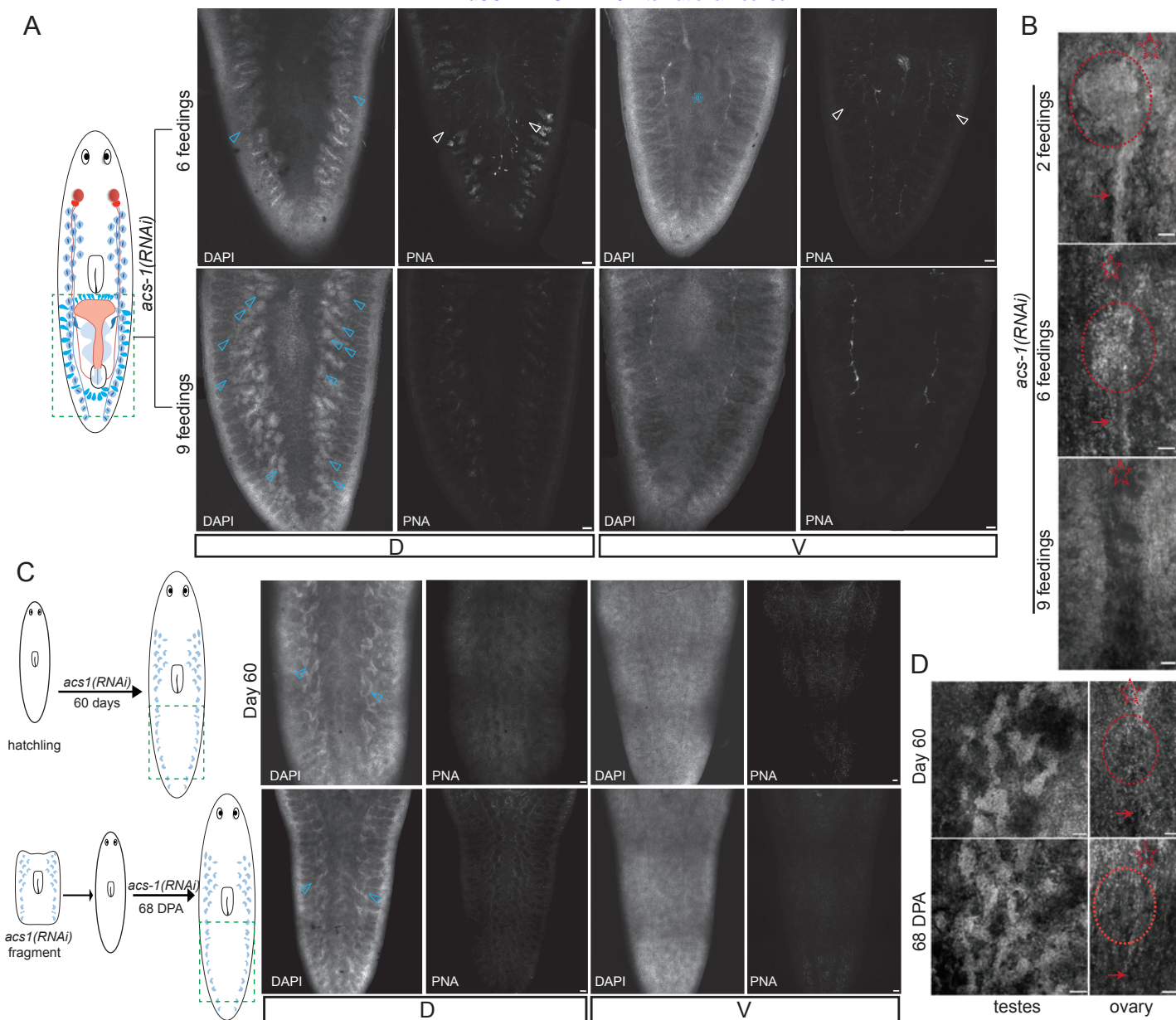


