

1 **Title:** General characterization of regeneration in *Aeolosoma viride*

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17

18 **Abstract**

19 Regeneration has long attracted scientists for its potential to restore lost, damaged or

20 aged tissues and organs. A wide range of studies have conducted on different model

21 organisms on both cellular and molecular levels. Current evidences suggest that a

22 variety of regenerative strategies are developed and used by different species, and

23 their regenerative strategies are highly correlated to their reproductive methods. Our

24 present work focused on the freshwater annelid *Aeolosoma viride*, which reproduces
25 by paratonic fission and is capable of complete regeneration. We found out that *A.*
26 *viride* can regenerate both anterior and posterior end, even with only 3 segments
27 remained. This process is characterized by epimorphosis that involves large amount of
28 cell proliferation which drives the formation of blastema. Cell proliferation and
29 regeneration successful ratio were significantly decreased when treated with
30 microtubule inhibitor taxol or *Avi-tubulin* dsRNA, which confirmed that cell
31 proliferation served as a key event during regeneration. Together, our data described
32 the regenerative processes of *A. viride*, which includes high level of cell proliferation
33 and the formation of blastema. Furthermore, our findings demonstrated *A. viride* as a
34 potential model for the study of regeneration.

35

36 **Keywords:** *Aeolosoma viride*, Epimorphic regeneration, Cell proliferation, Blastema

37

38 **Introduction**

39 An ability to regenerate lost body part is widely, but non-uniformly, distributed in the
40 animal kingdom [1-3]. Both the capacities and mechanisms of regeneration differ
41 between taxa. T. H. Morgan described the processes of regeneration in two
42 generalized terms, namely morphallaxis and epimorphosis [4]. Morphallaxis is

43 characterized by rearrangement of pre-existing cells and the absence of blastema [5].
44 Early regeneration in hydra is a classic example of morphallaxis as it requires
45 minimum cell proliferation and redistribution of pre-existing tissues. In epimorphosis,
46 on the other hand, undifferentiated cells or progenitor cells aggregate at the wound
47 sites, and these undifferentiated cells proliferate to form a blastema [6, 7]. Therefore,
48 cell proliferation is required for epimorphic regeneration, and application of drugs that
49 perturb cell cycle progression, such as nocodazole or hydroxyurea, would prevent
50 normal progression of regeneration [8].

51

52 Asexual reproduction is often compared to regeneration, since identical individuals
53 arise from fragments of somatic tissue in both processes [9]. Asexual reproduction in
54 annelids can be divided into two major types: architomy and paratomy. Architomy is
55 fission followed by fragmentation. Contrarily, paratomy required individuals to be
56 fully formed or developed prior to fission [10]. In paratomic reproduction,
57 reconstitution or rearrangement of somatic tissue typically occurs in a predictable
58 growth zone. Previous studies demonstrated that such a growth zones can also
59 regenerate lost body parts after injury in certain species reproduce by paratomic
60 fission [9, 11].

61

62 Paratomic fission has been described as a common character of annelids, as it is found
63 in taxonomic groups such as Sipuncula, Serpulidae, Aeolosomatidae, and Naididae
64 [12]. Regeneration is also wide-spreading among annelids [13, 14]. Nevertheless,
65 species differs in their regenerative capacities. For example, the naidid *Pristina leidy*
66 mostly reproduce by paratomic fission and is able to complete both anterior and
67 posterior regeneration [11]. In contrast, the capitellid polychaete *Capitella teleta*
68 mostly reproduce sexually and is only capable to partially regenerate its posterior end
69 [15]. Such a difference may reflect a connection between asexual reproduction by
70 paratomic fission and regeneration [9, 12, 16].

71

72 Aeolosomatidae are polychetae that mostly inhabit in fresh water environment [17].
73 Approximately 30 species have been identified in this family, and predominantly
74 reproduce by agametic reproduction [10, 18]. Most aeolosomatid species reproduce
75 by paratomic fission to create clonal individuals, and an ability to regenerate the
76 anterior and posterior segments after injury has been demonstrated in some species of
77 this family [19]. However, there is no detailed descriptive characterization of the
78 regeneration in this group of annelids. In this study, we provided a detailed
79 description of the regeneration process in *Aeolosoma viride*. It was observed that this
80 worm can restore its lost body parts, both anteriorly and posteriorly, within 3-5 days.

81 Furthermore, we demonstrated that a high level of cell proliferation occurs at the
82 wound site and that such a cell proliferation activity is critical for the completion of
83 regeneration. Using pulse-and-chase experiments, we were able to show that cell
84 migration has no particular role in anterior regeneration of *A. viride*. Finally, our study
85 demonstrated the potential of *A. viride* to be an excellent model for regenerative
86 studies.

87

88 **Results**

89 **General and reproductive characteristics of *A. viride***

90 *Aeolosoma viride* is a 2-3 mm length semi-transparent annelid contained 10 to 12
91 segments with pairs of chaetae at each side of the worms. The intact worm has a
92 ring-like mouth structure that can be observed at both dorsal and ventral side of the
93 peristomium (1st segment). Mouth is connected to an enlarged digestive tract located
94 at the center of the worm, which can be easily observed under a dissecting microscope.
95 *A. viride* exclusively perform paratonic fission to asexually reproduce one progeny
96 from its posterior end (Fig. 1). The paratonic fission started by extension of the
97 posterior end. Worms exceeding 12 segments with an unusual lengthy posterior region
98 could be easily observed (Fig. 1C). The anterior portion of the offspring gradually
99 developed and formed a head shape tilted apart from the parental worm (Fig. 1D). At

100 this stage, the parent and the progeny can be easily distinguished. Finally, the
101 offspring worm separates apart from the parental worm, and becomes an individual
102 worm (Fig. 1E).

103

104 **Anterior regeneration in *A. viride***

105 Anterior amputation is performed in front of the enlarged digestive tract. Immediately
106 after amputation, the worm severely twisted; fluids and food residues gushed from the
107 inner cavity. At 3 hours post amputation (hpa), the regenerating *A. viride* adhered on
108 the bottom of culture chamber, and its wounded area remained rough and uneven. The
109 rough wounded area became smoothed within 6 hpa. At 12 hpa, a small amount of
110 tissue became lighter and clearer in color, started to develop at the regenerating area.
111 This tissue will later be proven to be the regenerative blastema. The protruding
112 regenerative blastema expanded from the center of the wounded area and became
113 apparent from 24 to 48 hpa. Vertical contraction could be observed from 72 hpa, but
114 the regenerating worms were still unable to move freely. The reappearance of
115 ring-like structure characterized mouth formation, and a tubular structure (esophagus)
116 extended to connect with the enlarged digestive tract at 96 hpa. After 96 hours of
117 regeneration, the regenerating head area gradually bulged and became wider than
118 other body segments. The prostomium expanded from 96 to 120 hpa (Fig. 2B).

119 During the entire process of regeneration, the regenerating *A. viride* remain adhere on
120 the bottom of culture chamber. Most of them could swim freely around 120 hpa,
121 which was considered as an indicator for successful regeneration in *A. viride*.

