Systematically improved *in vitro* culture conditions reveal new insights into the reproductive biology of the human parasite *Schistosoma mansoni*

Jipeng Wang[1], Rui Chen[1], James J. Collins III[1*]

[1]Department of Pharmacology, UT Southwestern Medical Center, Dallas, Texas

*To whom correspondence should be addressed

James J. Collins III
UT Southwestern Medical Center
Department of Pharmacology
6001 Forest Park. Rd.
Dallas, TX 75390
JamesJ.Collins@UTSouthwestern.edu

ABSTRACT

Schistosomes infect over 200 million people. The prodigious egg output of these parasites is the sole driver of pathology due to infection, yet our understanding of their sexual reproduction is limited because egg production is not sustained for more than a few days *in vitro*. Here, we describe culture conditions that support schistosome sexual development and sustained egg production *in vitro.* Female schistosomes rely on continuous pairing with male worms to fuel the maturation of their reproductive organs. Exploiting these new culture conditions, we explore the process of male-stimulated female maturation and demonstrate that physical contact with a male worm, and not insemination, is sufficient to induce female development and the production of viable parthenogenetic haploid embryos. We further report the characterization of a novel nuclear receptor, that we call vitellogenic factor 1, that is essential for female sexual development following pairing with a male worm. Taken together, these results provide a platform to study the fascinating sexual biology of these parasites on a molecular level, illuminating new strategies to control schistosome egg production.

INTRODUCTION

Schistosomes are blood-dwelling parasitic flatworms that cause serious disease in millions of people in the developing world [1]. The pathology caused by these parasites is entirely due to the parasite's prodigious egg output [2]. Although the goal of the parasite is to pass these eggs from the host to ensure the continuity of the parasite's complex life cycle, approximately half of these eggs become trapped in host tissues inducing inflammation that represents the primary driver of disease [3]. Since parasites incapable of producing eggs produced little pathology in infected hosts, understanding the biology of schistosome egg production could suggest new therapeutic strategies against these devastating parasites.

Schistosomes are unique among flatworms as they do not sexually reproduce as hermaphrodites instead they have evolved separate male and female sexes [2, 4, 5]. This transition from hermaphroditism to dioecism has led to some intriguing biological phenomena, in particular the observation that female schistosomes rely on continuous pairing with a male worm to become sexually mature and produce eggs [2, 6-8]. Indeed, females grown in the absence of male worms are developmentally stunted and their reproductive organs are undeveloped. Upon pairing with a male worm, the female's sexual organs become mature and egg production commences. Interestingly, this process is reversible since females deprived of male contact will regress to an immature state [9]. Although a variety of molecules important for female reproduction have been characterized [10-12], the nature of the signal(s) from the male that stimulate female maturation and the female response to these signals remain poorly understood.

A major bottleneck for understanding the biology of egg production and female sexual development is that normal egg production ceases within days of removal of the parasite from the host even in the presence of male worms [13-16]. While work by numerous investigators have established robust conditions for the maintenance [17-20] and growth of adult-staged parasites [15, 21, 22], no *in vitro* conditions that sustain continuous egg production have been described. Here, we report conditions that support long-term schistosome egg production *in vitro* and allow for virgin female worms to become sexually mature after pairing with a male worm. As a proof of principle, we use these culture conditions to explore the process by which male worms stimulate female maturation. We find that direct contact with a male worm along the female worm's entire body is essential for sexual maturation and viable egg production. Interestingly, we demonstrate that in the absence of sperm transfer that contact with a male worm is sufficient for female worms to produce viable parthenogenetic haploid embryos. Capitalizing on these culture conditions we further report the characterization of a previously uncharacterized nuclear receptor that is essential for normal female development following pairing with a male worm. These studies provide new insights into the biology of schistosome egg production and lay the groundwork for the application of a growing molecular tool kit to understanding the fascinating sexual biology of these important pathogens.

RESULTS

Media containing ascorbic acid, red blood cells, and cholesterol supports egg production *in vitro*. The most successful systematic effort for culturing schistosomes *in vitro* are those of Basch [15, 21, 22]. While Basch's "medium 169" (BM169) was able to support the *in vitro* growth of larval parasites to adulthood [21], it was insufficient for maintaining sexually mature egg-laying female parasites [15, 22]. Nevertheless, given the success of BM169 to support parasite growth, we reasoned BM169 was the ideal place to begin optimizing conditions for egg

86 production. As previously reported [15], adult schistosomes recovered from mice and cultured in
87 BM169 progressively lost the ability to lay eggs with the morphological characteristics of those
88 laid *in vivo* or immediately *ex vivo* (Fig 1A).

89 A schistosome egg is constructed from cells derived from two organs: the ovary, that
90 contributes an oocyte, and the vitellaria, that provide 20-30 vitellocytes [2, 23]. Although female
91 worms paired with male worms retained the ability to produce oocytes during *in vitro* culture (Fig
92 S1A,B), we noted a rapid loss in the ability of cultured parasites to generate vitellocytes, consistent
93 to previous studies [14, 16]. Vitellocytes contain two types of large cytoplasmic inclusions: lipid
94 droplets and vitelline droplets that coalesce to form the eggshell [24, 25]. Using Fast Blue BB to
95 label vitelline droplets we found that vitellaria progressively ceased production of large numbers
96 of vitellocytes in BM169 (Fig 1B,C). Similarly, BODIPY 493/503 labeling found that the vitellaria
97 of females in BM169 possessed few lipid droplets at D20 (Fig 1C). Examination of a panel of
98 genes expressed in mature vitellocytes [26] by whole mount *in situ* hybridization (Fig 1D) and
99 quantitative PCR (Fig 1E) found a significant decrease in the expression of vitellaria-specific
100 transcripts during culture, similar to previous studies [16]. Thus, the capacity for vitellogenesis is
101 rapidly lost *in vitro*.

102 To improve the rate of vitellogenesis and egg production we examined supplements that
103 could potentially satisfy either known metabolic requirements for egg production (e.g., lipids[27])
104 or documented auxotropies of the worm (e.g., polyamines, fatty acids, sterols[28-30]). From these
105 analyses we observed no qualitative effects on egg production from supplements, including:
106 albumin (e.g., Lactalbumin or Linoleic Acid-Oleic Acid-Albumin), spermidine, commercially
107 available lipid supplements, commercially available antioxidant supplements, red blood cells
108 (RBCs), low density lipoprotein, L-carnitine, N-acetyl-cysteine, or sera from various species
109 (chicken, bovine, or horse). During this process, however, we came across a report detailing the
110 formation of abnormal eggs in schistosome-infected guinea pigs fed an Vitamin C (L-ascorbic acid)
111 deficient diet [31]. Strikingly, addition of ascorbic acid to BM169 led to a marked increase in
112 vitelline development (Fig 1C, S1C Fig) and the production of eggs morphologically similar to
113 those laid by parasites immediately *ex vivo* (Fig 1C).

114 Although L-ascorbic acid had profound effects on the quality of eggs generated *in vitro*,
115 the rate of egg production and development of the vitellaria remained inferior to that of fresh *ex*
116 *vivo* parasites (Fig 1C). Thus, we re-examined some of the previous assessed media supplements.
117 Given the critical role for lipid metabolism in egg production [27], we reasoned that adding
118 complex sources of lipids (and other nutrients) that the parasite encounters *in vivo* might act
119 synergistically with ascorbic acid. We found that supplementation with either red blood cells or a
120 commercial "cholesterol" concentrate containing purified Low Density Lipoprotein (LDL)
121 increased lipid stores along the intestine but had little effect on vitelline development or the
122 production of normal eggs (Fig 1C). However, combination of red blood cells, the cholesterol/LDL
123 concentrate, and ascorbic acid produced a dramatic increase in the rate of vitellogenesis (Fig 1C),
124 the rate of egg production (Fig 1C), and a marked increase in the expression of vitellaria-specific
125 transcripts (Fig 1D,E). From here on we refer to this formulation as ABC169 (Ascorbic Acid,
126 Blood Cells, Cholesterol and Basch Media 169).

127 In vertebrate cells, L-ascorbic acid acts not only as an antioxidant, but an essential co-factor
128 for a variety of enzymes [32]. Mammals deficient for L-ascorbic acid cannot perform key
129 enzymatic reactions (e.g., collagen hydroxylation) leading to symptoms commonly known as
130 scurvy [32]. Interestingly, the effects of vitamin C to prevent scurvy are stereoselective as D-

131 isoascorbic acid cannot replace L-ascorbic acid at equimolar concentrations [33]. Similarly, we
132 observed that D-isoascorbic acid could not replace L-ascorbic acid in egg production (Fig 1F),
133 suggesting that either L-ascorbic acid is selectively transported into cells or that it acts in a
134 stereoselective fashion to facilitate one or more enzymatic reactions within the cell.

135 **Parasites cultured in ABC169 produce eggs capable of developing to miracidia.** After an
136 initial peak, egg production in BM169 dropped precipitously and by D7 of culture parasites laid
137 ~13 egg-like masses per day (Fig 2A,B). We noted a similar peak in egg production using ABC169,
138 however, after D7 these parasites sustained production of ~44 morphologically normal eggs per
139 day (Fig 2A). Indeed, eggs laid in ABC169 possessed a lateral spine typical of *S. mansoni* eggs,
140 had smooth shells, and contained a "germinal disc" corresponding to the early embryo (Fig 2B).
141 Eggs freshly laid in ABC169 media were larger on average than those laid in BM169 (Fig 2C) and
142 contained similar numbers of nuclei as eggs laid by parasites freshly recovered from mice (Fig
143 2D). Due to a high concentration of phenolic proteins that originate in the vitelline droplets that
144 form the eggshell, schistosome eggshells are highly autofluorescent [23]. In contrast to the egg-
145 like masses from BM169, where parasites produce few vitelline droplets (Fig 1C), eggs from
146 parasites in ABC169 possessed autofluorescence comparable to those from parasites freshly *ex*
147 *vivo* (Fig 2E). Furthermore, nearly half of the eggs laid by parasites at D18-20 of culture in
148 ABC169 contained clusters of proliferative embryonic cells visualized by labeling with thymidine
149 analog EdU (Fig 2F, G). *S. mansoni* eggs are passed from the host to release larvae called miracidia
150 [2]. Approximately 10-20% of eggs laid on the first day cultured either in BM169 or ABC169
151 produced miracidia (S2 Fig). However, this rate dropped during time in culture (S2 Fig). While
152 eggs laid in BM169 after D7 were incapable of liberating miracidia, about 1-2% of eggs laid in
153 ABC169 produced viable and morphologically normal miracidia (Fig 2H, S1-2 Movie).
154 Furthermore, we found that miracidia recovered from eggs laid on D15 to D20 were capable of
155 penetrating *Biomphalaria glabrata* snails (n=214/217 total miracidia). However, after examining
156 more than 25 infected *B. glabrata* snails we only found two snails that shed viable cercariae (S3
157 Movie). Given the disparity between the rate of entering embryonic development (Fig 2G) and
158 the capacity for these embryos to mature to miracidia (S2 Fig), together with the limited ability of
159 these miracidia to generate cercariae after infecting snails, it is possible that cues from the host are
160 necessary for efficient development of *S. mansoni* embryos to infective miracidia. Further studies
161 exploring optimal growth conditions for schistosome egg development could address these issues.

162 **Immature virgin females sexually mature and produce viable eggs upon pairing with male**
163 **worms in ABC169 medium.** Studying the process by which male schistosomes stimulate female
164 development has traditionally been challenging since females experience incomplete development
165 following pairing with a male *in vitro* [34, 35]. To examine sexual maturation in ABC169, we
166 recovered immature virgin females from mice and paired these females with sexually mature virgin
167 male worms. The vitellaria of paired immature females grown in BM169 were poorly developed
168 (Fig 3A) and these parasites laid small numbers of morphologically abnormal eggs (Fig 3B).
169 However, the vitellaria of newly paired immature virgin females cultured in ABC169 developed
170 normal vitellaria (Fig 3A) and these parasites were capable of laying morphologically normal eggs
171 beginning between D6-D7 of culture (Fig 3B-C). These eggs could initiate embryogenesis (Fig
172 3D, 42.7% n=1010 from D16 to D22) and about 2% could develop to miracidia (eggs from D11-
173 14, n=2807 eggs). Thus, ABC169 supports female development following pairing with a male
174 worm.

175 **Physical contact with a male worm is sufficient for female worms to produce viable**
176 **parthenogenetic haploid embryos.** Several theories have been put forward to explain the
177 mechanism by which male worms stimulate female development [2, 36-38]. However, the
178 experiments supporting (or refuting) these hypotheses were conducted using sub-optimal culture
179 conditions [37] and in many cases have not been subject to extensive reproduction in the modern
180 literature. Thus, we were compelled to revisit key observations using ABC169. The prevailing
181 thought is that female development requires direct contact with a male worm [7, 37, 38]. The most
182 intriguing study supporting this hypothesis are those of Popiel and Basch [35]. These authors
183 observed that small segments of male worms could stimulate vitelline development. Interestingly,
184 vitelline development was confined to regions in direct contact with the male segment [35].
185 Consistent with these observations, we found a large fraction of small posterior fragments could
186 pair with immature females (Fig 4A,B, S4 Movie). These posterior segments often paired with
187 posterior regions of female worms, and consistent with observations of Popiel and Basch, vitelline
188 maturation occurred only in regions in direct contact the male segment (Fig 4B). Thus, pairing
189 with a male induces a signal that induces localized female vitelline maturation.