Figure 7

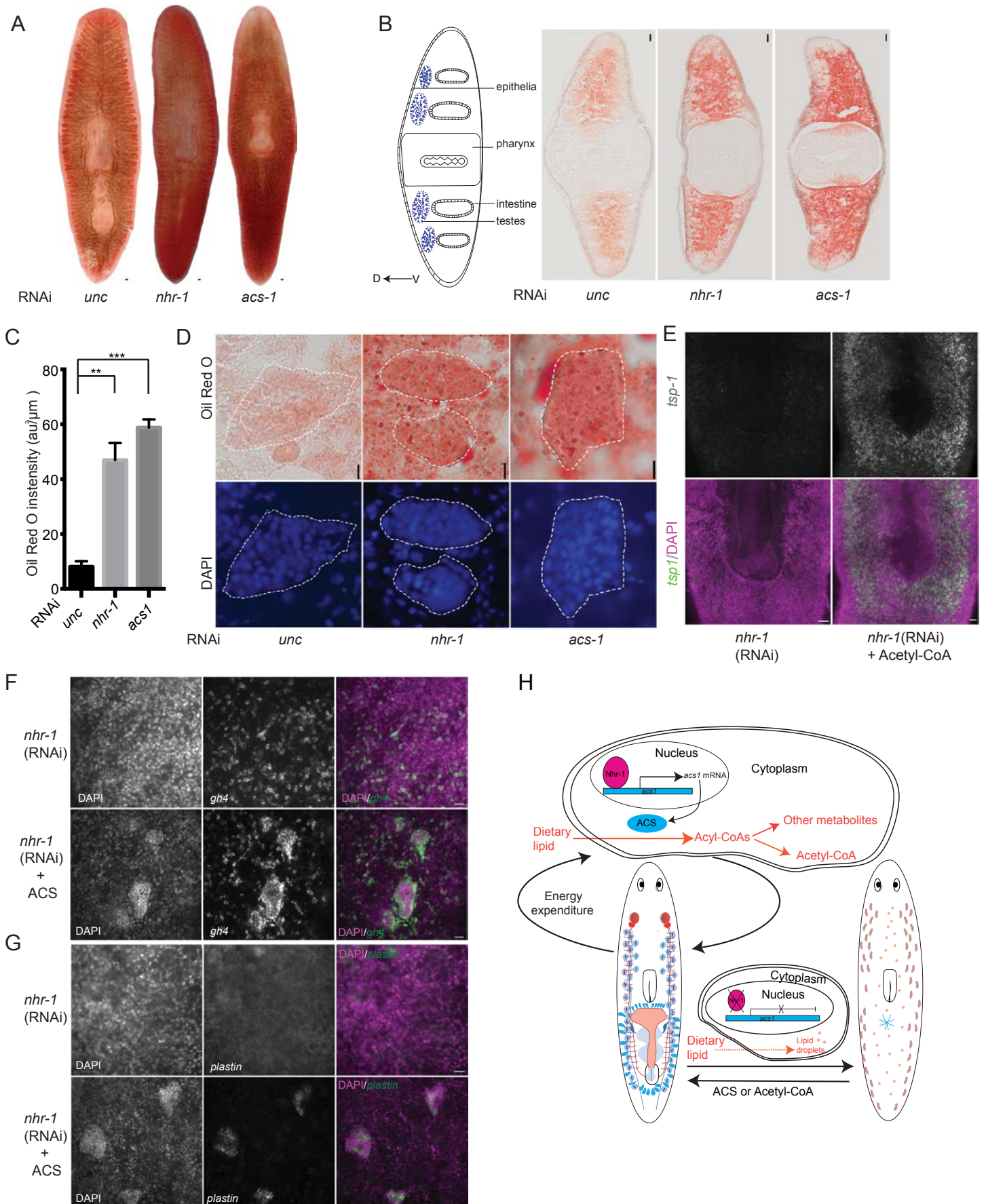