122

123 **The regenerative capacity of *A. viride***

124 For characterization of the regenerative capacity, either the anterior or the posterior
125 ends of worms were amputated. Amputated worms with three segments could still
126 complete regeneration, even the successful ratio was around 40%. Over 60% of
127 worms with 6 or 9 posterior segments completed their anterior regeneration at 5 day
128 post amputation (dpa) (Fig. 3). Posterior regeneration shared a similar pattern. Over
129 53.3% worms with three anterior segments completed their posterior regeneration.
130 Worms with 6 or 9 anterior segments increased successful ratio of posterior
131 regeneration over 80% at 3 dpa (Fig. 3). Since the anterior segments of *A. viride*
132 contain the mouth and the brain, their complexity and importance outweigh the
133 posterior segments. Therefore, all following experiments in this study were conducted
134 on the anterior end.

135

136 **Cell proliferation in the regenerative process of *A. viride***

137 EdU signal showed proliferating cells continuously present in *A. viride*. The worms

138 were also stained with a nuclear dye Hoechst 33342 to conform the EdU labeling (Fig.
139 4A). Intact and regenerating worms were incubated in EdU solution 12 hours prior to
140 fixation. Normally, the EdU⁺ cells were significantly detected at the posterior end of
141 intact worm, the area of asexual reproductive zone, which is the connecting interface
142 between the parent and the future offspring. The EdU signal was also detected at the
143 anterior mouth with minor EdU signals randomly distributed in intact and
144 regenerating worms from 0 to 12 hpa. However, signals posterior to the amputation
145 site quickly diminished after 24 hpa. During regeneration, the EdU signal became
146 concentrated at the regenerating blastema, and the strongest signal was observed at 24
147 and 48 hpa, then gently decreased from 72 to 120 hpa. At 96 and 120 hpa, EdU⁺ cell
148 formed a ring-like shape at the center of head, which indicated the development of
149 mouth in the regenerating worms (Fig. 4C).

150

151 **Cell proliferation is required for anterior regeneration**

152 *Aeolosoma viride* were treated with taxol, an inhibitor of cell proliferation that works
153 by interfering with the normal function of microtubules. As previous result has shown,
154 the largest amount of proliferating cells was detected at 48 hpa (Fig. 4C). However,
155 the EdU signal in the blastema were apparently reduced by 25 μ M of taxol at 48 hpa,
156 which indicated that the proliferating blastema was not properly formed at anterior

157 regenerating site (Fig. 5A). The regeneration successful ratio of *A. viride* treated with
158 25 μ M taxol decreased 80% at 5 dpa and 45% at 7 dpa (Fig. 5B). Also, the bulged
159 head of the regenerating *A. viride* was absent after taxol treatment, and only a tiny
160 blastema was observed at 5 and 7 dpa (Fig. 5C).

161

162 To confirm the importance of cell proliferation on regeneration in *A. viride*, we also
163 used gene-specific dsRNA to perform RNA interference. Both feeding and
164 microinjecting dsRNA successfully reduced the expression of *Avi-tubulin* mRNA by
165 50% compared to *yfp* dsRNA (MOCK) (Fig. 6A). Western blot was conducted to
166 further confirm this result. The predicted molecular weight of tubulin protein is 48.8
167 kDa. *Avi-actin* served as loading control, and its predicted molecular weight is 42.1
168 kDa. The result showed a significant reduction of tubulin protein expression
169 comparing to MOCK RNAi (Fig. 6B). In addition, worms treated with *yfp* dsRNA
170 regenerated normally. But, the regeneration of *Avi-tubulin* RNAi treated animals was
171 significantly inhibited at 5 dpa. The group of feeding dsRNA reduced successful ratio
172 of regeneration by 45%, whereas the group of injecting dsRNA reduced by 35% (Fig.
173 6C).

174

175 **Cell migration in the regeneration of *A. viride***

176 Some speculated that the source of stem or progenitor cells in the regenerative area
177 could be migration [20]. Stem cell migration not only is fundamental to embryonic
178 development, but also indispensable to adult tissue homeostasis and repair [21].

179

180 To examine the role and importance of cell migration during anterior regeneration of
181 *A. viride*, pulse-chase experiment with EdU labeling was performed. Only a few EdU⁺
182 cells were detected at the surface of the newly regenerated tissue at 24, 72 and 120
183 hpa (Fig. 7). However, the distribution of EdU⁺ cells around non-regenerated area was
184 evident from 24 to 120 hpa.

185

186 **Discussion**

187 *Aeolosoma viride* is the first species in Aeolosomatidae used as a model for detailed
188 regeneration study. In this study, we had carefully evaluated the regenerative capacity
189 of *A. viride*. Regeneration of *A. viride* followed by either anterior and posterior
190 amputation could be completely restored within 5 days and 3 days respectively. Most
191 worms could survive and regenerate with 3 segments. Anterior regeneration showed
192 lower successful ratio than posterior regeneration. It is reasonable to explain that the
193 anterior regeneration is involved the restoration of a more complex structure including
194 head and mouth. Even so, compared with earthworm *Enchytraeus japonensis* that

195 could regenerate into complete individual from 6 fragments [22], and *Eisenia fetida*
196 that could only partially regenerate [23], *A. viride* clearly demonstrated a stronger
197 regenerative ability.

198

199 Sexual and asexual reproduction including budding, paratonic fission, and
200 fragmentation have all been documented in annelid reproduction [24]. Previous study
201 has recorded the regenerative capacity and reproductive strategies of many annelids,
202 and discovered that most annelids capable of head regeneration reproduce by asexual
203 reproduction [25]. *Aeolosoma viride* had been recorded that its reproduction is
204 exclusively asexual by paratonic fission [18]. Our study provided detailed imaging on
205 the entire process, and confirmed the idea that animals with asexual reproductive
206 characters typically demonstrated strong regenerative capacity.

207

208 Cell proliferation is generally considered to be the key event that occurs at the area of
209 regeneration [26, 27]. The contribution of cell proliferation to regeneration differs
210 across metazoan models [8]. In this study, large amount of EdU⁺ cells present at
211 blastema during regeneration. Combined with the changes in morphology, we
212 observed that anterior amputation in *A. viride* is followed by the formation of
213 blastema with great quantity of cell proliferation during regeneration. Also, significant

214 decrease of proliferating cells was observed at the blastema after treated with mitotic
215 inhibitor taxol. Together, these data demonstrated the importance of cell proliferation
216 in the regenerative process of *A. viride*.

217

218 Although variation exists in regeneration ability and mechanism, the widespread
219 phylogenetic distribution of blastema formation in annelid suggested an evolutionary
220 ancient origin of regeneration process. Even though, recent studies indicated that
221 regeneration could not be solely classified as epimorphic or morphallatic, several
222 segmented worm species including *Branchiomma luctuosum* and *C. teleta* have
223 shown limited evidence for morphallaxis [28, 29]. Morphallaxis is characterized by
224 rearrangement of pre-existing tissues, which strongly depend on stem cells or
225 remaining undifferentiated cells migrating to the injured site [30]. Our data supported
226 the hypothesis that *A. viride* carried out anterior regeneration by epimorphosis which
227 is consistent with most other annelids [29, 31-34]. Recent findings inferred that
228 regeneration in annelids involves both cell proliferation and cell migration [35]. And
229 the cellular source of blastema originated from the migration of stem cells [20].
230 According to our pulse-chase experiment, EdU⁺ cells were limited in the anterior
231 regenerating site, which means that only a small amount of proliferating cells
232 migrated into the wounded area during anterior regeneration. This allowed us to

233 hypothesize that cell migration served no specific or limited role during anterior
234 regeneration in *A. viride*. There was no evident morphallaxis occurred, however,
235 further experiment with stem cell markers and live-imaging are needed for a definite
236 conclusion.