190 While culturing male posterior segments with female worms we observed that these female
191 worms laid morphologically normal eggs (Fig 4C). However, examination of these eggs found that
192 they contained no embryos capable of incorporating EdU (Fig 4C). To explore this observation in
193 more detail we examined the ovaries of the female worms paired with male segments and found
194 no evidence of mature oocyte production (Fig 4D). Since the schistosome ovary is located anterior
195 to the vitellaria, and this region was not in contact with male posterior segments, we reasoned that
196 ovaries, like the vitellaria, might also require local contact with a male worm to mature and begin
197 oocyte production. To test this model, we amputated males behind the testes (Fig 4E) and paired
198 the decapitated and castrated fragments with immature female worms. Since these large posterior
199 fragments typically ensheathed the entire female worm (Fig 4E), we reasoned that this pairing
200 might be sufficient to stimulate oogenesis. Consistent with this model, we found that ovaries of
201 females paired with decapitated males produced oocytes (Fig 4F). Furthermore, these parasites
202 had fully developed vitellaria along their entire length (Fig 4G) and laid morphologically normal
203 eggs (Fig 4H).

204 Despite the fact that females paired with castrated male segments had no chance of being
205 inseminated, we observed that eggs laid by these parasites possessed the ability to initiate
206 embryogenesis (Fig 4H) and could even give rise to miracidia (0.23%, n=4704 eggs). Previous
207 studies have suggested that female schistosomes can produce parthenogenetic offspring containing
208 a haploid set of maternal chromosomes when mated with males of distantly related schistosome
209 species [39, 40]. From these experiments it is not clear if simply coming into contact with a male
210 schistosome of another species is sufficient to induce the production of parthenogenetic offspring
211 or if parthenogenesis occurs only following the transfer of sperm [39, 40]. Therefore, we examined
212 the karyotypes of eggs laid by females paired with castrated males. Unlike the diploid karyotypes
213 of embryos from eggs laid by freshly *ex vivo* parasites (2n=16), mitotic cells from eggs laid by
214 females paired with castrated males were haploid containing only 8 chromosomes (Fig 4I, S3 Fig).
215 These results suggest that only contact with a male worm along the entire length of the female
216 body, and not insemination, is sufficient for the production of viable haploid embryos. These data
217 highlight the value of ABC169 media to enhance our understanding of schistosome reproductive
218 biology.

219

220 **A novel nuclear receptor is required for the maturation of female vitellaria following pairing**
221 **with a male worm.**

222 The ability of virgin females to mature upon pairing with a male in ABC169 (Fig 3A) provides an
223 opportunity to discover novel regulators of male induced female maturation. In virgin female
224 worms, specialized stem cells located in primordial ovaries and vitellaria differentiate to oocytes
225 or vitellocytes, respectively, after paring with a male worm. Thus, the stem cells in the primordial
226 reproductive organs must be capable of responding to external cues instructing these cells to
227 produce differentiated progeny (i.e., oocytes or vitellocytes). Therefore, characterizing genes
228 expressed in these stem cells could provide clues about molecules essential for maturation
229 following pairing.

230 To discover genes expressed in the reproductive stem cells, we queried our previously
231 published dataset of genes expressed in the mature female vitellaria [26] and performed *in situ*
232 hybridization on mature and immature female worms. From this analysis, we identified
233 *Smp_248100* (previously known as *Smp_212440*), an uncharacterized protein that shares similarity
234 to members of the nuclear receptor family of ligand-activated transcription factors. Canonical
235 nuclear receptors contain two key domains: a highly-conserved N-terminal DNA-binding domain
236 (DBD) and a C-terminal ligand-binding domain (LBD)[41]. Primary sequence analysis confirmed
237 that Smp_248100 contained a DBD with high amino acid identity to DBDs from other vertebrate
238 and invertebrate nuclear receptors (Fig 5A and S4A Fig). However, examination of the
239 Smp_248100 C-terminus failed to identify a canonical LBD. Interestingly, the C-terminus of
240 Smp_248100 shared stretches of high amino acid identity with orthologous proteins from other
241 parasitic flatworms (S4B Fig), leaving open the possibility that this protein contains a divergent
242 LBD.

243 By colorimetric *in situ* hybridization, we detected *Smp_248100* expression exclusively in
244 the vitellaria of mature and immature female (Fig 5B). We failed to detect high-levels of specific
245 expression in male parasites (Fig 5B). In immature females, the proliferative stem cells of the
246 primordial vitellaria, also known as S1 cells [24], lie along the intestine in the posterior of the
247 worm [16]. Thus, based on the expression of *Smp_248100* in immature females we reasoned that
248 this gene is expressed in the S1 stem cells of the primordial vitellaria. To examine this model, we
249 used a proliferative cell marker, *Histone H2B*, to label the proliferative S1 cells [26]. Double
250 fluorescent *in situ* hybridization (FISH) on immature female worms found that most *H2B* $^{+}$ cells
251 along the gut also expressed *Smp_248100* (Fig 5C), indicating *Smp_248100* is expressed in S1
252 cells within the primordial vitellaria. In mature females, we similarly detected *Smp_248100* in
253 *H2B*$^{+}$ S1 cells, however, we also detected *Smp_248100* expression in *H2B*$^{-}$ cells adjacent to *H2B*$^{+}$
254 cells (Fig 5D). Since we did not detect *Smp_248100* expression in fully mature vitellocytes with
255 in the vitelline duct (Fig 5D), we suggest that *Smp_248100*$^{+}$ *H2B*$^{-}$ cells are likely the immediate
256 differentiation progeny of the S1 cells.

257 Based on the expression pattern, we hypothesized that *Smp_248100* may play a role in
258 vitelline cell development. To examine this possibility, we performed RNA interference (RNAi)
259 on immature virgin female worms in ABC169 and examined whether these parasites could become
260 sexually mature and commence egg-laying upon pairing with male worm. We observed majority
261 of the *Smp_248100(RNAi)*-treated female parasites failed to produce mature vitellocytes as
262 measured by Fast Blue BB staining (Fig 6A, B, S5 Fig) and *in situ* hybridization using the
263 vitellocyte marker *superoxide dismutase* [26] (Fig 6B). Consistent with the effects of
264 *Smp_248100* RNAi treatment on vitelline cell development, we noted a significant decline in the

265 rate of egg production compared to controls (Fig 6C). Since *Smp_248100* is expressed in the
266 proliferative S1 cells, we reasoned that the *Smp_248100(RNAi)* may result in either defects stem
267 cell maintenance or a defect in the ability of these cell to differentiate. To distinguish between
268 these possibilities, we examined cell proliferation in the region of the vitellaria following
269 *Smp_248100* dsRNA treatment. We observed no difference in cell proliferation in the region of
270 the vitellaria in *Smp_248100(RNAi)* parasites (Fig 6D), suggesting that these gene is dispensable
271 for S1 cell maintenance. Thus, *Smp_248100* appears to be a key regulator of stem cell
272 differentiation in the schistosome vitellaria. We also examined the effects of *Smp_248100* dsRNA
273 treatment on ovary development. Consistent with a lack of robust *Smp_248100* expression in the
274 ovary, we detected no defect in the ability of *Smp_248100* RNAi-treated parasites to produce
275 differentiated oocytes (Fig 6E). Given this specific role for Smp_248100 in vitelline cell
276 development we propose this protein be called Vitellogenic Factor 1 (VF1).

277 **DISCUSSION**

278 Here we describe culture conditions that support sustained schistosome sexual
279 development i*n vitro*. Of all the components of ABC media, L-ascorbic acid appeared to have the
280 most profound effect sexual development and egg production. Since the effects of L-ascorbic acid
281 are stereoselective (Fig 1F), it is likely its effects are related to either the transport of L-ascorbic
282 acid into the cell or its activity on specific biological molecules. One potential target of L-ascorbic
283 acid is the tyrosinase enzymes present in mature vitellocytes. During the production of
284 schistosome egg shells, tyrosinases in the vitellocytes are essential for crosslinking eggshell
285 proteins that coalesce to form the eggshell [42, 43]. *In vitro* studies on tyrosinases suggest that L-
286 ascorbic acid can enhance the activity of tyrosinases by reducing copper molecules in the active
287 site of the enzyme [44]. Consistent with the model that L-ascorbic acid potentiates schistosome
288 tyrosinase activity, inhibition of tyrosinase activity in female schistosomes results in eggshell
289 deformations [43] that appear similar to eggs from schistosomes grown *in vitro* without L-ascorbic
290 acid (Fig 1A). Future studies aimed at determining the function of vitamin C in schistosomes
291 could suggest new approaches to blunt egg production.

292 Our studies confirmed the importance direct physical contact with a male in inducing
293 female sexual maturation. Indeed, we found that contact with a castrated male along the entire
294 length of the female body was sufficient to induce sexual maturation and the production of
295 parthenogenetic haploid embryos (Fig 4I). The phenomenon of parthenogenetic sexual
296 reproduction in *Schistosoma mansoni* has been observed several times in studies performing
297 intraspecies crosses (e.g., female *S. mansoni* x male *S. japonicum*) inside mammal hosts [39, 40,
298 45]. However, given the propensity of certain schistosome species to form intraspecies hybrids
299 [46], it is challenging to distinguish between genetic hybridization and true parthenogenesis. Since
300 our studies were conducted entirely under *in vitro* conditions using virgin females and castrated
301 males we believe these are the strongest evidence to date that female schistosomes require only
302 contact with a male worm to generate viable haploid progeny. As studies in the field continue to
303 explore the genetics of schistosomes in regions endemic for multiple schistosome species [47], it
304 will be interesting to see if the production of parthenogenetic haploid offspring is common in
305 natural schistosome populations.

306 Here we demonstrate an essential role for the nuclear receptor VF1 in the differentiation of
307 schistosome vitellocytes. Several nuclear receptors (e.g, the estrogen receptor) are activated by
308 sex hormones and go on to elicit transcriptional responses key to sexual development [48]. Given
309 this, it is tantalizing to speculate that that VF1 executes a pro-vitellogenic transcriptional program

310 in response to activation by a schistosome sex hormone. Since our analyses sequence failed to
311 identify a canonical LBD in the C-terminus of VF1, it is possible that VF1 lacks the capacity to
312 bind traditional hormone receptor ligands (e.g., steroids). However, the possibility remains that
313 VF1 is activated by a non-canonical nuclear receptor ligand. Indeed, examination of VF1
314 orthologs in other parasitic flatworms uncovered a region of extensive amino acid identity in the
315 C-termini of these proteins (S4B Fig). Since these other flatworms also possess vitellaria, and the
316 evolution of vitellaria is a relatively recent innovation in flatworm biology [4, 49], it is possible
317 that during vitellaria evolution these VF1 proteins adapted to being activated by a non-canonical
318 ligand. In addition to their ability bind ligands, nuclear receptors are notorious for their propensity
319 to bind DNA as either homo- or heterodimers [41]. Thus, VF1 may lack ligand-binding activity
320 but retain the ability to heterodimerize with other ligand-activated nuclear receptors. One set of
321 potential heterodimerization partners for VF1 are the schistosome RXR nuclear receptors. Indeed,
322 RXR proteins heterodimerize with the variety of NRs [41] and the two schistosome RXR proteins
323 are known to bind the promoters of genes encoding egg shell proteins in the vitellaria [50, 51].
324 Thus, there is the possibility these nuclear receptors collaborate to coordinate pro-vitellogenic cell
325 fates.

326 What role might VF1 play in male-induced female maturation? Clearly VF1 is essential
327 for vitelline cell development following pairing with a male worm. However, we did not detect
328 *vf1* expression in the ovary and our RNAi experiments suggest that oogenesis is not affected in
329 *vf1(RNAi)* parasites. Thus, VF1 is not likely to be a global regulator in the female response to
330 pairing. A more likely scenario is that VF1 is activated by pairing, resulting in transcriptional
331 changes important to vitellogenesis in the primordial vitellaria. Based on this model, we would
332 also speculate there are ovary-specific transcriptional regulators that are activated upon pairing
333 within the primordial ovary. Capitalizing on ABC media, and modern molecular approaches,
334 future studies should be aimed at uncovering the general upstream signaling programs that activate
335 factors, such as VF1, in the primordial reproductive organs.