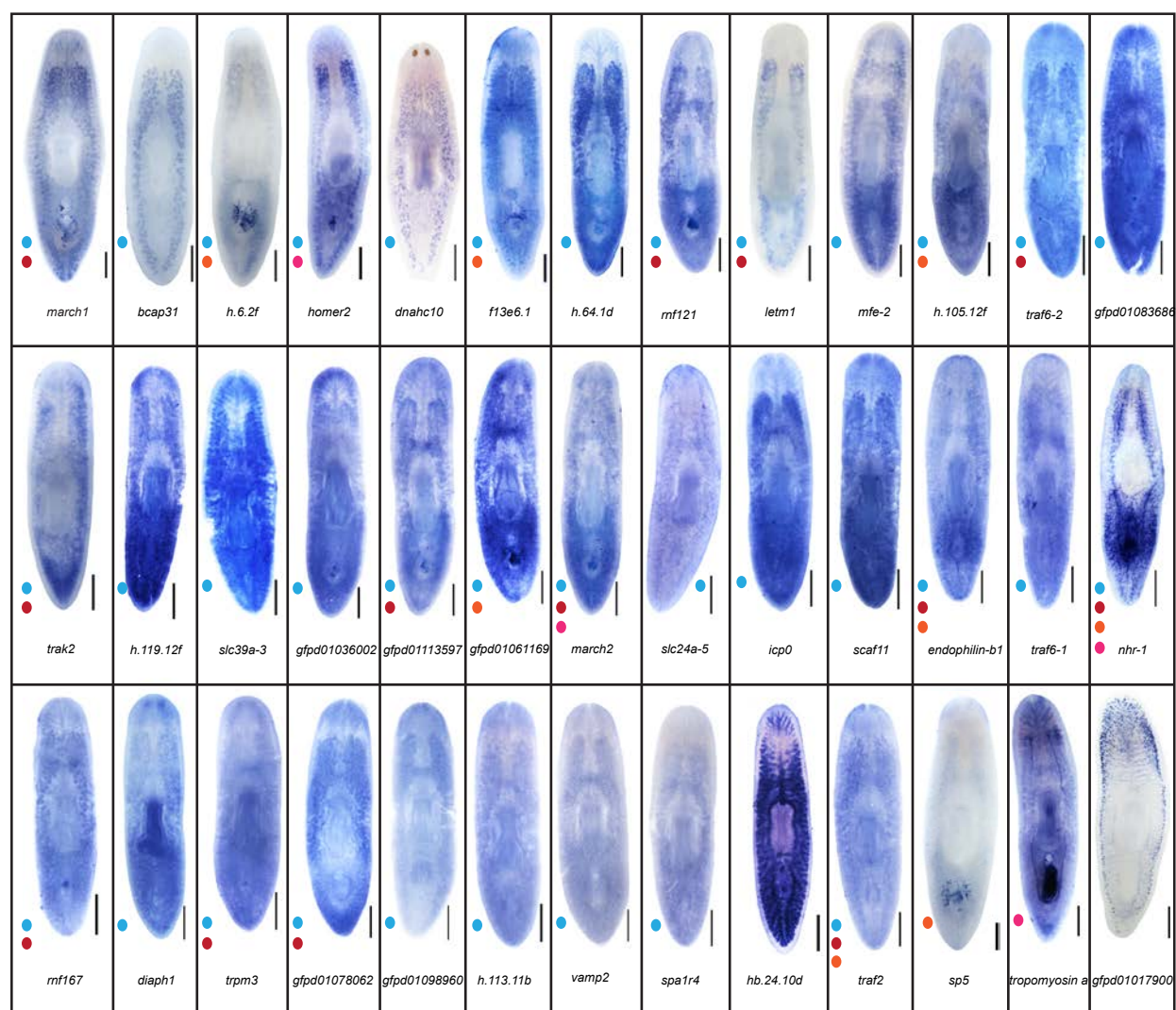