237

238 Limited regeneration studies conducted on annelids model organisms. Hydra and
239 planarian are both considered as the master of regeneration, and often used as the
240 model organisms for regeneration studies [7, 36-39]. But, there are specific
241 restrictions on regeneration studies regarding these commonly used animals. Both
242 planarian and hydra lack sophisticated organ systems and a complex central nervous
243 system, which is far different from vertebrate animals. Hydra regeneration is also
244 classified as morphallactic, which means that they could regenerate under limited cell
245 proliferation [40, 41]. And based on current studies, none of the known vertebrate
246 could regenerate mainly through morphallaxis. Epimorphosis is the major type of
247 regeneration observed in vertebrate animals. Salamander, *Xenopus* and zebrafish are
248 all prominent examples of animal models used in the study of epimorphic
249 regeneration. However, regeneration in these animals is limited to specific organs or
250 tissues. Some might even have restriction on early stage of life [42-44]. Certain
251 species of echinoderms could completely regenerate from a piece of tissues [45], but

252 echinoderm shared very different characteristic from vertebrate animals, such as body
253 symmetry, lack of head, digestive, and circulatory system. Due to the limitations that
254 current models retained, we believe that annelids can serve as different model
255 organism for the study of regeneration. Besides an evolutionary advantage, *A. viride*
256 also possess similar level of experimental advantages compare to many current model
257 organisms. Small molecular inhibitors, molecular tools were both applicable on this
258 worm. First, *A. viride* is easy to maintain and raise in laboratory. Second, *A. viride* has
259 small and transparent body which make them easy to be manipulated and observed
260 during the experimental processes. Last but not least, RNA interference was used in
261 the study of molecular biology to knock down or silence specific mRNA expression.
262 Feeding method commonly designed for *C. elegans* and microinjecting method
263 designed for animal embryo and adult were both functional in this newly introduced
264 model animal [46]. In addition to its regeneration capacity, this small annelid has
265 relative short lifespan and reproductive cycle which is suited for study on aging
266 [47-49].

267

268 In conclusion, we examined the regenerative capacity in *A. viride*, especially on
269 anterior regeneration. In addition to strong regeneration ability, *A. viride* has several
270 characters that may make them valuable as a potential animal model for regeneration

271 research.

272

273 **Materials and Methods**

274 **Animals**

275 *Aeolosoma viride* was cultured in artificial spring water (ASW, 48 mg L⁻¹ NaHCO₃,
276 24 mg L⁻¹ CaSO₄•2H₂O, 30 mg L⁻¹ MgSO₄•7H₂O, and 2 mg L⁻¹ KCl in distilled water,
277 pH = 7.4) at 25°C under 12 hours of day-night cycles. Grounded oat meal serves as
278 major food source. Approximately 500 ± 200 worms were fed with 20 mg powdered
279 oats 3 to 5 times per week. Prior to all experiment, worms were starved in ASW
280 overnight.

281

282 **Regeneration experiment**

283 *Aeolosoma viride* without offspring was selected for the following experiment. To
284 analyze the capacity of regeneration, amputation was carried out at the desired
285 segment in *A. viride* (Fig. 1A). The detail morphological changes of regenerating
286 worms were observed with an Olympus DP80 microscope for the following 3 to 168
287 hours. The successful ratio of anterior or posterior regeneration was determined from
288 morphology and behavior with a dissecting microscope (WILD M8, Leica) at 1 to 7
289 days post-amputation (dpa).

290

291 In the inhibitory experiment, all procedures were similar as described earlier, but the
292 animal were transferred into fresh ASW containing 25 μ M taxol (Sigma-Aldrich)

293

294 **EdU labeling**

295 Worms were treated with EdU (100 μ g ml⁻¹) for 12 or 24 hours prior to amputation;
296 12 to 120 hours prior to fixation. The animals were fixed with 4% paraformaldehyde
297 (PFA) at 4°C overnight. Whole-mount immunohistochemistry detection was processed
298 using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) according to the
299 manufacturer's protocol.

300

301 **RNA interference (RNAi)**

302 *Avi-tubulin* was cloned by our lab (NCBI # AQP09899.1). The RNA interference
303 protocol was modified as described previously [50]. Partial sequence of yellow
304 fluorescent protein (*yfp*, as MOCK group) or *Avi-tubulin* were constructed with L4440
305 vector and transformed into an RNase III deficient strain competent cell HT115 (DE3)
306 (Yeastern Biotech). *A. viride* was fed with bacteria containing dsRNA for 3 days.

307

308 Microinjecting method was modified based on previous study [51]. The *yfp* or

309 *Avi-tubulin* dsRNA were *in vitro* transcribed with T7 polymerase (Ambion). Each
310 individual animal was injected 50 ng of dsRNA through microinjector (Promega) for
311 3 consecutive days. Worms were then collected for RNA extraction, protein extraction
312 or regeneration study afterward.

313

314 **RNA exaction and quantitative real-time RT-PCR (qRT-PCR)**

315 The total RNAs were extracted from 20 worms by using TRIzol and
316 then reverse-transcribed to cDNA by using SuperScript III Kit (Invitrogen).

317 Transcriptional levels were determined by Bio-Rad iCycler™ (Bio-Rad) using SYBR
318 green system. The primers used to amplify *Avi-tubulin* were 5'-

319 GGTAACAACCTGGGCTAAGGG-3' and 5'-GCGAAGCCAGGCATGAAGAA-3'.

320 *Avi-actin* was used as internal control with specific primers:

321 5'-ATGGAGAAGATCTGGCATCA-3' and 5'-GGAGTACTTGCGCTCAGGTG-3'

322 designed from *Avi-tublin* (NCBI # AQP09898.1). Relative quantification of gene

323 expression was calculated by using the $\Delta\Delta C_t$ method. Three technical replicates were

324 used in each real-time PCR reaction, and a no-template blank was served as negative

325 control.

326

327 **Western blotting**

328 Animals were homogenized in RIPA buffer (50 mM Tris-HCl (pH 7.0), 150 mM NaCl,
329 1% Triton X-100, 0.5% C₂₄H₄₀O₄, 0.1% SDS with protease inhibitor cocktail
330 (Sigma-Aldrich) and DNase (Promega)). The homogenized samples were mixed with
331 sample buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM
332 2-mercaptoethanol and 0.1% bromophenol blue), separated by 7.5% polyacrylamide
333 gel, and transferred to a PVDF membrane (Millipore). After blocking with 5% BSA at
334 25°C for 1 h, the PVDF membrane was incubated with either anti-acetylated α -tubulin
335 (1:8000, GeneTex) or anti- α -actin (1:8000, Santa Cruz) antibodies in the blocking
336 solution at 25°C for 1 h, rinsed three times with PBST, and then incubated with either
337 HRP-conjugated goat anti-mouse IgG (1:3000, Chemicon-Millipore) for α -tubulin or
338 HRP-conjugated goat anti-rabbit IgG (1:8000, Chemicon-Millipore) for α -actin.
339 Finally, patterns were detected on the PVDF membrane by ECL (Biomax).

340

341 **Statistics**

342 Data were tested for significance using one-way analysis of variance with a Scheffe's
343 method post *hoc* test or two-tailed unpaired student's t-test. Probability values of $p <$
344 0.05 were regarded as statistically significant.