336 During his extensive studies to develop *in vitro* culture conditions for *S. mansoni*, Paul
337 Basch lamented that the production of viable eggs *in vitro* "remains a formidable challenge" [22].
338 We find three additions to Basch's base media formulation (ascorbic acid, blood cells, and LDL)
339 can sustain the production of viable eggs for several weeks *in vitro*. Although ABC169 does not
340 fully replicate the *in vivo* reproductive potential of the parasite, it allows us to recapitulate
341 important aspects of schistosome sexual biology *in vitro* and thus represents a significant
342 improvement over existing culture methods. Taken together, the ability to sustain sexual maturity
343 *in vitro,* coupled with a growing understanding of the molecular programs associated with distinct
344 reproductive states [52, 53], and tools such as RNAi, will expedite our understanding of the
345 molecular factors governing parasite sexual development and egg production, perhaps suggesting
346 new therapeutic opportunities.
347
348
349
350
351

352 **Materials and Methods**

353 **Parasites and *in vitro* maintenance.** Adult NMRI *Schistosoma mansoni* (6-7 weeks post infection)
354 were recovered from infected mice by perfusion through hepatic portal vein with 37°C DMEM
355 (Mediatech, Manassas, VA) plus 5% serum and heparin (200–350 U/ml). Single sex infections
356 were obtained by infecting mice with male or female cercariae recovered from NMRI
357 *Biomphalaria glabrata* snails infected with single miracidia. Following recovery from the host,
358 worms were rinsed several times in DMEM + 5% serum before placing into culture media. The
359 base medium for these studies was BM169 [21] with the addition of 1x Antibiotic-Antimycotic
360 (Gibco/Life Technologies, Carlsbad, CA). Although Basch included red blood cells in BM169, it
361 has been noted that erythrocytes, which schistosomes consume *in vivo*, are not sufficient to sustain
362 egg production [16, 22, 27] and were initially omitted from the base medium. We explored sera
363 from a variety of sources (horse, fetal bovine, chicken) and determined that newborn calf serum
364 (Sigma-Aldrich, St. Louis, MO) both performed well and was cost efficient. For ABC169, BM169
365 was supplemented with 200 µM ascorbic acid (Sigma-Aldrich, St. Louis, MO), 0.2% V/V bovine
366 washed red blood cells (10% suspension, Lampire Biological Laboratories, Pipersville, PA) and
367 0.2% V/V Porcine cholesterol concentrate (Rocky Mountain Biologicals, Missoula, MT). Ascorbic
368 acid was added from a 500 mM stock dissolved in BM169 that was stored at 4ºC in the dark for
369 less than 2 weeks. For regular maintenance, 4-6 worm pairs were cultured at 37ºC in 5% CO_2 in 3
370 ml media in a 12-well plate; media was changed every other day. To count egg-laying rates,
371 individual worm pairs were maintained in 24 well plates in 1 ml of media and eggs were removed
372 for counting every 24 h during a media change. Worms that separated or became ill during
373 cultivation were excluded from analysis.

374 **Molecular biology.** For quantification of gene expression, RNA was reverse transcribed (iScript,
375 Biorad) and Quantitative PCR was performed using an Applied Biosystems Quantistudio3
376 instrument and PowerUp SYBR Green Master mix (ThermoFisher, Carlsbad, CA). Gene
377 expression was normalized to the expression of a proteasome subunit (Smp_056500) and relative
378 gene expression values and statistical analyses were performed using the Quantistudio Design and
379 Analysis software (ThermoFisher, Carlsbad, CA). The heatmap depicting relative gene expression
380 was generated using R. Oligonucleotide sequences were listed in S1 table.

381 **Parasite labeling and imaging.** Whole-mount *in situ* hybridization was performed as previously
382 described [26, 54]. ImageJ was used to quantify egg size, autofluorescence, and nuclei number
383 from images of eggs acquired on a Nikon A1+ laser scanning confocal microscope. Like
384 previously described diazo salts [55, 56], Fast Blue BB strongly labeled the polyphenol rich
385 vitelline droplets of mature vitellocytes and could be visualized as both by bright field and
386 fluorescence microscopy. For Fast Blue BB staining, female worms were separated from males
387 using 0.25% tricaine in BM169 and fixed for 4 hrs in 4% Formaldehyde in PBS+0.3% Triton X-
388 100 (PBSTx). Parasites were washed in PBSTx for 10 mins, stained in freshly-made filtered 1%
389 Fast Blue BB in PBSTx for 5 mins and rinsed in PBSTx 3 times. Worms were incubated with 1
390 µg/ml DAPI in PBSTx for 2hrs, cleared in 80% glycerol in PBS and mounted on slides. For Fast
391 Blue BB/BODIPY 493/503 staining, parasites were processed similarly expect detergents were
392 omitted from all buffers; parasites were labeled with 1 µg/ml BODIPY 493/503 (ThermoFisher,
393 Carlsbad, Ca) in PBS for 1hr following Fast Blue BB staining. For EdU labeling, eggs within 24-
394 48 hours of being laid were incubated in Medium 199 supplemented with 10 µM 5-Ethynyl-2′-
395 deoxyuridine (EdU) and incubated overnight. The following day the eggs were collected by
396 centrifugation at 10,000g for 1min, fixed in 4% formaldehyde in PBSTx for 4hrs, EdU was

397 detected as previously described [54], and washed in PBSTx. *in situ* hybridizations and colormetic
398 detection of Fast Blue BB were imaged using a Zeiss AxioZoom V16 equipped with a transmitted
399 light base and a Zeiss AxioCam 105 Color camera. All other images were acquired using a Nikon
400 A1+ laser scanning confocal microscope.

401 **Egg culture, miracidia hatching, and labeling.** Eggs were collected on 10 µm cell strainer rinsed
402 into Medium 199 (Corning) containing 10% Fetal Bovine Serum and 1x Antibiotic-Antimycotic
403 (Gibco/Life Technologies, Carlsbad, CA) for 7 days at 37ºC in 5% CO_2. After culture, eggs were
404 collected on 10 µm cell strainer and rinsed with artificial pond water 1-2 times and placed in
405 artificial pond water under light. A small aliquot of eggs was counted to determine the total number
406 of eggs examined. Active miracidia were observed and counted under light microscopy every 30
407 mins for up to 4hrs. Miracidia fixation and labeling was performed as previously described [57].

408 **Karyotype analysis**
409 Karyotypes from schistosome embryos were determined using a modified version of a previously
410 published method [58]. Briefly, eggs were incubated in M199 for two days after deposition and
411 then incubated in 5 µM Nocodazole for 1 hour at 37ºC. Eggs were pelleted at 20,000 g for 1 min,
412 rinsed in DI water, pelleted, resuspended in 1ml of water, and incubated for 20 min at RT.
413 Following centrifugation, the pelleted eggs were resuspended in 1 ml of fixative (3:1
414 methanol:acetic acid) for 15 minutes. Eggs were again pelleted and resuspended in 100 µl of
415 fixative and the eggshells were disrupted using a Kontes pellet pestle (DWK Life Sciences,
416 Rockwood, TN). The disrupted eggshells were allowed to settle for 10 min, the supernatant was
417 collected, and centrifuged for 8 min at 240 g. All but ~20 µl of supernatant was removed and the
418 remaining liquid was pipetted dropwise on to a glass slide (Superfrost Plus, ThermoFisher,
419 Carlsbad, CA) freshly dipped in PBS. After air-drying chromosomes were labeled with
420 Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and imaged on a Nikon A1+
421 laser scanning confocal microscope with a 60x/1.4 NA objective.
422
423 **RNA Interference**
424 Double stranded RNA was generated as previously described [54, 59]. For dsRNA treatment 12
425 immature female from single-sex infections were soaking in 1 ml Basch 169 supplemented with
426 1% FBS containing 60 µg/ml of dsRNA for 24 hrs. Then 3 virgin female worms were co-cultured
427 with 3 adult male worms in 3mL ABC169 media with 10% FBS supplemented with 60 µg/ml of
428 dsRNA on day 1, 2, 6 and 10. At D4, unpaired female worms were discarded. At D14, parasites
429 were pulsed for 4 hours with EdU and processed as previously described [54]. Eggs laid between
430 D12 to D14 were collected to determine egg-laying rates.
431
432
433 **ACKNOWLEDGMENTS.** We dedicate this manuscript to the memory of Paul Basch. We thank
434 Melanie Cobb, Michael Reese and members of Collins laboratory for comments on the manuscript.
435 Mice and *B. glabrata* snails were provided by the NIAID Schistosomiasis Resource Center of the
436 Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract
437 HHSN272201000005I for distribution through BEI Resources. The work was supported by the
438 National Institutes of Health (R01 R01AI121037), the Wellcome Trust (107475/Z/15/Z) and the
439 Welch Foundation (I-1948-20180324).
440

441 **REFERENCES**

442 1. Hotez PJ, Fenwick A. Schistosomiasis in Africa: an emerging tragedy in our new global
443 health decade. PLoS Negl Trop Dis. 2009;3(9):e485. Epub 2009/09/30. doi:
444 10.1371/journal.pntd.0000485. PubMed PMID: 19787054; PubMed Central PMCID:
445 PMC2746322.
446 2. Basch PF. Schistosomes: Development, Reproduction, and Host Relations. New York:
447 Oxford University Press; 1991. 248 p.
448 3. Pearce EJ, MacDonald AS. The immunobiology of schistosomiasis. Nat Rev Immunol.
449 2002;2(7):499-511. doi: 10.1038/nri843. PubMed PMID: 12094224.
450 4. Collins JJ, 3rd. Platyhelminthes. Curr Biol. 2017;27(7):R252-R6. doi:
451 10.1016/j.cub.2017.02.016. PubMed PMID: 28376328.
452 5. Grevelding CG. *Schistosoma*. Curr Biol. 2004;14(14):R545. doi:
453 10.1016/j.cub.2004.07.006. PubMed PMID: 15268869.
454 6. Severinghaus AE. Sex Studies on *Schistosoma japonicum*. Quarterly Journal of
455 Microscopical Science. 1928;71:653-702.
456 7. LoVerde PT. Sex and schistosomes: an interesting biological interplay with control
457 implications. J Parasitol. 2002;88(1):3-13. doi: 10.1645/0022-
458 3395(2002)088[0003:PASASA]2.0.CO;2. PubMed PMID: 12053976.
459 8. Wendt GR, Collins JJ, 3rd. Schistosomiasis as a disease of stem cells. Curr Opin Genet
460 Dev. 2016;40:95-102. doi: 10.1016/j.gde.2016.06.010. PubMed PMID: 27392295; PubMed
461 Central PMCID: PMCPMC5135665.
462 9. Clough ER. Morphology and reproductive organs and oogenesis in bisexual and
463 unisexual transplants of mature *Schistosoma mansoni* females. J Parasitol. 1981;67(4):535-9.
464 Epub 1981/08/01. PubMed PMID: 7264839.
465 10. LoVerde PT, Andrade LF, Oliveira G. Signal transduction regulates schistosome
466 reproductive biology. Curr Opin Microbiol. 2009;12(4):422-8. Epub 2009/07/07. doi: S1369-
467 5274(09)00082-4 [pii]
468 10.1016/j.mib.2009.06.005. PubMed PMID: 19577949; PubMed Central PMCID: PMC2740793.
469 11. Beckmann S, Quack T, Burmeister C, Buro C, Long T, Dissous C, et al. *Schistosoma*
470 *mansoni*: signal transduction processes during the development of the reproductive organs.
471 Parasitology. 2010;137(3):497-520. Epub 2010/02/19. doi: S0031182010000053 [pii]
472 10.1017/S0031182010000053. PubMed PMID: 20163751.
473 12. Grevelding CG, Langner S, Dissous C. Kinases: Molecular Stage Directors for
474 Schistosome Development and Differentiation. Trends Parasitol. 2018;34(3):246-60. Epub
475 2017/12/26. doi: 10.1016/j.pt.2017.12.001. PubMed PMID: 29276074.
476 13. Michaels RM, Prata A. Evolution and characteristics of *Schistosoma mansoni* eggs laid *in*
477 *vitro*. J Parasitol. 1968;54(5):921-30. PubMed PMID: 5761119.
478 14. Irie Y, Tanaka M, Yasuraoka K. Degenerative changes in the reproductive organs of
479 female schistosomes during maintenance *in vitro*. J Parasitol. 1987;73(4):829-35. PubMed
480 PMID: 3625433.
481 15. Basch PF, Humbert R. Cultivation of *Schistosoma mansoni in vitro*. III. implantation of
482 cultured worms into mouse mesenteric veins. J Parasitol. 1981;67(2):191-5. PubMed PMID:
483 7241279.
484 16. Galanti SE, Huang SC, Pearce EJ. Cell death and reproductive regression in female
485 *Schistosoma mansoni*. PLoS Negl Trop Dis. 2012;6(2):e1509. doi:

486 10.1371/journal.pntd.0001509. PubMed PMID: 22363825; PubMed Central PMCID:
487 PMCPMC3283563.
488 17. Senft AW, Weller TH. Growth and regeneration of *Schistosoma mansoni* in vitro. Proc
489 Soc Exp Biol Med. 1956;93(1):16-9. PubMed PMID: 13370563.
490 18. Cheever AW, Weller TH. Observations on the growth and nutritional requirements of
491 *Schistosoma mansoni in vitro*. Am J Hyg. 1958;68(3):322-39. PubMed PMID: 13594932.
492 19. Robinson DL. Egg-laying by *Schistosoma mansoni in vitro*. Ann Trop Med Parasitol.
493 1960;54:112-7. PubMed PMID: 14437934.
494 20. Clegg JA. *in vitro* Cultivation of *Schistosoma mansoni*. Exp Parasitol. 1965;16:133-47.
495 PubMed PMID: 14281210.
496 21. Basch PF. Cultivation of *Schistosoma mansoni in vitro*. I. Establishment of cultures from
497 cercariae and development until pairing. J Parasitol. 1981;67(2):179-85. Epub 1981/04/01.
498 PubMed PMID: 7241277.
499 22. Basch PF. Cultivation of *Schistosoma mansoni in vitro*. II. production of infertile eggs by
500 worm pairs cultured from cercariae. J Parasitol. 1981;67(2):186-90. PubMed PMID: 7241278.
501 23. Ashton PD, Harrop R, Shah B, Wilson RA. The schistosome egg: development and
502 secretions. Parasitology. 2001;122(Pt 3):329-38. PubMed PMID: 11289069.
503 24. Erasmus DA. *Schistosoma mansoni*: development of the vitelline cell, its role in drug
504 sequestration, and changes induced by Astiban. Exp Parasitol. 1975;38(2):240-56. PubMed
505 PMID: 1175727.
506 25. Smyth JD, Clegg JA. Egg-shell formation in trematodes and cestodes. Exp Parasitol.
507 1959;8(3):286-323. PubMed PMID: 13663910.
508 26. Wang J, Collins JJ, 3rd. Identification of new markers for the *Schistosoma mansoni*
509 vitelline lineage. Int J Parasitol. 2016;46(7):405-10.
510 27. Huang SC, Freitas TC, Amiel E, Everts B, Pearce EL, Lok JB, et al. Fatty acid oxidation
511 is essential for egg production by the parasitic flatworm *Schistosoma mansoni*. PLoS Pathog.
512 2012;8(10):e1002996. doi: 10.1371/journal.ppat.1002996. PubMed PMID: 23133378; PubMed
513 Central PMCID: PMC3486914.
514 28. Meyer F, Meyer H, Bueding E. Lipid metabolism in the parasitic and free-living
515 flatworms, Schistosoma mansoni and Dugesia dorotocephala. Biochim Biophys Acta.
516 1970;210(2):257-66. PubMed PMID: 4319989.
517 29. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, et al. The
518 genome of the blood fluke *Schistosoma mansoni*. Nature. 2009;460(7253):352-8. Epub
519 2009/07/17. doi: nature08160 [pii]
520 10.1038/nature08160. PubMed PMID: 19606141.
521 30. Michael AJ. Polyamines in Eukaryotes, Bacteria, and Archaea. J Biol Chem.
522 2016;291(29):14896-903. doi: 10.1074/jbc.R116.734780. PubMed PMID: 27268252; PubMed
523 Central PMCID: PMCPMC4946907.
524 31. Krakower C, Hoffman WA, Axtmayer JH. Defective granular eggshell formation by
525 *Schistosoma mansoni* in experimentally infected guinea pigs on a vitamin C deficient diet. The
526 Journal of Infectious Diseases. 1944;74(3):178-83. doi: 10.1093/infdis/74.3.178.
527 32. Arrigoni O, De Tullio MC. Ascorbic acid: much more than just an antioxidant. Biochim
528 Biophys Acta. 2002;1569(1-3):1-9. PubMed PMID: 11853951.
529 33. Goldman HM, Gould BS, Munro HN. The antiscorbutic action of L-ascorbic acid and D-
530 isoascorbic acid (erythorbic acid) in the guinea pig. Am J Clin Nutr. 1981;34(1):24-33. doi:
531 10.1093/ajcn/34.1.24. PubMed PMID: 7446456.

532 34. Shaw JR. *Schistosoma mansoni*: pairing in vitro and development of females from single
533 sex infections. Exp Parasitol. 1977;41(1):54-65. PubMed PMID: 838033.
534 35. Popiel I, Basch PF. Reproductive development of female *Schistosoma mansoni* (Digenea:
535 Schistosomatidae) following bisexual pairing of worms and worm segments. J Exp Zool.
536 1984;232(1):141-50. doi: 10.1002/jez.1402320117. PubMed PMID: 6502090.
537 36. Ribeiro-Paes JT, Rodrigues V. Sex determination and female reproductive development
538 in the genus *Schistosoma*: a review. Rev Inst Med Trop Sao Paulo. 1997;39(6):337-44. PubMed
539 PMID: 9674285.
540 37. Popiel I. Male-stimulated female maturation in *Schistosoma*: A review. J Chem Ecol.
541 1986;12(8):1745-54. doi: 10.1007/BF01022380. PubMed PMID: 24305892.
542 38. Kunz W. Schistosome male-female interaction: induction of germ-cell differentiation.
543 Trends Parasitol. 2001;17(5):227-31. Epub 2001/04/27. doi: S1471-4922(01)01893-1 [pii].
544 PubMed PMID: 11323306.
545 39. Basch PF, Basch N. Intergeneric reproductive stimulation and parthenogenesis in
546 *Schistosoma mansoni*. Parasitology. 1984;89 (Pt 2):369-76. PubMed PMID: 6504564.
547 40. Tchuente LA, Imbert-Establet D, Southgate VR, Jourdane J. Interspecific stimulation of
548 parthenogenesis in Schistosoma intercalatum and S. mansoni. J Helminthol. 1994;68(2):167-73.
549 Epub 1994/06/01. PubMed PMID: 7930460.
550 41. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The
551 nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835-9. PubMed PMID:
552 8521507.
553 42. Eshete F, LoVerde PT. Characteristics of phenol oxidase of *Schistosoma mansoni* and its
554 functional implications in eggshell synthesis. J Parasitol. 1993;79(3):309-17. PubMed PMID:
555 8501588.
556 43. Fitzpatrick JM, Hirai Y, Hirai H, Hoffmann KF. Schistosome egg production is
557 dependent upon the activities of two developmentally regulated tyrosinases. FASEB J.
558 2007;21(3):823-35. doi: 10.1096/fj.06-7314com. PubMed PMID: 17167065.
559 44. Ramsden CA, Riley PA. Tyrosinase: the four oxidation states of the active site and their
560 relevance to enzymatic activation, oxidation and inactivation. Bioorg Med Chem.
561 2014;22(8):2388-95. doi: 10.1016/j.bmc.2014.02.048. PubMed PMID: 24656803.
562 45. Imbert-Establet D, Xia M, Jourdane J. Parthenogenesis in the genus Schistosoma:
563 electrophoretic evidence for this reproduction system in S. japonicum and S. mansoni. Parasitol
564 Res. 1994;80(3):186-91. Epub 1994/01/01. PubMed PMID: 8036230.
565 46. Southgate VR, Jourdane J, Tchuente LA. Recent studies on the reproductive biology of
566 the schistosomes and their relevance to speciation in the Digenea. Int J Parasitol.
567 1998;28(8):1159-72. Epub 1998/10/08. PubMed PMID: 9762560.
568 47. Catalano S, Sene M, Diouf ND, Fall CB, Borlase A, Leger E, et al. Rodents as Natural
569 Hosts of Zoonotic Schistosoma Species and Hybrids: An Epidemiological and Evolutionary
570 Perspective From West Africa. J Infect Dis. 2018;218(3):429-33. Epub 2018/01/25. doi:
571 10.1093/infdis/jiy029. PubMed PMID: 29365139.
572 48. Couse JF, Korach KS. Exploring the role of sex steroids through studies of receptor
573 deficient mice. J Mol Med (Berl). 1998;76(7):497-511. Epub 1998/07/11. PubMed PMID:
574 9660168.
575 49. Hyman L. The Invertebrates: Platyhelminthes and Rhynchocoela The Acoelomate
576 Bilateria. New York: McGraw-Hill Book Company, Inc.; 1951. 550 p.

577 50. Fantappie MR, Freebern WJ, Osman A, LaDuca J, Niles EG, LoVerde PT. Evaluation of
578 Schistosoma mansoni retinoid X receptor (SmRXR1 and SmRXR2) activity and tissue
579 distribution. Mol Biochem Parasitol. 2001;115(1):87-99. Epub 2001/05/30. PubMed PMID:
580 11377743.
581 51. Freebern WJ, Osman A, Niles EG, Christen L, LoVerde PT. Identification of a cDNA
582 encoding a retinoid X receptor homologue from *Schistosoma mansoni*. Evidence for a role in
583 female-specific gene expression. J Biol Chem. 1999;274(8):4577-85. Epub 1999/02/13. PubMed
584 PMID: 9988692.
585 52. Lu Z, Sessler F, Holroyd N, Hahnel S, Quack T, Berriman M, et al. A gene expression
586 atlas of adult *Schistosoma mansoni* and their gonads. Sci Data. 2017;4:170118. doi:
587 10.1038/sdata.2017.118. PubMed PMID: 28829433; PubMed Central PMCID:
588 PMCPMC5566097.
589 53. Lu Z, Sessler F, Holroyd N, Hahnel S, Quack T, Berriman M, et al. Schistosome sex
590 matters: a deep view into gonad-specific and pairing-dependent transcriptomes reveals a
591 complex gender interplay. Sci Rep. 2016;6:31150. doi: 10.1038/srep31150. PubMed PMID:
592 27499125; PubMed Central PMCID: PMCPMC4976352.
593 54. Collins JJ, III, Wang B, Lambrus BG, Tharp ME, Iyer H, Newmark PA. Adult somatic
594 stem cells in the human parasite *Schistosoma mansoni*. Nature. 2013;494(7438):476-9. doi:
595 10.1038/nature11924. PubMed PMID: 23426263; PubMed Central PMCID: PMC3586782.
596 55. Johri LN, Smyth JD. A histochemical approach to the study of helminth morphology.
597 Parasitology. 1956;46(1-2):107-16. PubMed PMID: 13322460.
598 56. Wang J, Wang S, Liu X, Xu B, Chai R, Zhou P, et al. Intake of Erythrocytes Required for
599 Reproductive Development of Female *Schistosoma japonicum*. PLoS One.
600 2015;10(5):e0126822. doi: 10.1371/journal.pone.0126822. PubMed PMID: 25978643; PubMed
601 Central PMCID: PMCPMC4433235.
602 57. Collins JJ, III, King RS, Cogswell A, Williams DL, Newmark PA. An atlas for
603 *Schistosoma mansoni* organs and life-cycle stages using cell type-specific markers and confocal
604 microscopy. PLoS Negl Trop Dis. 2011;5(3):e1009. Epub 2011/03/17. doi:
605 10.1371/journal.pntd.0001009. PubMed PMID: 21408085; PubMed Central PMCID:
606 PMC3050934.
607 58. Short RB, Grossman AI. Conventional giemsa and C-banded karyotypes of *Schistosoma*
608 *mansoni* and *S. rodhaini*. J Parasitol. 1981;67(5):661-71. PubMed PMID: 7299577.
609 59. Collins JJ, III, Hou X, Romanova EV, Lambrus BG, Miller CM, Saberi A, et al. Genome-
610 Wide Analyses Reveal a Role for Peptide Hormones in Planarian Germline Development. PLoS
611 Biol. 2010;8(10):e1000509.