Figure S1

A



B

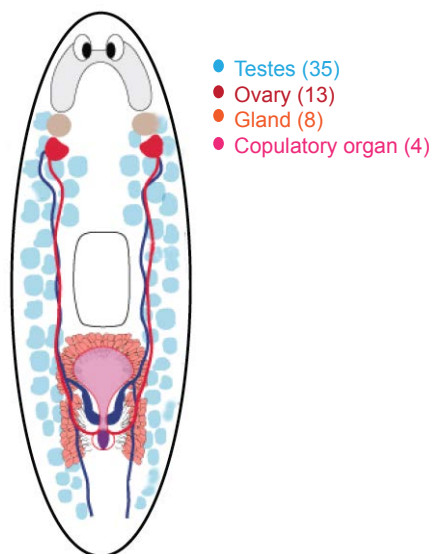


Figure S2

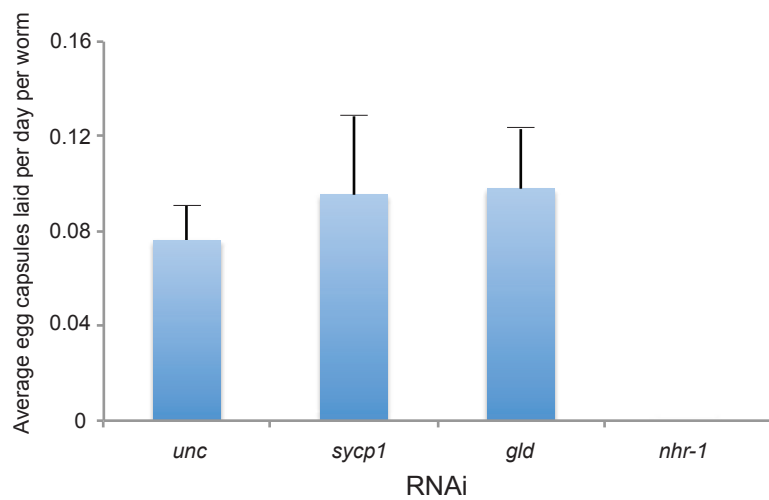
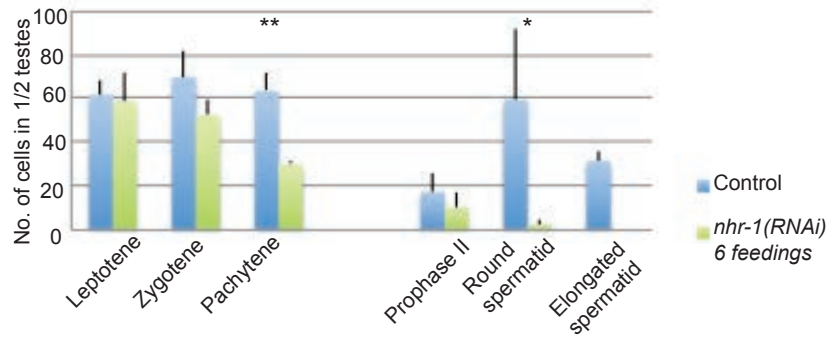
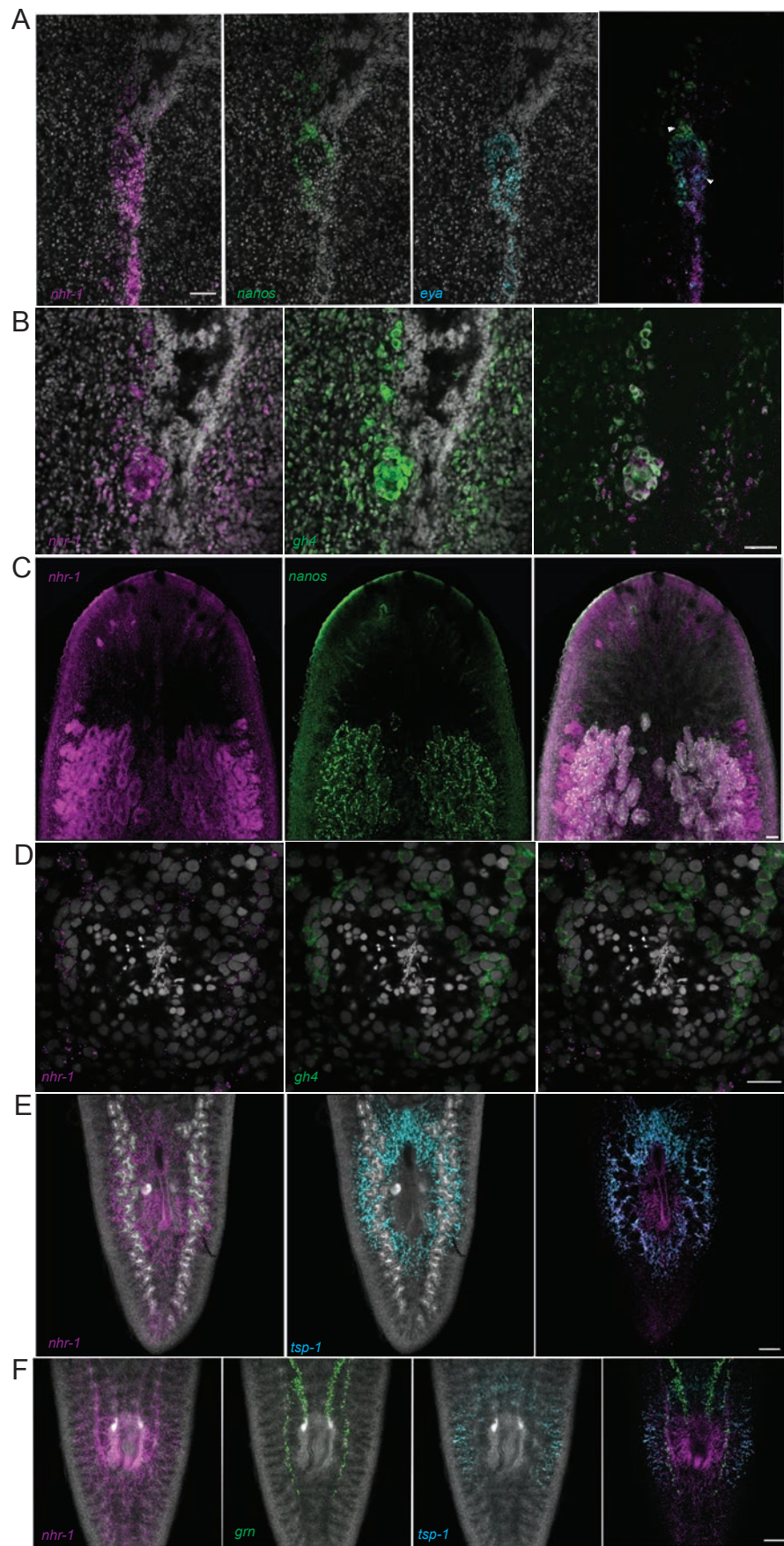