345

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487

488 **Availability of data and materials**

489 The datasets generated and/or analysed during the current study are available from the
490 corresponding author on reasonable request.

491

492 **Author contributions**

493 All authors designed the experiments. CC, SF and YH carried out the experiments.
494 CC and SF interpreted the data. CC and SF wrote the manuscript. JC reviewed this
495 manuscript.

496

497 **Competing interests**

498 The authors declare no competing interests.

499

500 **Figure legends**

501 **Figure 1. The morphology and paratonic fission of intact *A. viride*.** (A) The first
502 segment of intact *A. viride* has a prostomium and a peristomium with a mouth.
503 Average *A. viride* contains 10 to 12 segments with pairs of chaetae. The transparent
504 body has an enlarged digestive tract located at the center of its body. The pygidium is
505 located on the last segment of the posterior end. The red dashed line indicated the
506 amputation site in front of enlarged digestive tract. (B) After the worm was amputated
507 at the site indicated in (A), the two fragments will individually proceed anterior or
508 posterior regeneration (indicated by white arrows). (C-E) The process of paratonic
509 fission separated an intact worm into two individuals. Worms with unusual lengthy
510 posterior region could be easily observed (Fig. 1C). The anterior portion of the
511 offspring gradually developed, and tilted apart from the parental worm (Fig. 1D). The
512 offspring worm break apart from the parental worm, and become an individual worm
513 (Fig. 1E). The white arrow indicated interface between parent and offspring worm.
514 Scale bar: 1 mm.

515

516 **Figure 2. Anterior regeneration in *A. viride*.** The worms were amputated in front of
517 the enlarged digestive tract, and the external morphology was observed in intact (A)

518 and 0 to 120 hpa (B) during regeneration. The intact worm has a mouth that can be
519 observed at both dorsal and ventral side of the peristomium at the first segment.
520 During anterior regeneration, the rough wounded area became smooth during wound
521 closure within 3 to 6 hpa. The protruding regenerative blastema became apparent from
522 24 to 48 hpa in most regenerating *A. viride*. Mouth formation, could be observed
523 around 96 hpa. After 96 hours of regeneration, the cephalic shape started to bulging.
524 Then, the prostomium expanded from 96 to 120 hpa. The amputation site was labeled
525 by black dotted line and the black arrow indicated the re-opening of mouth. Scale bar:
526 50 μm .

527

528 **Figure 3. Minimum segments required for successful regeneration in *A. viride*.**

529 Worms were amputated into different number of segments. The successful ratio of
530 either anterior or posterior regeneration in amputated worms regenerating from
531 different segments were observed at 5 or 3 dpa. The minimum segments required for
532 successful regeneration in *A. viride* was 3. All data represented the mean \pm s.d. from
533 at least three independent duplicate experiments (n = 10). One-way ANOVA was
534 performed to determine the significance of success rates compared to each group. *: p
535 <0.05 ; **: $p < 0.01$.

536

537 **Figure 4. Cell proliferation was detected by EdU labeling during anterior**
538 **regeneration in *A. viride*.** Worms were incubated in 100 $\mu\text{g ml}^{-1}$ EdU for 12 hours
539 prior to collecting the specimen at different time points after amputation. (A) EdU
540 signal (green) reveals cell proliferation conformed by nuclei stained with Hoechst
541 33342 (blue). The white dotted line indicates the colocalization cells in three channels.
542 Scale bar: 10 μm . (B) The worms were labeled with Hoechst 33342 and EdU in intact
543 worm. Figure on the right were magnified pictures of the head or tail regions. The
544 yellow arrow indicates the asexual reproductive zone. Since the asexual reproductive
545 zone undergo continued cell proliferation to produce progeny, the area showed
546 stronger signal of Hoechst 33342 which indicates the presence of larger amount of
547 cells. H: head, T: tail. (C) Cell proliferation was detected on anterior regenerating site
548 at different time points. Prior to amputation, the head, mouth showed the strongest
549 EdU signal. Minor proliferating cells randomly distributed throughout the body. After
550 12 hpa, EdU signal became focused at the wounded site. Edu signal completely
551 diminished post the amputation site after 24 hpa. The EdU signal peaked at 24 and 48
552 hpa, then gradually diminished. The amputation site is labeled by yellow dotted line.
553 Red triangle indicated the reappeared mouth. Scale bar: 100 μm .

554

555 **Figure 5. The inhibitory effect of taxol on cell proliferation and anterior**

556 **regeneration in *A. viride*.** (A) *A. viride* was incubated in EdU 12 hours prior to
557 fixation at 48 hpa. The amount of proliferating cells at the blastema was decreased
558 after 25 μ M taxol treatment. (B, C) The regeneration successful ratio was examined
559 from 1 to 7 dpa. Taxol treated worms showed delay and significant decrease compare
560 to the control group at 7 dpa. (D) The head morphology of regenerating worms was
561 obviously affected by taxol treatment. Head formation could be clearly observed in
562 regenerating worm incubated in 0.5% ethanol at 5 dpa, however, only a piece of
563 protruding tissue could be observed in the taxol treated group. At 7 dpa, the ethanol
564 treated group demonstrated normal regeneration, but the taxol treated group barely
565 started the formation of a regenerative blastema. Scale bar: 100 μ m. All data
566 represented the mean \pm s.d. from at least three independent duplicate experiments.
567 Significant differences relative to control group (0.5% ethanol) were denoted by *. *: p
568 <0.05 ; **: $p < 0.01$; ***: $p < 0.001$ using two-tailed unpaired student's t-test.

569

570 **Figure 6. The inhibitory effect of *Avi-tubulin* microRNA on anterior regeneration.**

571 Either the expression level of *Avi-tubulin* mRNA (A) or protein (B) expression levels o
572 were detected to assay the knock-down efficiency. The inhibitory effect of
573 regeneration successful ratio by *Avi-tubulin* RNAi was observed at 120 hpa (C).
574 One-way ANOVA was performed to determine the significance of mRNA, protein

575 expression or successful ratio compared to control group. **: $p < 0.01$.

576

577 **Figure 7. Limited cell migration is observed at anterior regenerating site of *A.***

578 *viride*. The proliferating cells were incorporated by EdU for 24 hours prior to

579 amputation in intact and regenerating worms. At 24, 72, 120 hours post amputation,

580 minor EdU⁺ cell migrated to regenerating area. Although limited signals could be

581 detected at the surface of regenerating worms, the number of proliferating cells

582 significantly differed from the area behind amputation site. The white dotted line

583 indicates the reappearance of enlarged head. The yellow bracket indicated the

584 enlarged area for the figure on the right side. Scale bar: 100 μm .