612
613

614 **FIGURE LEGENDS**
615
616 **Fig 1. ABC169 supports the maintenance of schistosome vitellaria.**
617 (A) Morphological changes of eggs laid by worm pairs maintained in BM169. After D6 parasites
618 began laying abnormally formed eggs (arrow). These eggs were small, usually did not contain a
619 lateral spine, and did not possess a smooth surface.
620 (B) Fast Blue BB staining (brownish-red labeling) showing loss of mature vitellocytes during
621 culture in BM169. Representative images from three experiments with n > 10 parasites.
622 (C) Confocal slice showing Fast Blue BB and BODIPY labeling in the vitellaria of freshly
623 recovered parasites and in schistosomes at D20 of cultivation in BM169 supplemented with RBCs
624 (Red Blood Cells), LDL, and/or ascorbic acid. Representative images from three experiments with
625 n > 10 parasites.
626 (D) Whole-mount *in situ* hybridization showing expression of vitellaria-enriched genes in freshly
627 perfused female worms and parasites cultured in ABC169 or BM169 for 20 days. Representative
628 images from three experiments with n ≥ 9 parasites.
629 (E) Heat-map showing relative expression of vitellaria-enriched transcripts in freshly perfused
630 female worms ("Fresh") and parasites cultured in ABC169 or BM169 for 20 days. Each column
631 represents an independent biological replicate; samples are normalized to the expression of an
632 arbitrarily chosen biological replicate from the BM169 group. Changes in expression between
633 BM169 and ABC169 were statistically significant ($p<0.05$, T-test).
634 (F) Morphology of eggs laid by paired adult females in medium ABC169 supplemented with L-
635 ascorbic acid or D-isoascorbic acid on D20. Representative of 3 experiments.
636 Scale bars: A, B, D, F 100µm; C 25µm.
637
638 **Fig 2. Eggs laid in ABC169 develop and hatch.**
639 (A) Rate of egg production per worm pair in BM169 or medium ABC169 during *in vitro* culture.
640 Dotted line indicates the point at which parasites beginning laying morphologically abnormal eggs.
641 n ≥ 19 worm pairs examined in 4 experiments. Error bars represent 95% confidence intervals.
642 (B) Morphology of eggs (Left, bright field; Right, DIC) laid by paired adult females in BM169 or
643 medium ABC169 on D20. Red arrows show early embryos in eggs laid in ABC169.
644 (C-E) Quantification of the (C) size, (D) number of DAPI-labeled nuclei, and (E) autofluorescence
645 intensity from eggs laid by freshly perfused female worms (Fresh, n=41), parasites cultured in
646 BM169 (n=61), or ABC169 (n=59) on D20. **** $p<0.0001$, T-Test. Error bars represent 95%
647 confidence intervals.
648 (F) EdU-labeled embryonic cells of an egg laid by a paired adult female in ABC169 on D20.
649 (G) Percentage of eggs with clusters of cycling EdU^+ embryonic cells from eggs laid between
650 D16 and D22 by paired adult females in BM169 or ABC169. >2000 eggs from 6 experiments.
651 Error bars represent 95% confidence interval.
652 (H) Miracidia from eggs laid in ABC169. Left, miracidium (pseudocolored red) inside an egg
653 laid on D20 of culture in ABC169. Right, hatched miracidium from D20 egg labeled with DAPI
654 and phalloidin. These miracidia appear grossly normal in morphology possessing 2 pairs of
655 flame cells (arrows).
656 Scale bars: B, F, H 20 µm; C-E 50 µm.
657
658 **Fig 3. ABC169 supports the maturation of immature female schistosomes following pairing**
659 **with a male worm.**

660 (A)Vitellaria development visualized by Fast Blue BB labeling in immature female schistosomes
661 in the presence or absence of male worms in BM169 or ABC169 at D20 of culture. Black pigment
662 in ABC169 parasites represents digested RBCs.
663 (B) Eggs laid by immature female in BM169 or ABC169 on day 20 after pairing with a male.
664 (C) Proportion of females laying eggs (left axis, magenta) and number of eggs laid per worm pair
665 (right axis, green) from D1 to D20 following pairing of immature females with male worms in
666 ABC169. n=31 worm pairs examined in 4 separate experiments. Error bars represent 95%
667 confidence intervals.
668 (D) Eggs laid by previously immature females commence embryonic development. Left,
669 representative developing EdU-labeled embryo from egg laid in ABC169 on D20.
670 Scale bars: A,B 100 µm; D 20 µm.
671
672 **Fig 4. Using ABC169 to study schistosome male-induced female sexual maturation.**
673 (A) Pairing of immature females with amputated male segments. Left, cartoon showing
674 approximate positions of male amputations. Most often we observed posterior segments pairing
675 with posterior region of immature females, shown to right.
676 (B) Fast Blue BB labeling showing female vitellaria development after pairing with posterior
677 male segments. Left panels, female after separation from male segment showing vitellaria
678 development in posterior region. Right panel, female before separation from male segment
679 showing vitellaria development is restricted to paired region. Representative of > 40 female
680 parasites examined in 4 separate experiments.
681 (C) Eggs laid by immature female worms paired with male segments are morphologically normal
682 (left) but do not contain developing embryos as measured by EdU labeling (right). n > 500 eggs
683 examined in 4 separate experiments.
684 (D) DAPI staining to examine ovary development in immature females paired with intact male
685 worms or an amputated posterior male segment. Females paired with intact males possess
686 differentiated oocytes (DO) (n=23), whereas females paired with male segments produce no
687 differentiated oocytes (n=41).
688 (E) Pairing of immature females with decapitated and castrated male segments. Left, cartoon
689 showing approximate positions of amputation, removing both the head and testes. These
690 decapitated and castrated segments paired with female along most of the female body, shown to
691 right.
692 (F) DAPI staining to examine ovary development in immature females paired with decapitated and
693 castrated male segments. These ovaries contain differentiated oocytes (DO). Representative
694 images from n = 22 parasites examined in 3 separate experiments.
695 (G) Fast Blue BB showing vitellaria development in females after pairing with decapitated and
696 castrated male segments. Representative images n=22 parasites from 3 separate experiments.
697 (H) EdU labeling showing embryonic development in eggs from females paired with decapitated
698 and castrated male segments. 273/602 eggs laid between D16-D22 contained clusters of EdU^+ cells.
699 (I) DAPI labeling of metaphase spreads from eggs laid by (left) fresh *ex vivo* female parasites
700 paired with intact males and (right) immature female parasites paired with decapitated and
701 castrated male segments. Embryonic cells from fresh *ex vivo* parasites were diploid (2n=16),
702 whereas those from unfertilized females are haploid (n=8).
703 Scale bars: A, B 500 µm; C, D, F, H 50 µm, G 100 µm, I 10 µm.
704
705 **Fig. 5. *Smp_248100* is expressed in the primordial vitellaria.**

706 (A). A schematic diagram of protein product encoded by *Smp_248100*. This protein contains a
707 ZnF-C4 domain similar to other nuclear receptor proteins.
708 (B). Whole-mount *in situ* hybridization showing expression of *Smp_248100* in a mature female
709 (left), immature female (middle) or male worm (right). *Smp_248100* highly expressed in female
710 worms exclusively in the region of vitellaria. Representative images from three experiments with
711 n >35 parasites. Scale bar 100 μm.
712 (C-D). Two-color FISH for *Histone H2B* (green) and *Smp_248100* (magenta) mRNAs on (C)
713 immature females or (D) sexually mature females. In immature virgin females, *Smp_248100* was
714 only expressed in $H2B^+$ cells. In adult female, *Smp_248100* was expressed in $H2B^+$ cells as well
715 as some $H2B^-$ cells. We did not detect *Smp_248100* expression in mature vitellocytes in the
716 vitelline duct (bounded by dashed yellow lines). Representative images from n >30 parasites
717 examined in 3 separate experiments.
718 Scale bars: B, 100 μm; C, 50 μm.

719 **Fig. 6. *Smp_248100* is specifically required for vitellaria maturation following pairing with**
720 **a male worm.**
721 (A). qPCR showing decreased *Smp_248100* transcript levels in *Smp_248100(RNAi)* versus
722 *control(RNAi)*. * $p<0.05$. Error bars indicate 95% confidence intervals calculated based on 4
723 separate experiments.
724 (B). Loss of mature vitellaria in *Smp_248100(RNAi)* worms. Fast Blue BB labeling following
725 control or *Smp_248100* RNAi treatment (top). Whole-mount *in situ* hybridization showing
726 expression of *sod* gene (a marker for vitellocytes) in control RNAi or *Smp_248100(RNAi)* worms
727 (bottom). *Smp_248100(RNAi)* parasites produce few vitellocytes. Representative images from 4
728 separate experiments.
729 (C). Plot showing egg production per worm pair between D12-D14 of RNAi treatment.
730 Representative images from n > 146 parasites for each treatment examined in 6 separate
731 experiments. *** $p<0.001$, T-Test. Error bars represent 95% confidence intervals.
732 (D). EdU labeling of proliferative cells following control or *Smp_248100* RNAi treatment. We
733 observed no significant difference in the number of EdU-labeled nuclei between *control(RNAi)*
734 and *Smp_248100(RNAi)* animals within the vitellaria. Representative images from n > 41 parasites
735 for each treatment examined in 4 separate experiments. ns, $p>0.05$, T-Test. Error bars represent
736 95% confidence intervals.
737 (E) DAPI labeling showing ovaries from control and *Smp_248100* RNAi-treated female
738 parasites at D14 after pairing with a male worm. Anterior towards the top, arrow indicates
739 differentiated oocytes. Representative images from 4 separate experiments.
740 Scale bars: B, 100 μm; D,E 50 μm.

741

742 **S1 Fig. Reproductive changes of paired adult female parasites during *in vitro* culture.**
743 Ovaries of females in (A) BM169 or (B) ABC169 between D0 to D20 of in vitro culture labeled
744 with DAPI. Differentiated oocytes are present in the posterior regions (right) of ovaries during in
745 vitro culture regardless of culture condition.
746 (C) Fast Blue BB labeling showing the maintenance of mature vitellocytes during culture in
747 BM169. Representative images from three biological replicates with n > 10 parasites. Scale bars:
748 100 μm.
749

750 **S2 Fig. Hatching ratio of eggs laid on different days by worm pairs cultured in BM169 or**
751 **ABC169.** Data points represent mean values from 4 independent experiments. Error bars
752 represent 95% confidence intervals.
753
754 **S3 Fig. Karyotype analysis on eggs.** Plot showing number of chromosomes from karyotypes
755 obtained from eggs laid by freshly perfused worm pairs on D1 of culture ("D1 Fresh eggs", blue)
756 or females paired with decapitated and castrated male segments ("unfertilized eggs", red).
757
758 **S4 Fig. The protein alignment of Smp_248100 and the most similar proteins from other**
759 **organisms.** (A) Sequence alignment showing Smp_248100 shares a conserved DBD with other
760 vertebrate and invertebrate nuclear receptors. Unlike the non-parasitic groups, parasitic
761 flatworms shared a conserved histidine residue (indicted in yellow) in the position of the second
762 conserved cystine (indicted in red) in the second zinc finger. (B) Full-length alignment showing
763 C-terminus of Smp_248100 shares stretches of high amino acid identity with orthologous
764 proteins from other parasitic flatworms. Protein identities: Human GCR, Human glucocorticoid
765 receptor (P04150); Human SF1, Human steroidogenic factor 1(Q13285); Cel DAF-12,
766 *Caenorhabditis elegans* nuclear hormone receptor family member daf-12 (NP_001041239); Dme
767 HR96, *Drosophila melanogaster* hormone receptor-like in 96 (NP_524493); Smed HR96,
768 *Schmidtea mediterranea* (dd_Smed_v6_14067_0_3). Emu, *Echinococcus multilocularis*
769 (EmuJ_001078800.1); Tso, *Taenia solium* (TsM_000102500); Ovi, *Opisthorchis viverrini*
770 (T265_09674); Csi, *Clonorchis sinensis* (csin106676); Sman, *Schistosoma mansoni*
771 (Smp_248100).
772
773 **S5 Fig**. **Fast Blue B staining of the dsRNA-treated worms at D14.** Plot showing percentage of
774 Fast Blue BB positive labeling in *Smp_248100 (RNAi)* versus *control (RNAi).* Representative
775 images from n > 39 parasites for each treatment examined in 4 separate experiments. ****
776 p<0.0001, T-Test. Error bars represent 95% confidence intervals.
777