Figure 6B

A





FigureS5

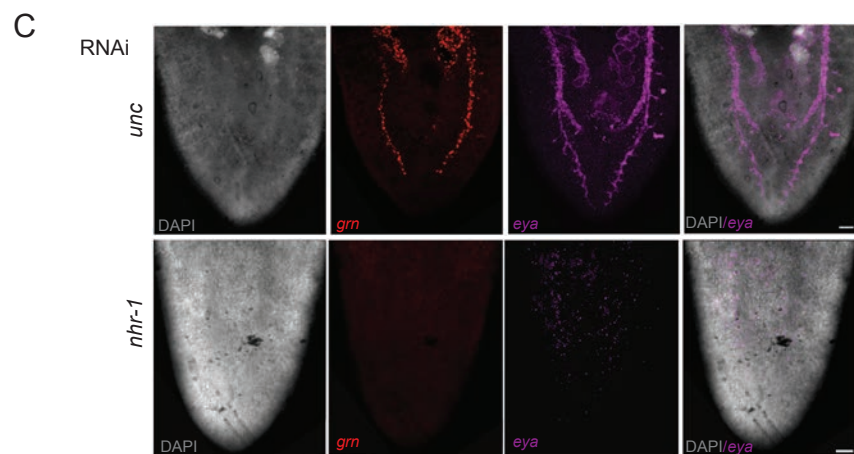
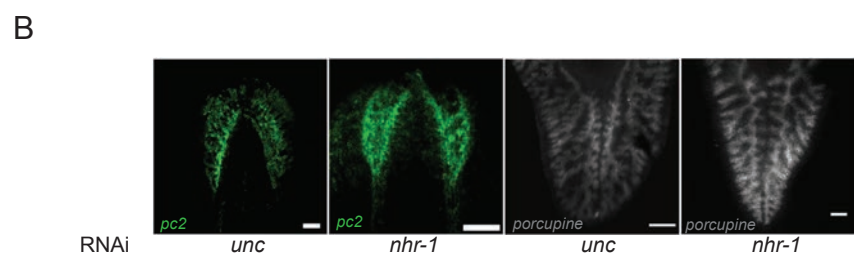
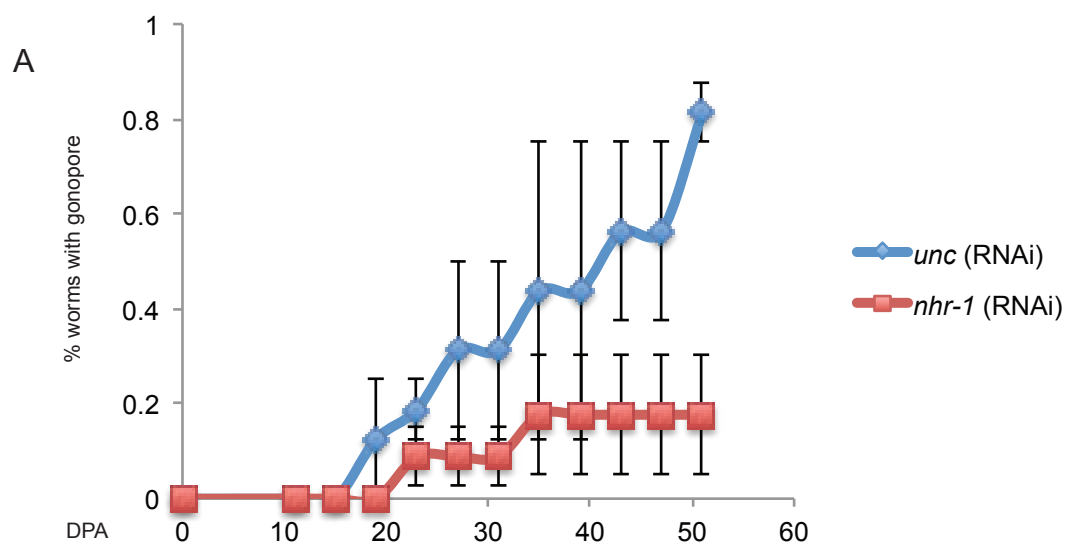