Figure 1

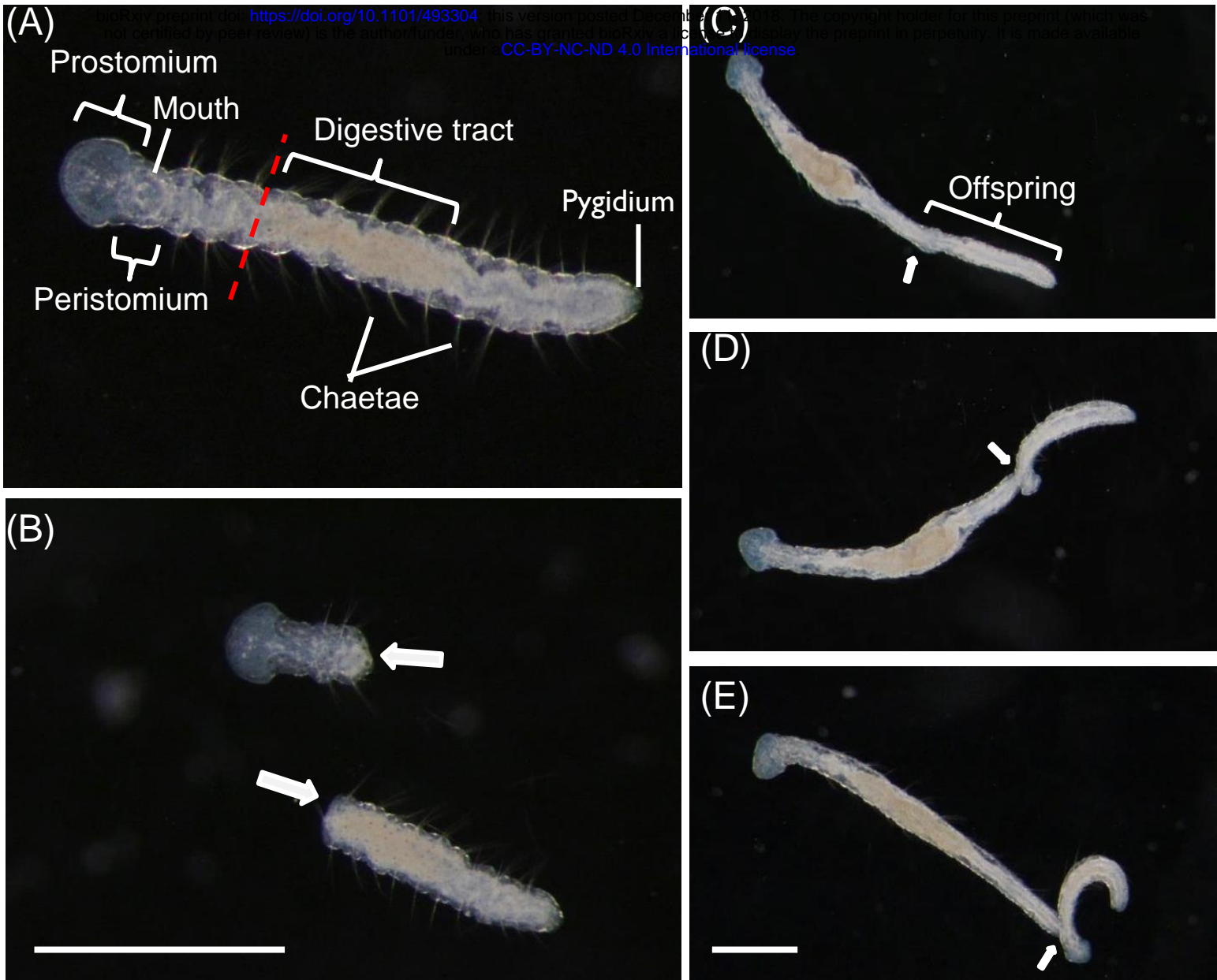


Figure 2

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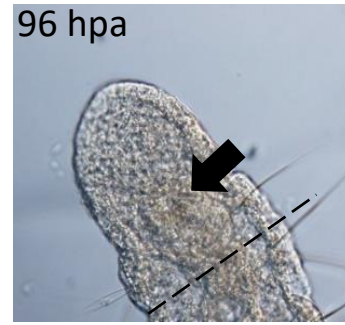
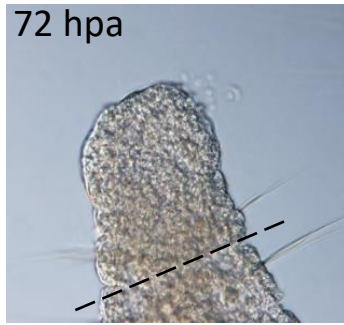
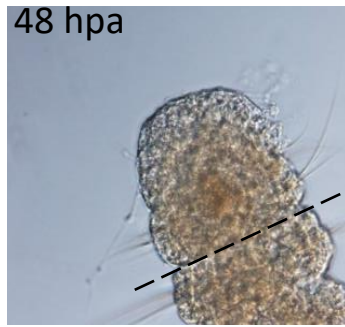
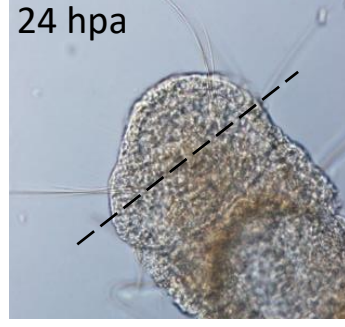
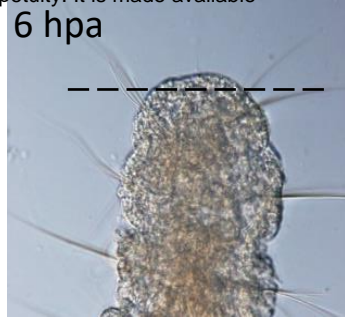
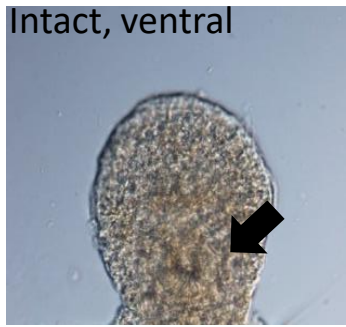