Fig. 1

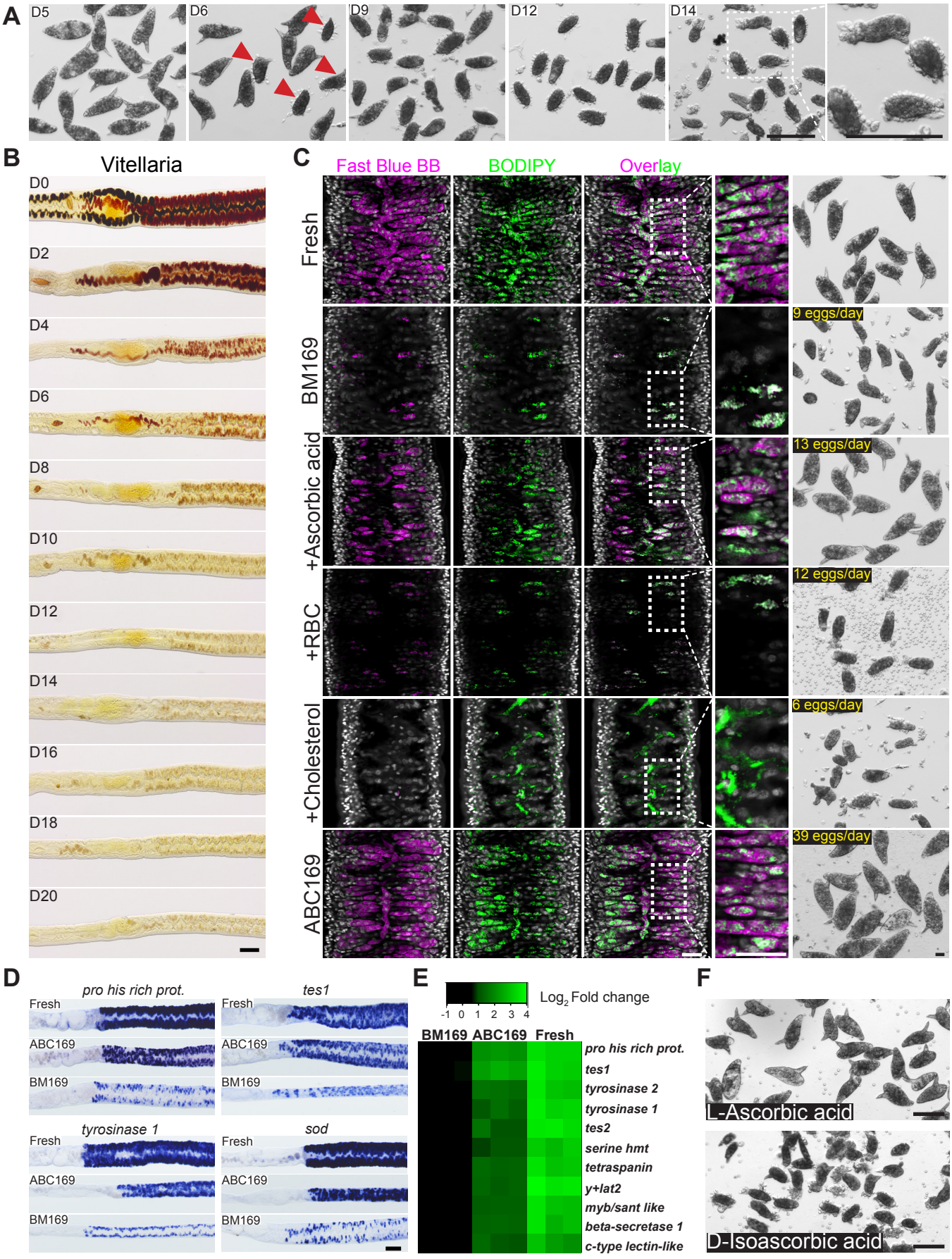

A
D5
D6
D9
D12
D14
B
Vitellaria
D0
D2
D4
D6
D8
D10
D12
D14
D16
D18
D20
C
Fast Blue BB
BODIPY
Overlay
Fresh
BM169
+Ascorbic acid
+RBC
+Cholesterol
ABC169
9 eggs/day
13 eggs/day
12 eggs/day
6 eggs/day
39 eggs/day
D
pro his rich prot.
tes1
tyrosinase 1
sod
Fresh
ABC169
BM169
E
Log2 Fold change
-1 0 1 2 3 4
BM169 ABC169 Fresh
pro his rich prot.
tes1
tyrosinase 2
tyrosinase 1
tes2
serine hmt
tetraspanin
y+lat2
myb/sant like
beta-secretase 1
c-type lectin-like
F
L-Ascorbic acid
D-Isoascorbic acid

Fig. 2

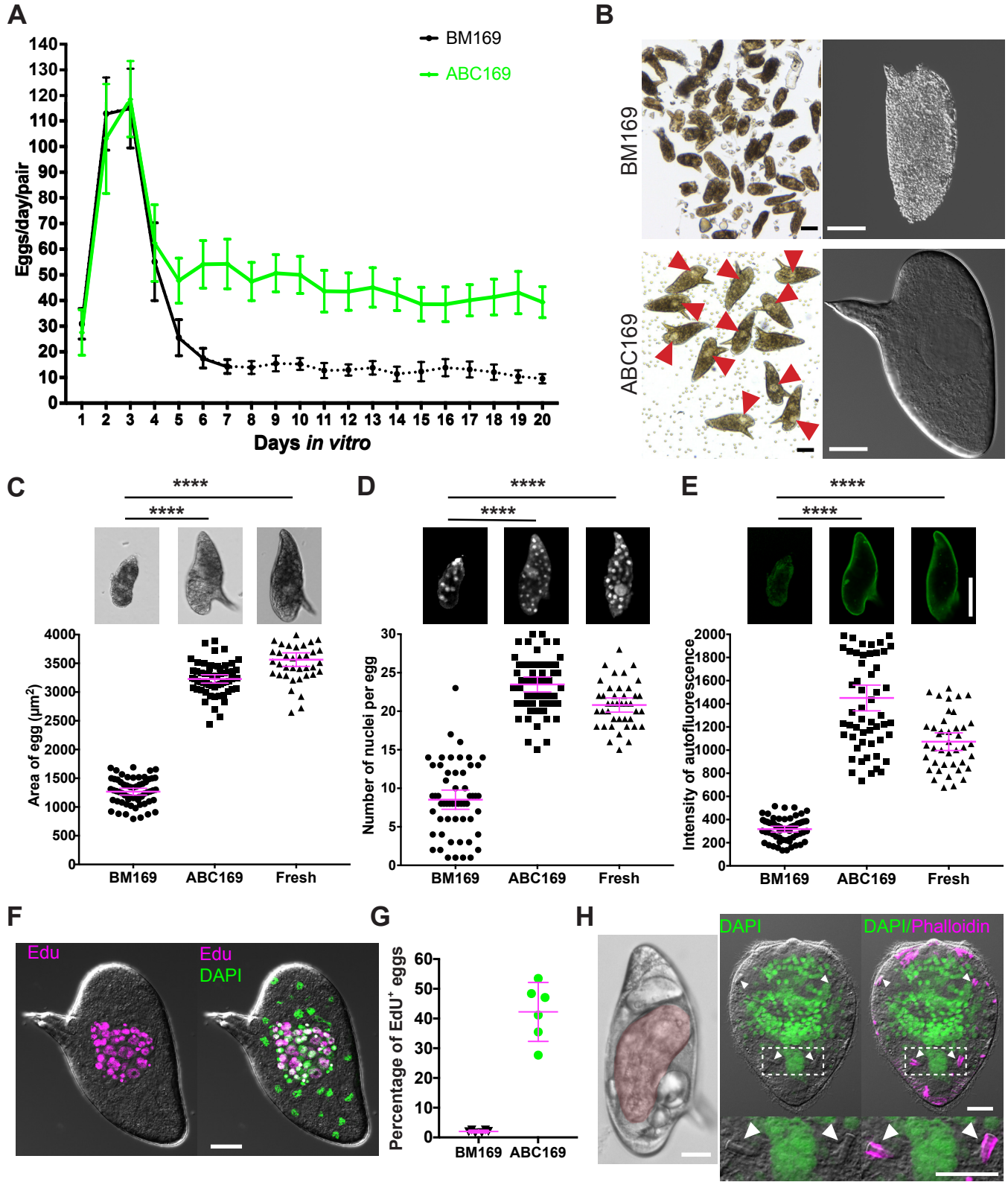

A
BM169
ABC169
Eggs/day/pair
Days in vitro
B
BM169
ABC169
C
****
****
Area of egg (μm²)
BM169
ABC169
Fresh
D
****
****
Number of nuclei per egg
BM169
ABC169
Fresh
E
****
****
Intensity of autofluorescence
BM169
ABC169
Fresh
F
Edu
Edu
DAPI
G
Percentage of EdU⁺ eggs
BM169
ABC169
H
DAPI
DAPI/Phalloidin

Fig. 3

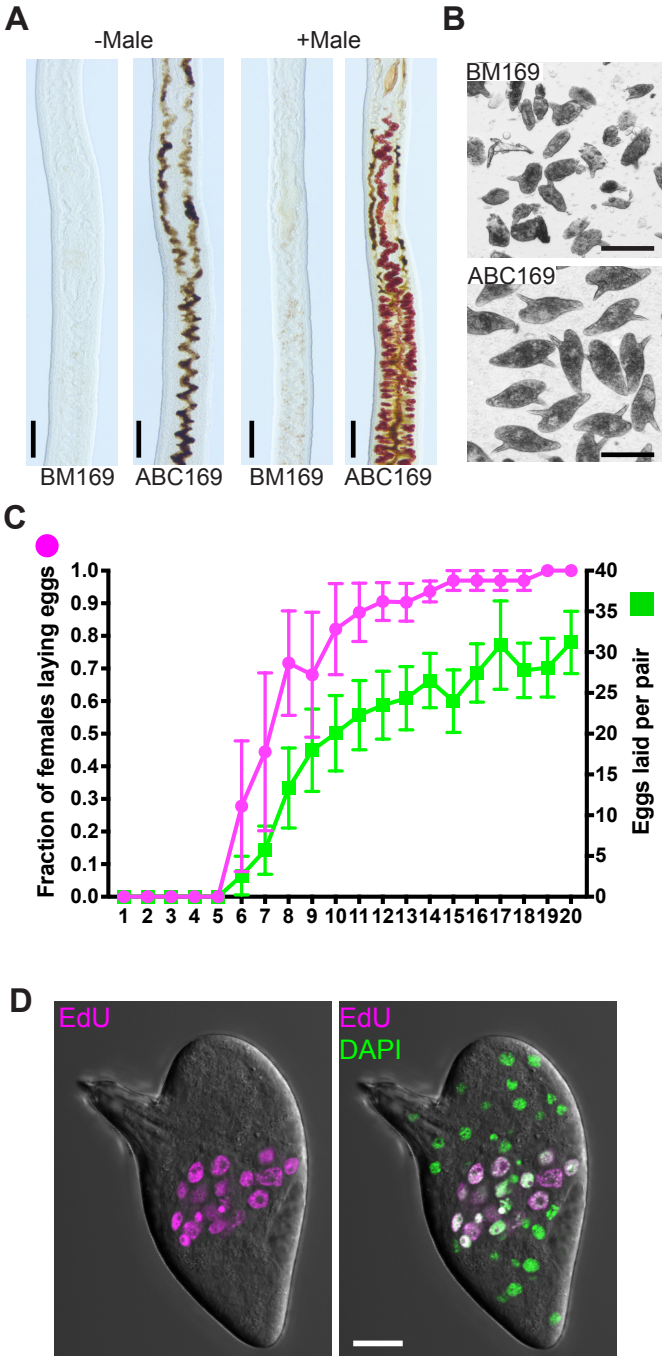

A
-Male
+Male
BM169
ABC169
BM169
ABC169
B
BM169
ABC169
C
Fraction of females laying eggs
Eggs laid per pair
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
D
EdU
EdU
DAPI

Fig. 4

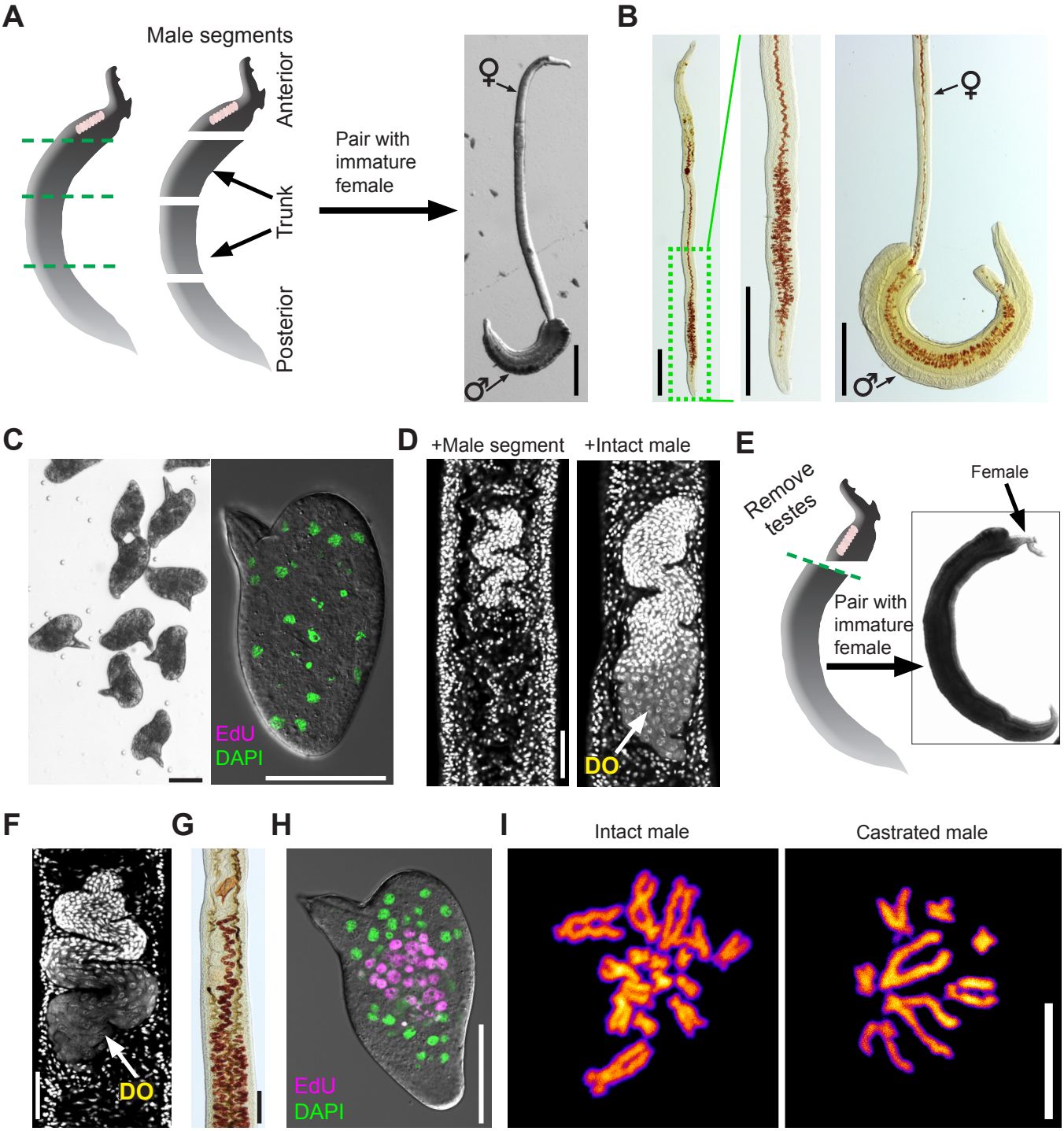

A
Male segments
Anterior
Trunk
Posterior
Pair with
immature
female
B
C
EdU
DAPI
D
+Male segment
+Intact male
DO
E
Remove
testes
Female
Pair with
immature
female
F
DO
G
H
EdU
DAPI
I
Intact male
Castrated male