Figure S7

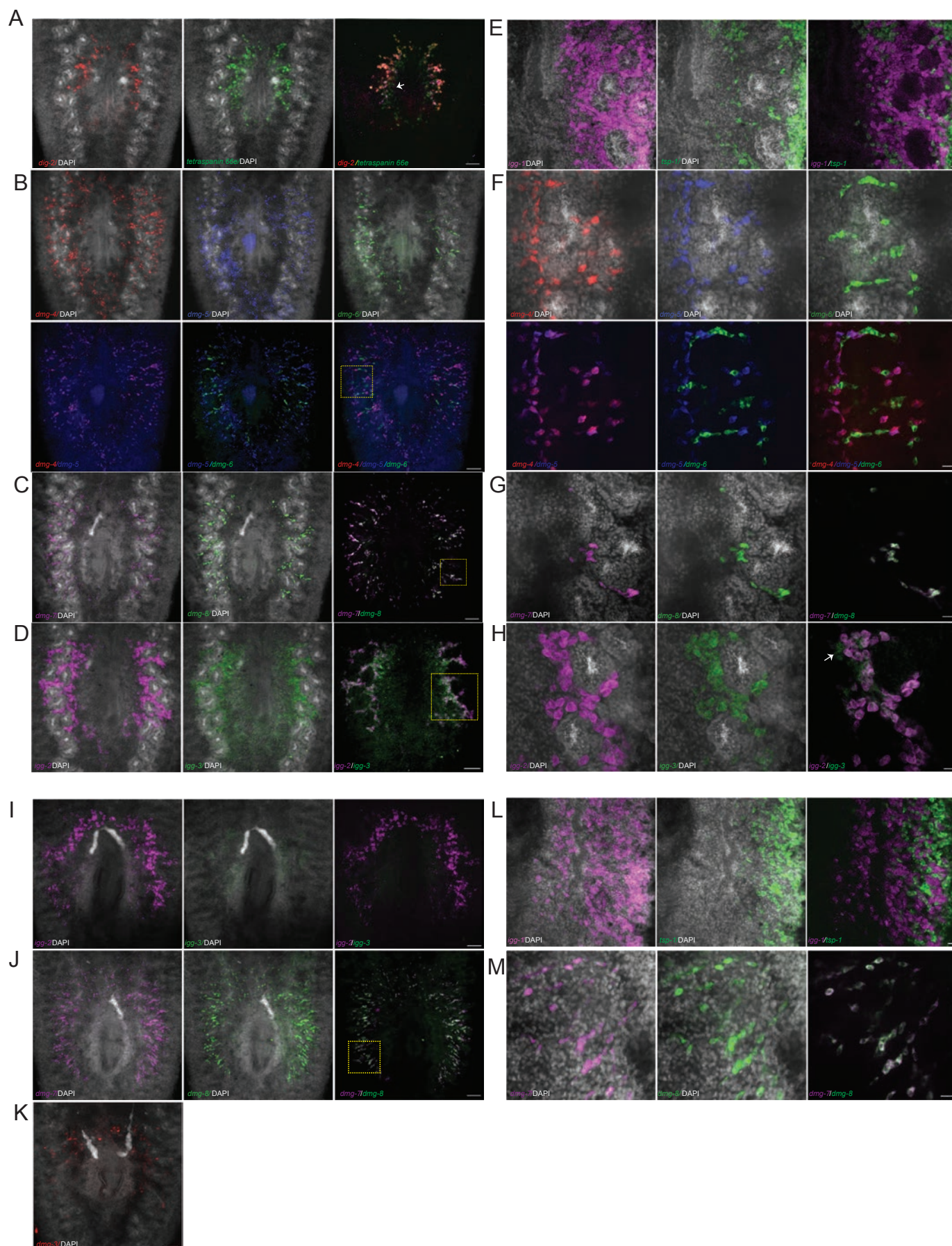


Figure S8