Figure 3

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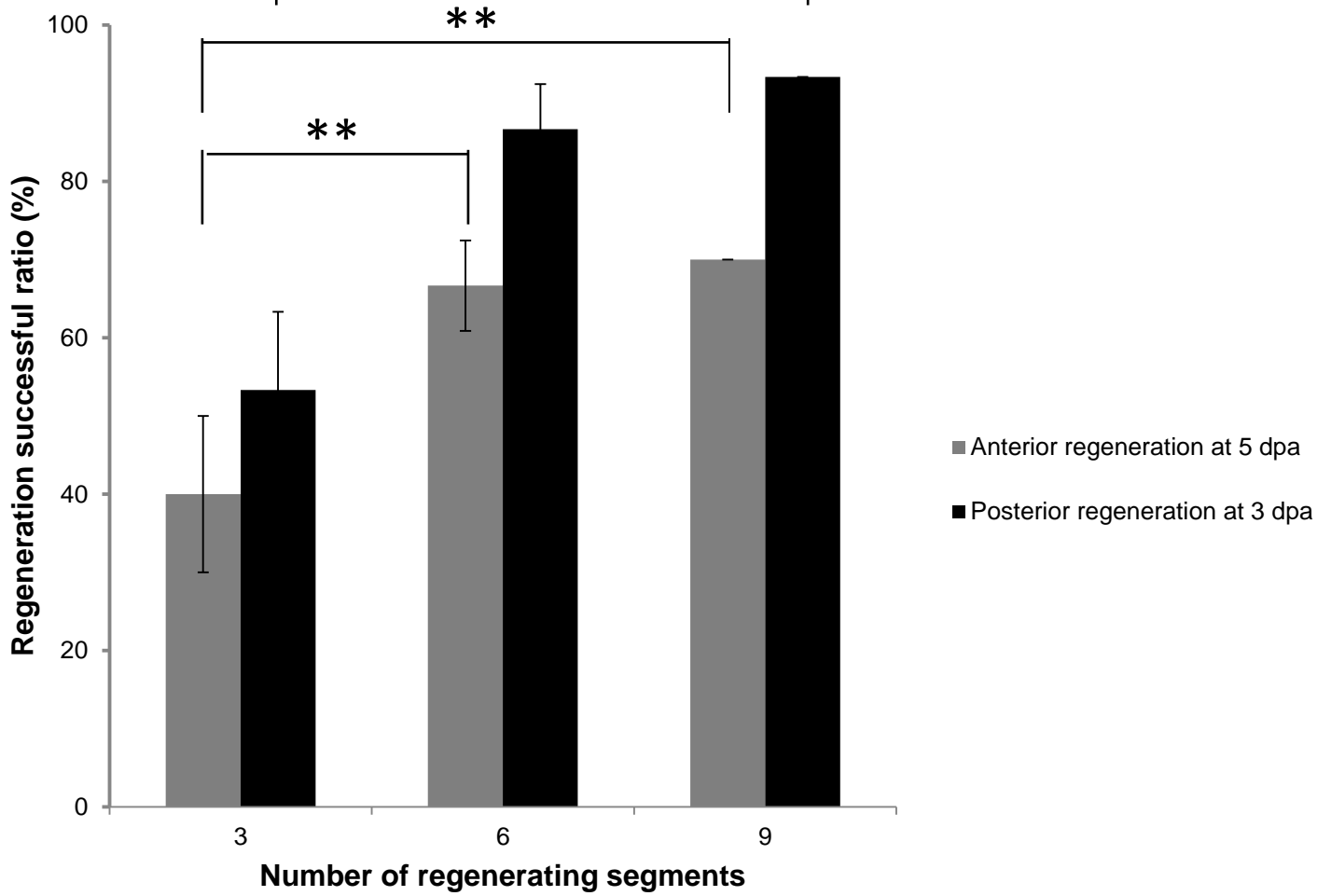
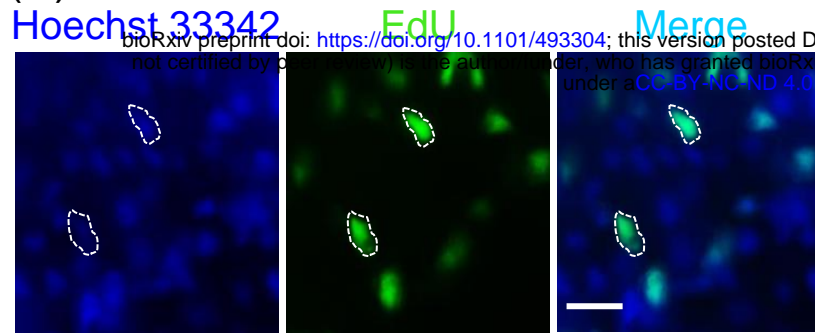
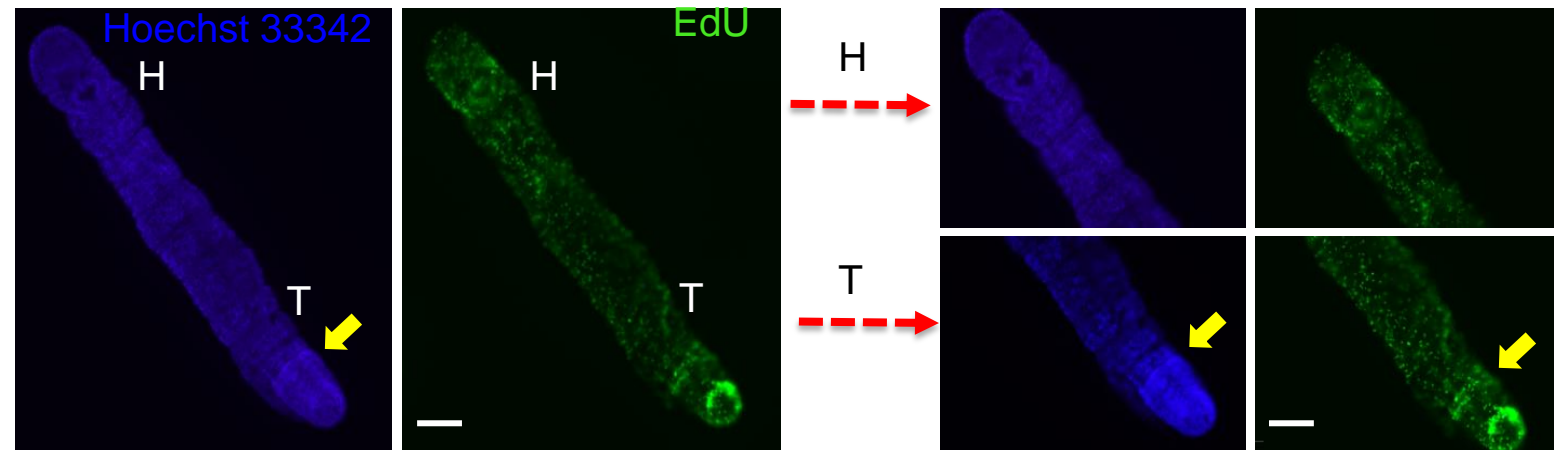


Figure 4

(A)



(B)



(C)