Fig. 5

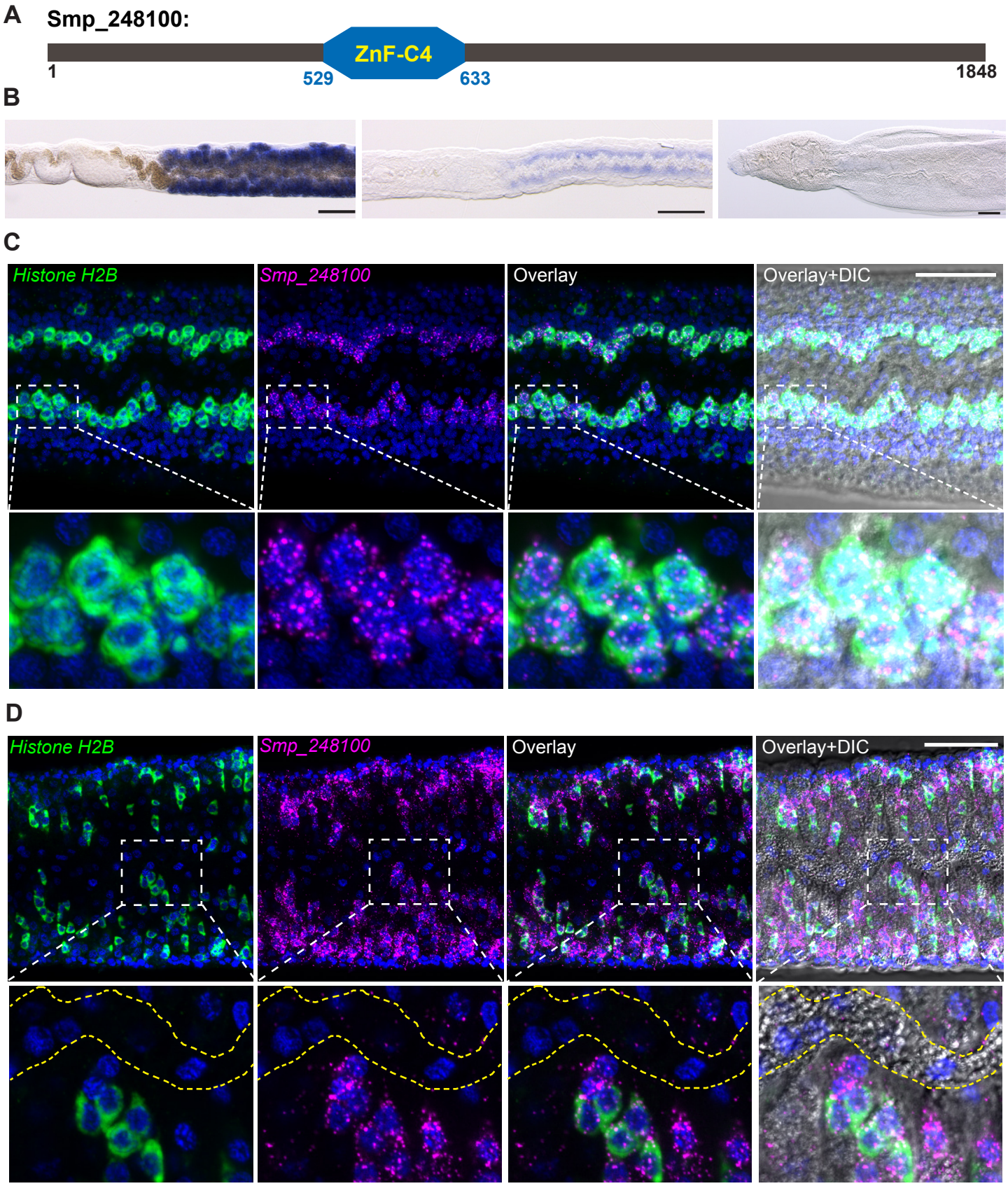
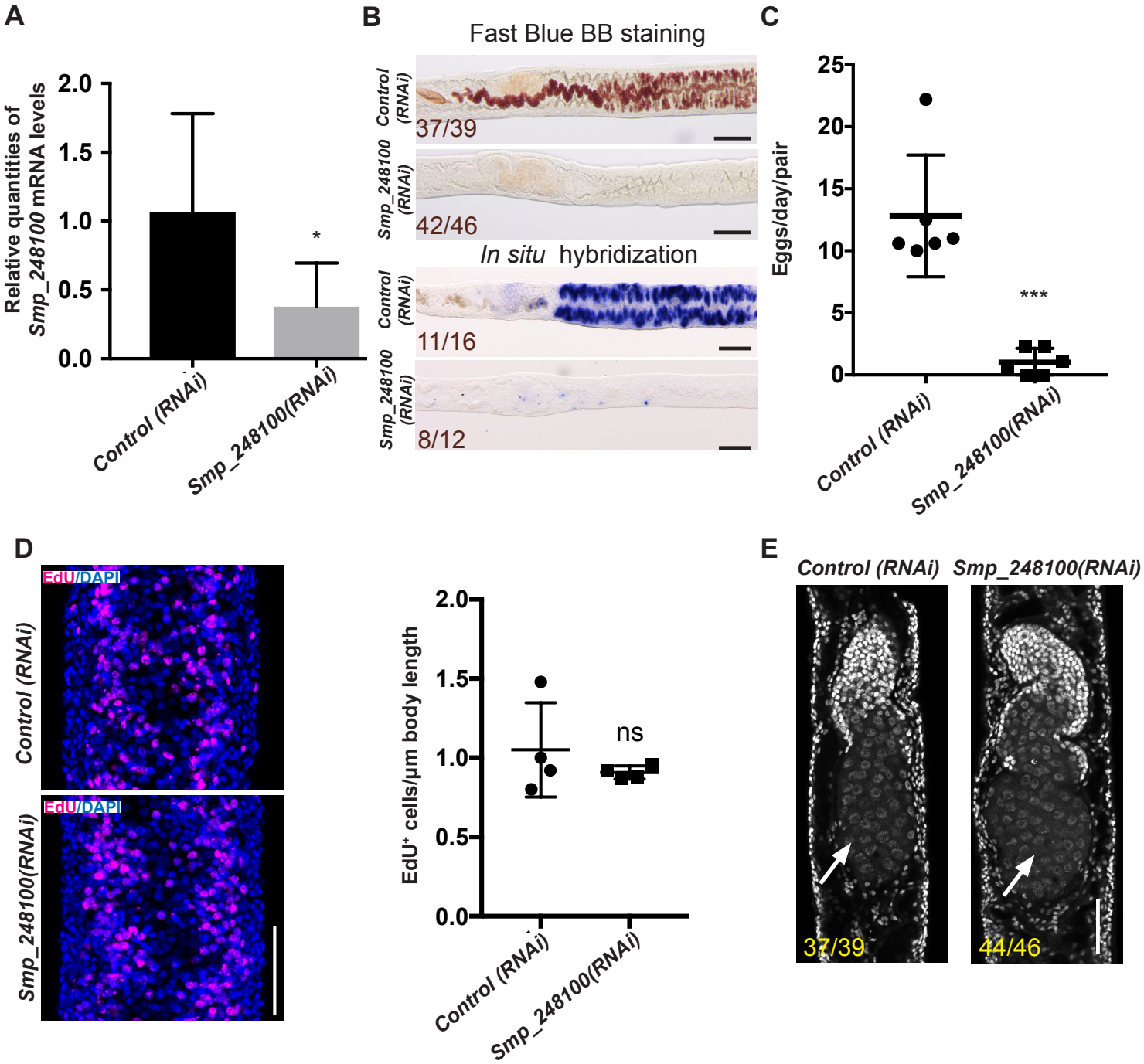
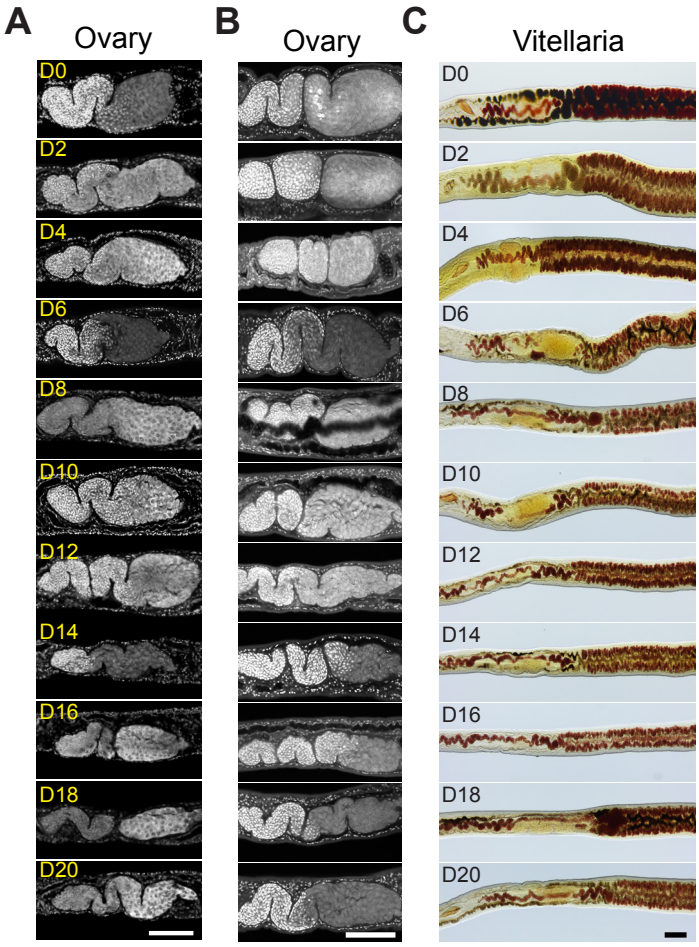

A
Smp_248100:
ZnF-C4
1
529
633
1848
B
C
Histone H2B
Smp_248100
Overlay
Overlay+DIC
D
Histone H2B
Smp_248100
Overlay
Overlay+DIC