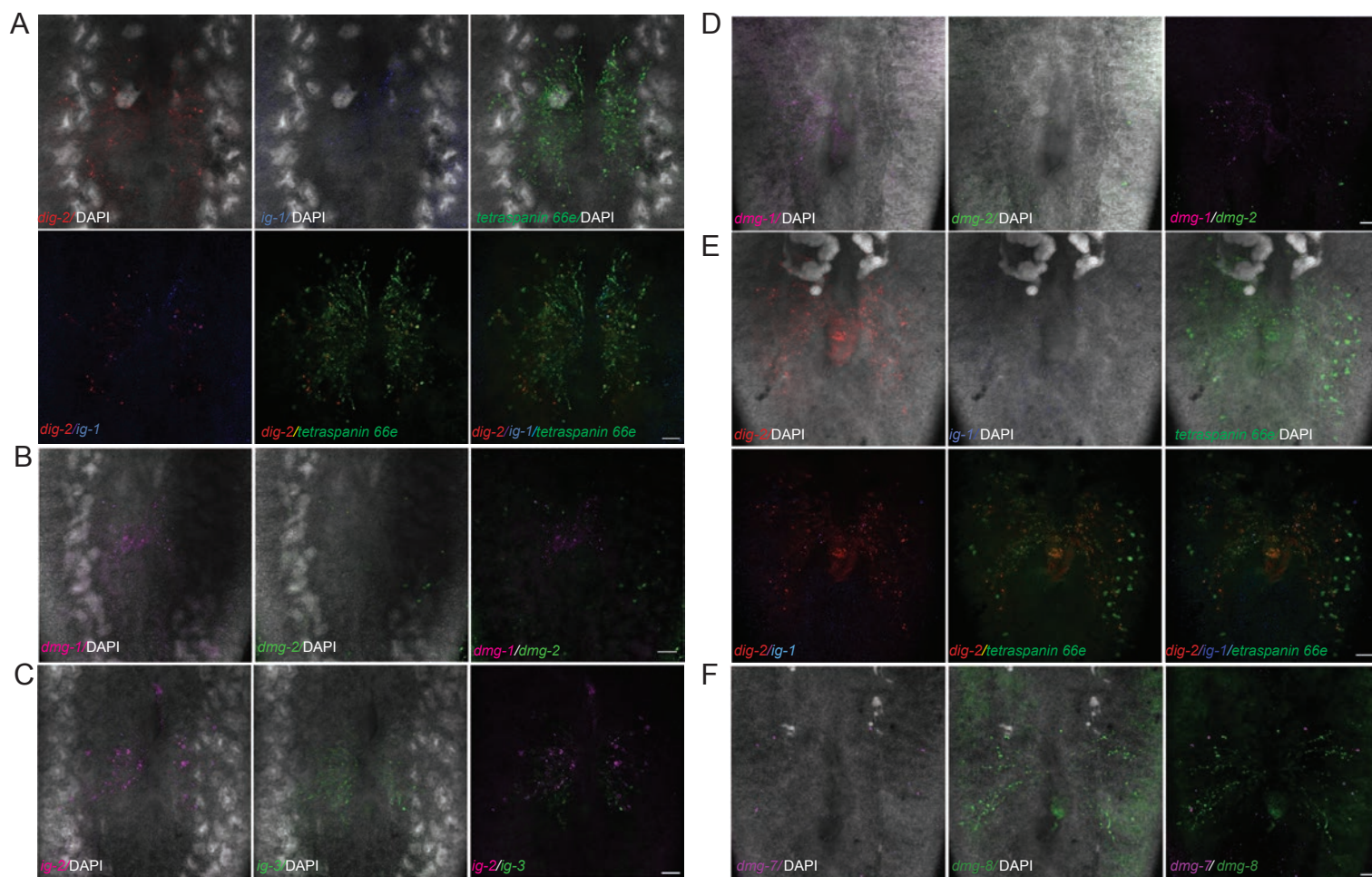


Figure S9

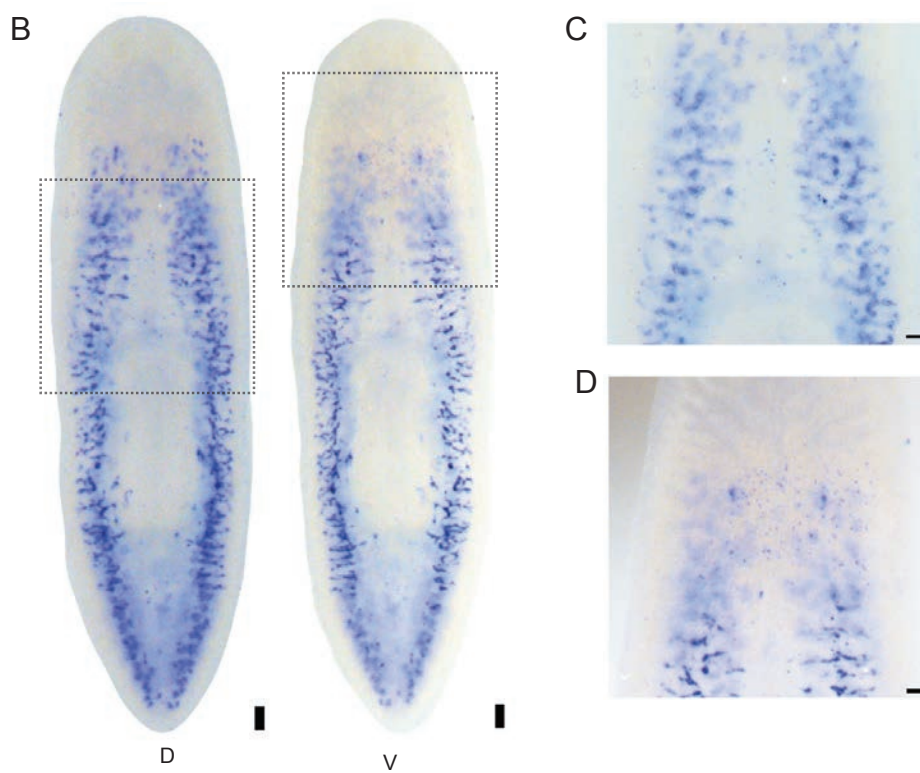