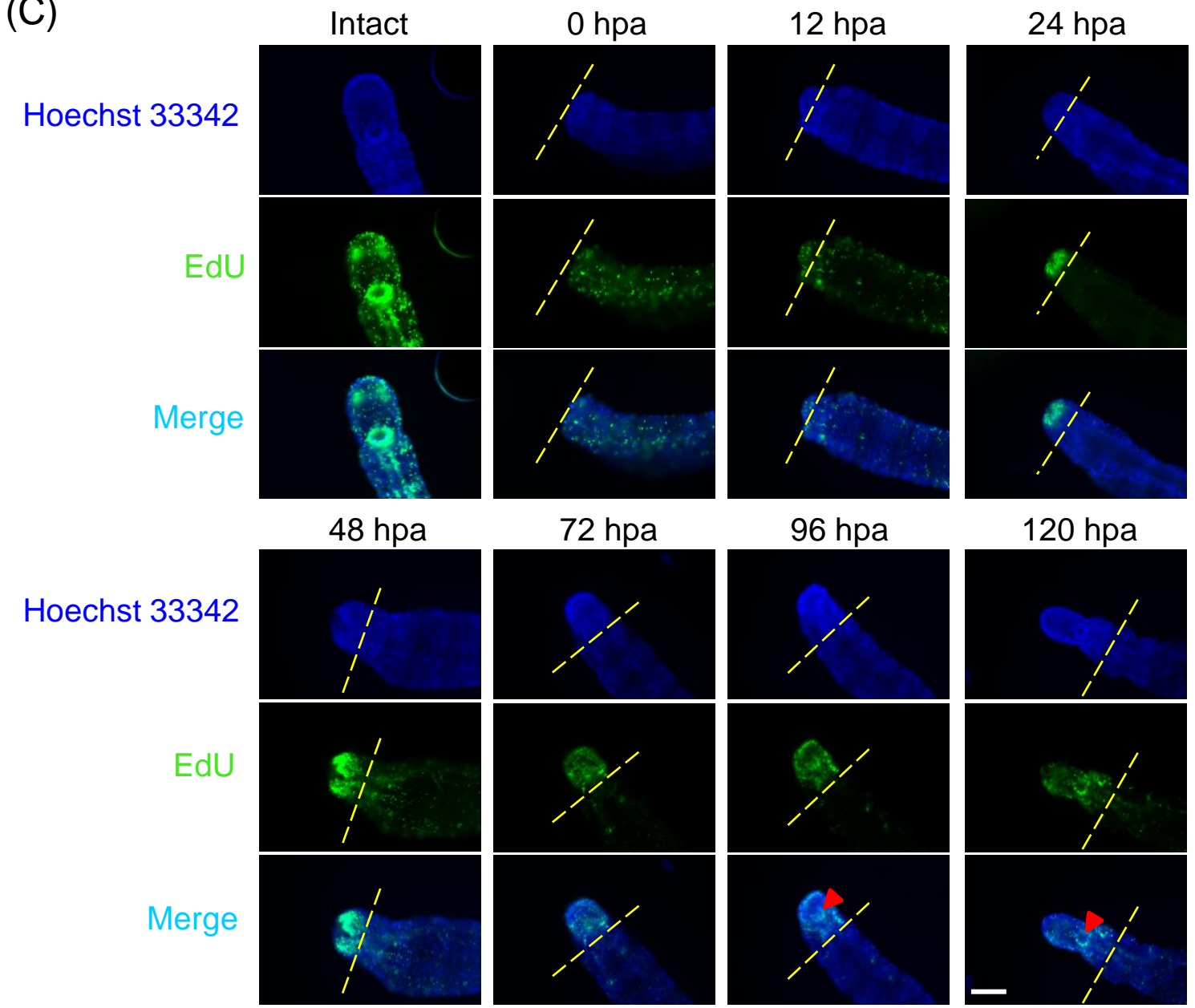
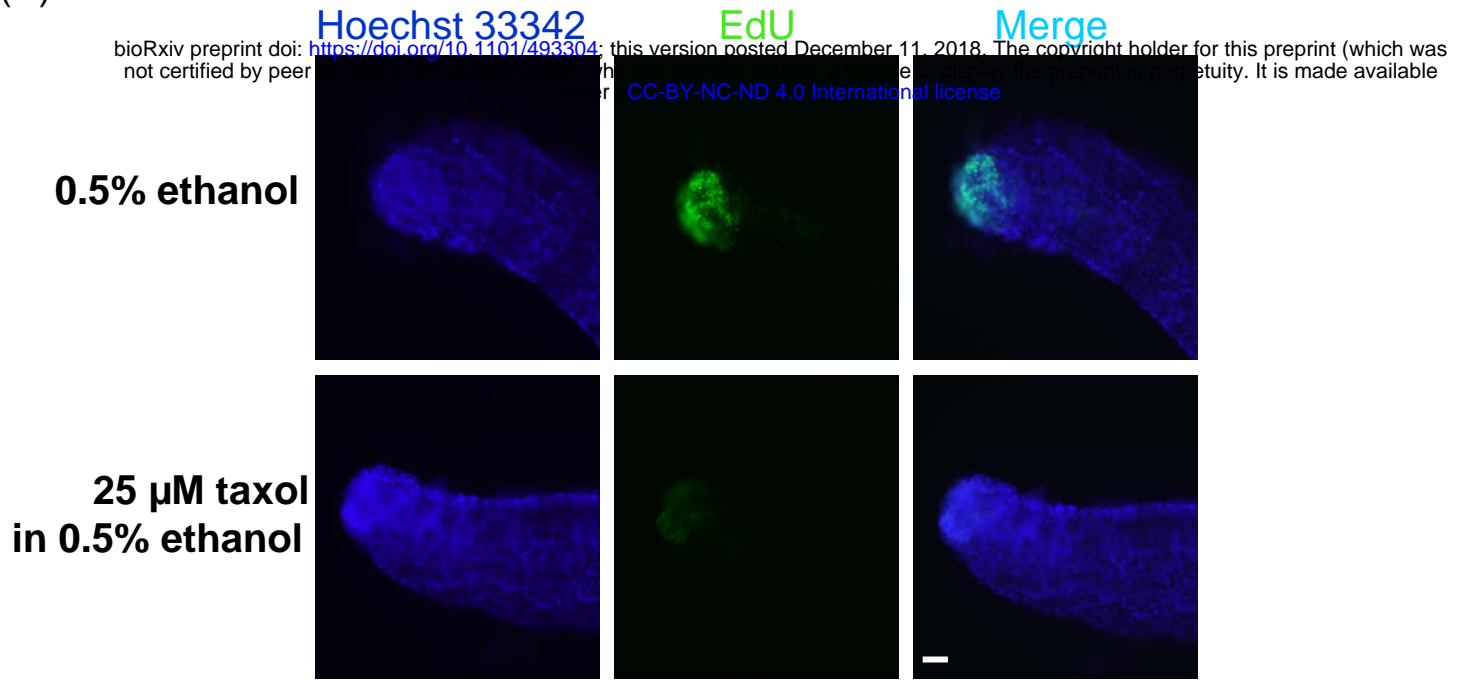
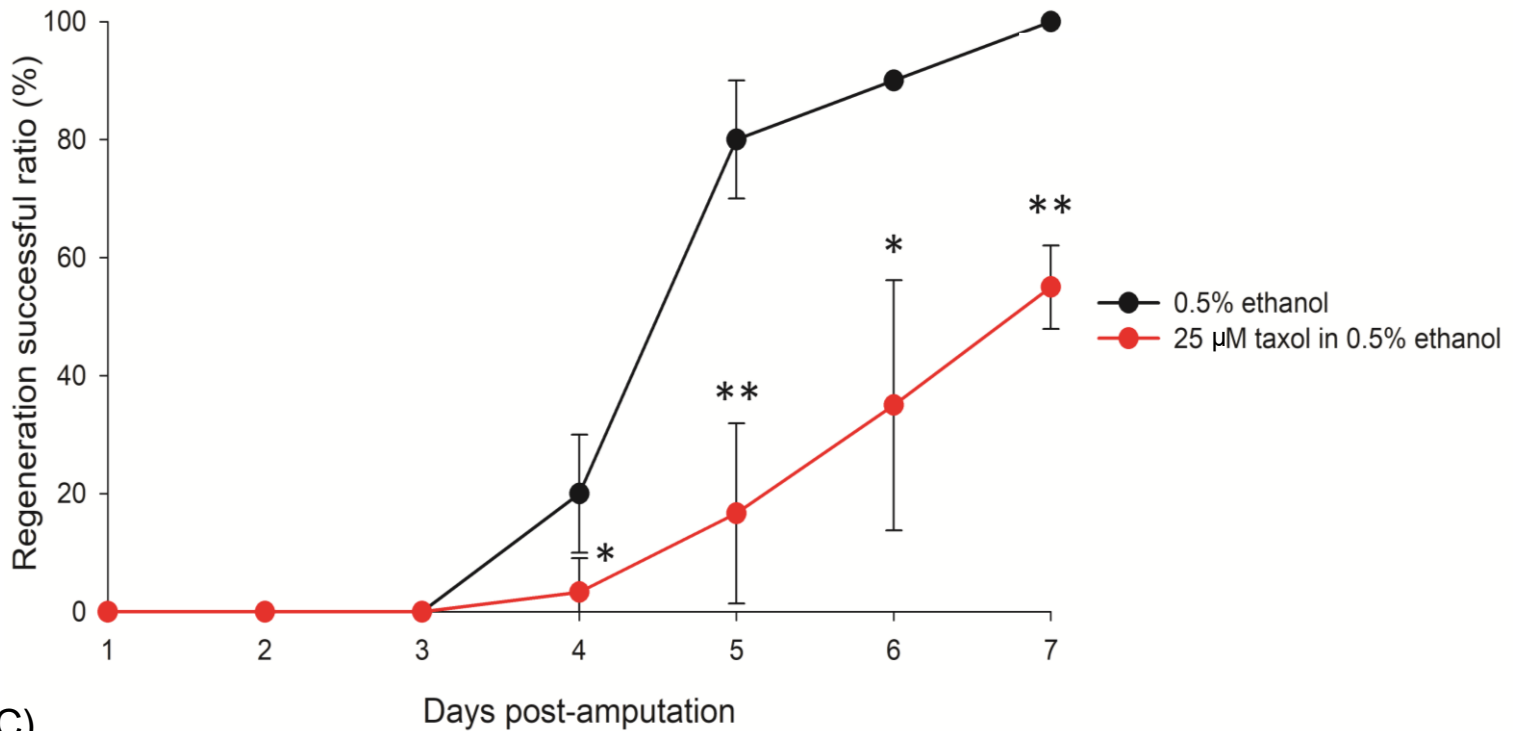