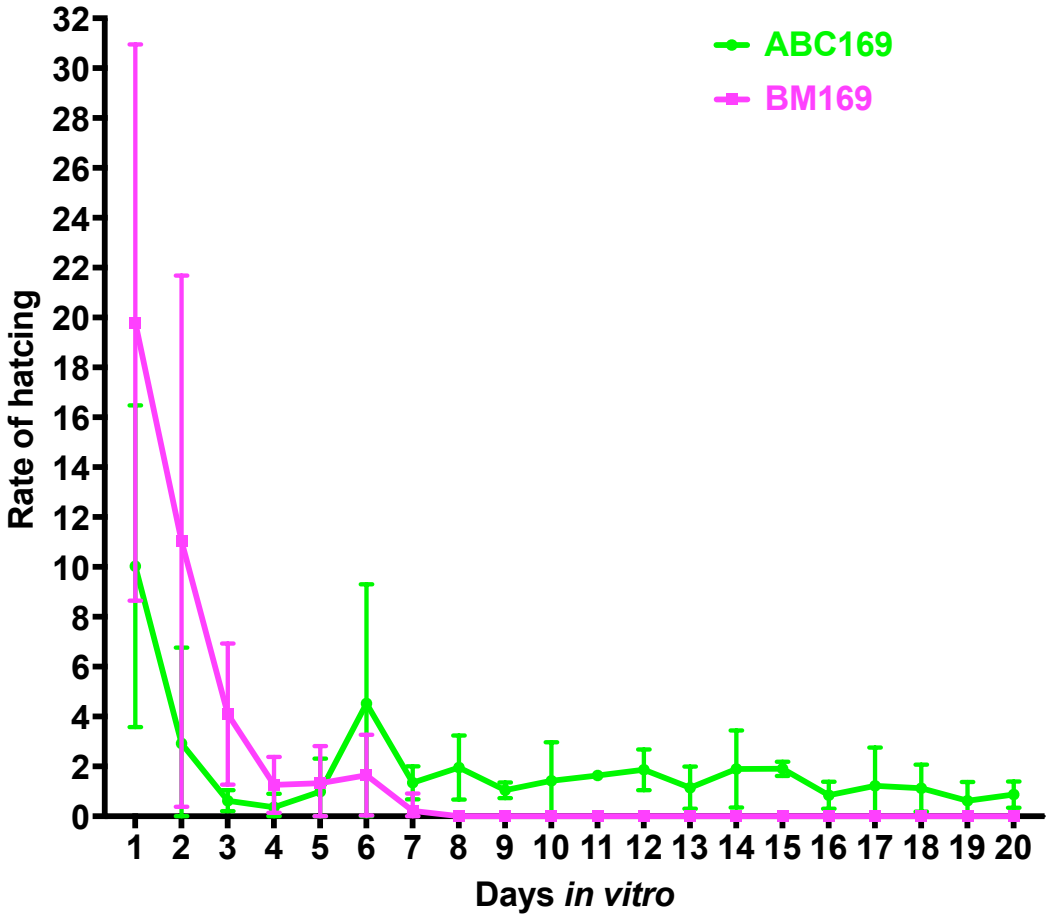
Fig. 6


A
Relative quantities of Smp_248100 mRNA levels
2.0
1.5
1.0
0.5
0.0
*
Control (RNAi)
Smp_248100(RNAi)
B
Fast Blue BB staining
Control (RNAi)
37/39
Smp_248100 (RNAi)
42/46
In situ hybridization
Control (RNAi)
11/16
Smp_248100 (RNAi)
8/12
C
Eggs/day/pair
25
20
15
10
5
0
***
Control (RNAi)
Smp_248100(RNAi)
D
EdU/DAPI
Control (RNAi)
EdU/DAPI
Smp_248100(RNAi)
EdU⁺ cells/µm body length
2.0
1.5
1.0
0.5
0.0
ns
Control (RNAi)
Smp_248100(RNAi)
E
Control (RNAi)
Smp_248100(RNAi)
37/39
44/46

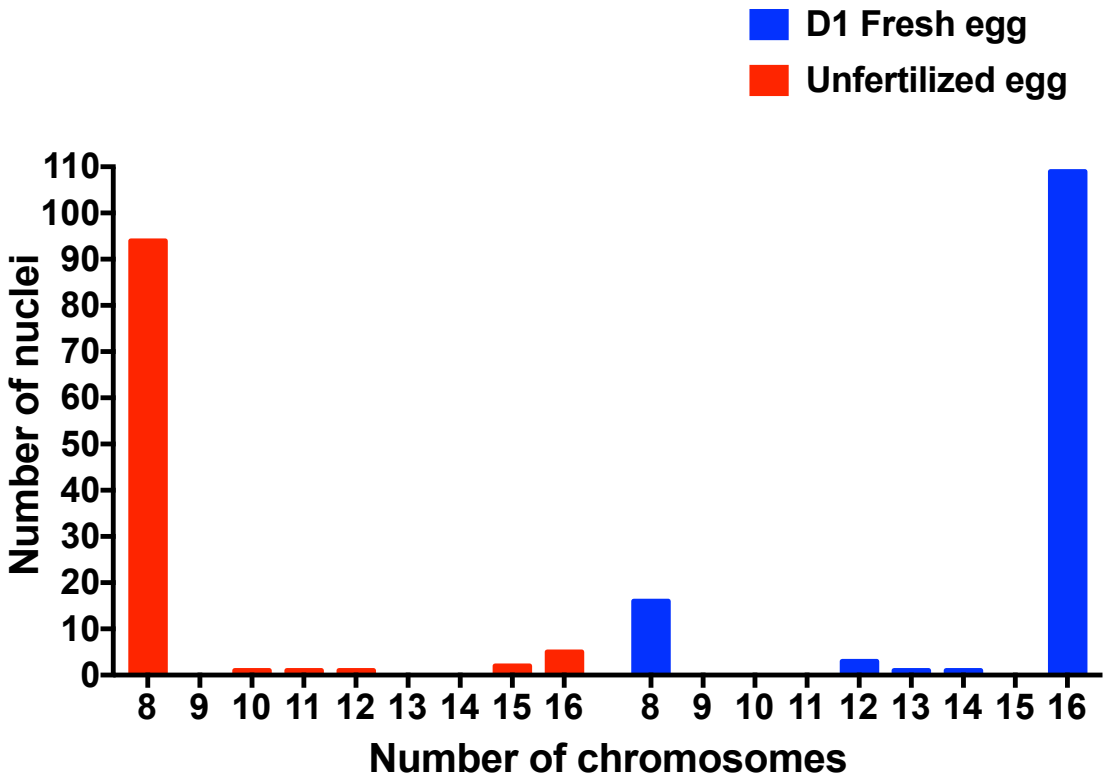
S1 Fig


A
Ovary
B
Ovary
C
Vitellaria
D0
D2
D4
D6
D8
D10
D12
D14
D16
D18
D20

S2 Fig

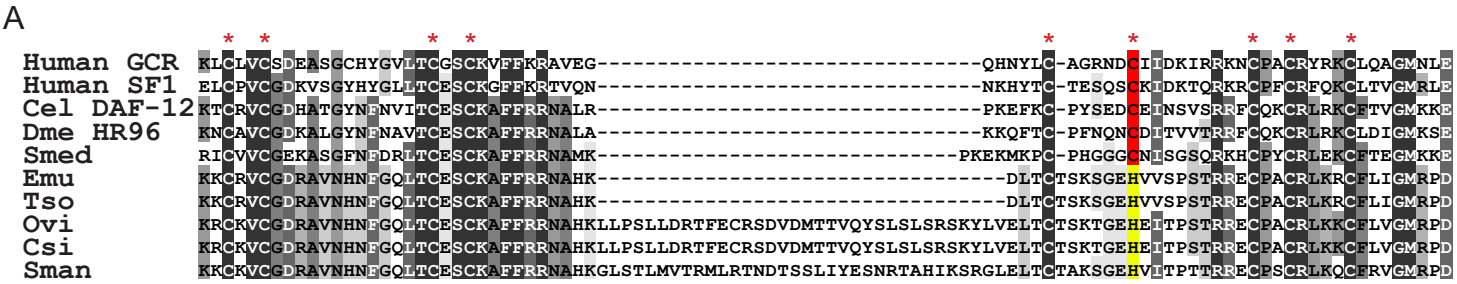

ABC169
BM169
Rate of hatcing
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Days *in vitro*

S3 Fig

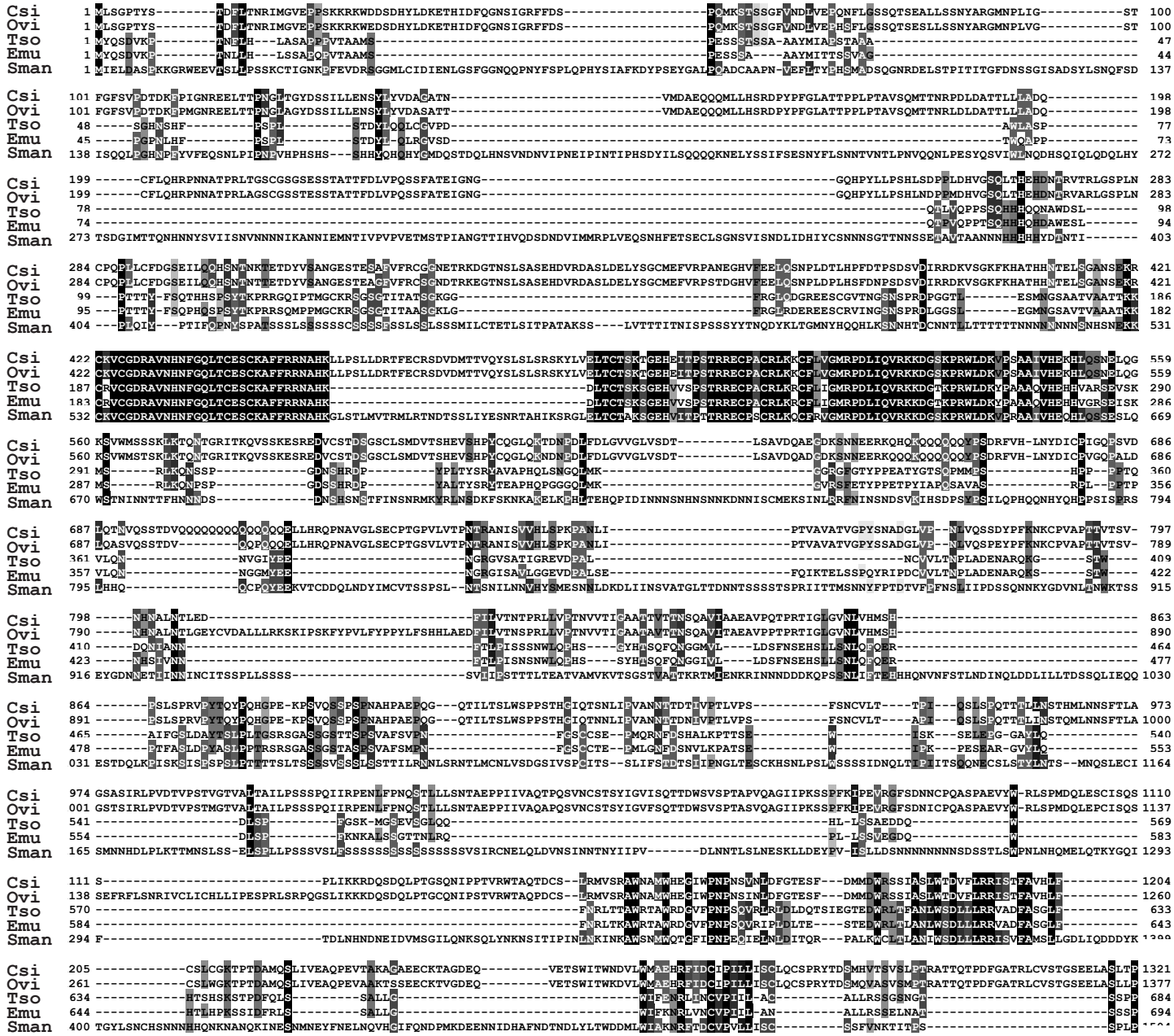

D1 Fresh egg
Unfertilized egg
Number of nuclei
110
100
90
80
70
60
50
40
30
20
10
0
8 9 10 11 12 13 14 15 16
8 9 10 11 12 13 14 15 16
Number of chromosomes

S4 Fig

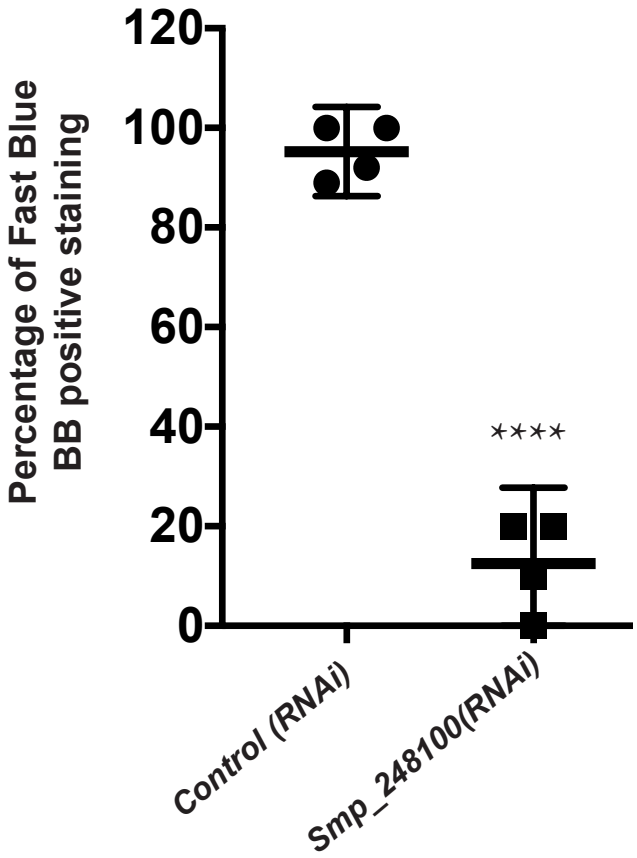
A


Human GCR
Human SF1
Cel DAF-12
Dme HR96
Smed
Emu
Tso
Ovi
Csi
Sman

B


Csi
Ovi
Tso
Emu
Sman

S5 Fig


Percentage of Fast Blue BB positive staining
120
100
80
60
40
20
0
****
Control (RNAi)
Smp_248100(RNAi)