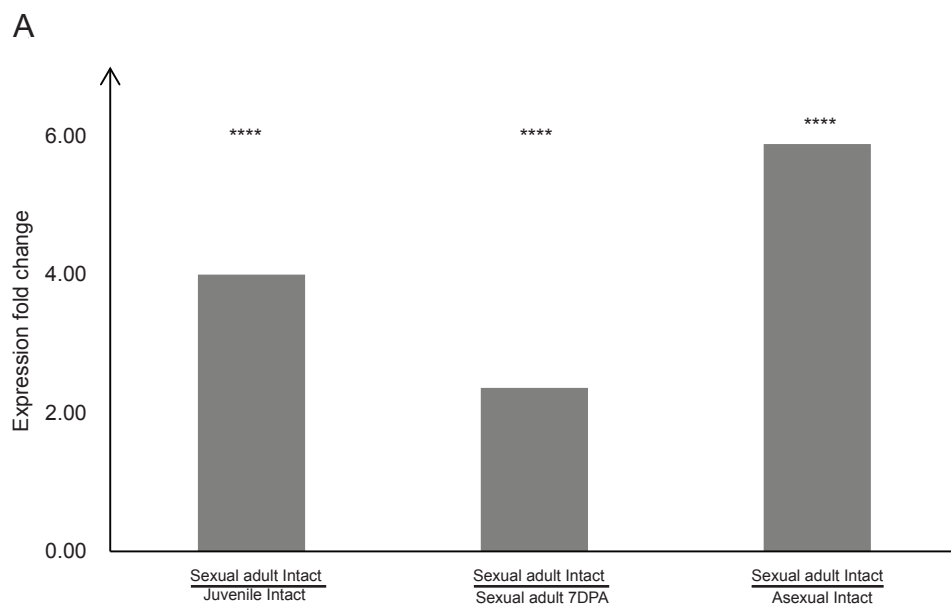


Figure S10