Figure 5

(A)



(B)



(C)

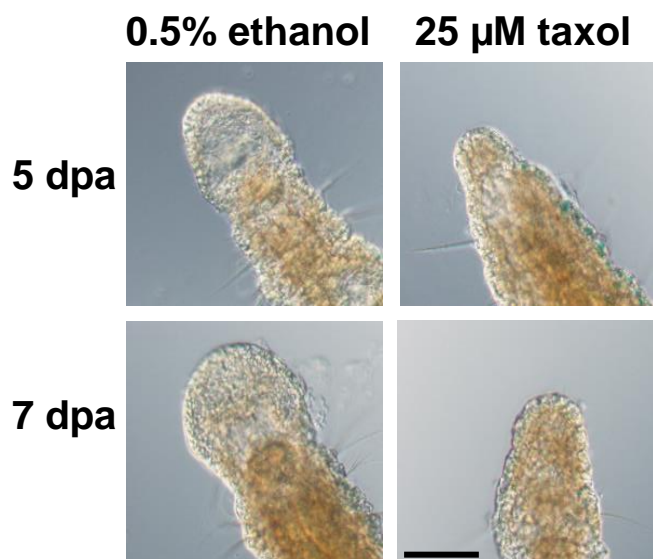
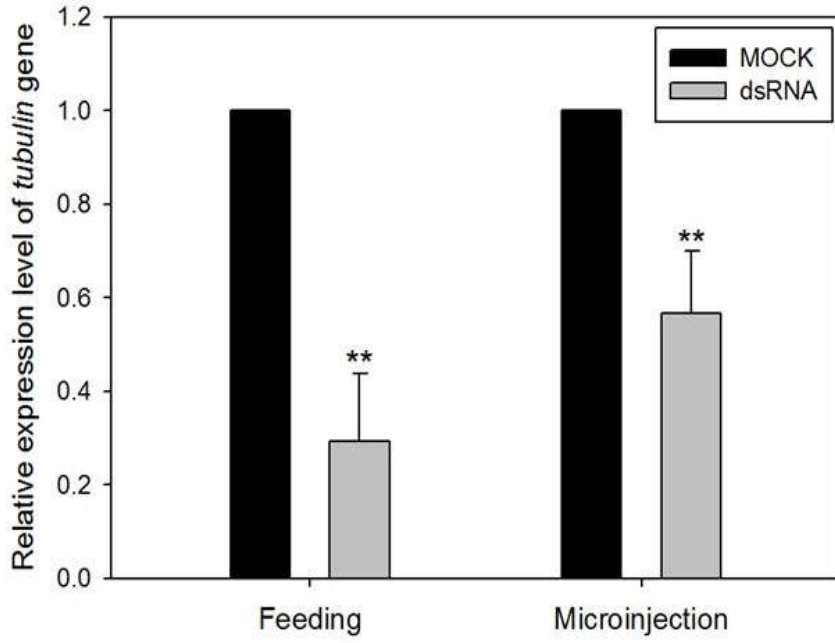


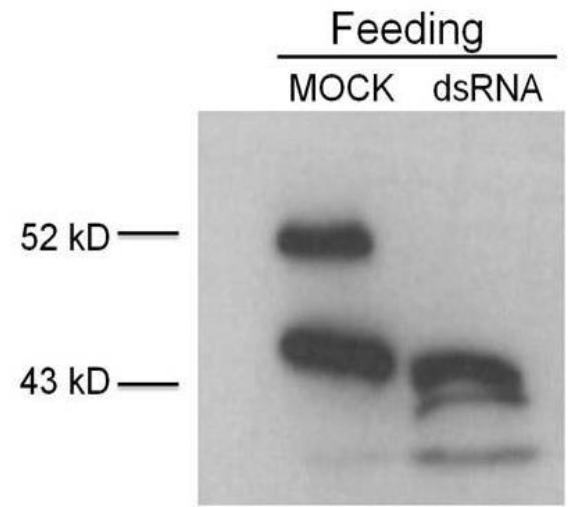
Figure 6

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(A)



(B)



(C)

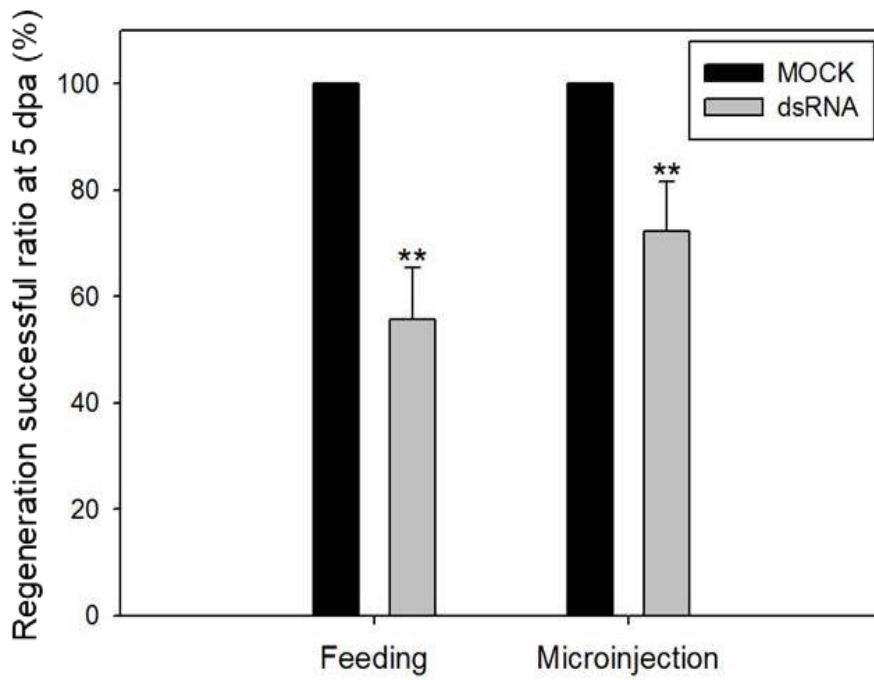


Figure 7

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