1 **Regeneration leads to global tissue rejuvenation in aging sexual planarians**

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12   **Abstract**

13   An ability to delay aging—or to reverse the negative effects of aging—could prevent age-related

14   disease and greatly enhance quality of life in old age. However, whether it is possible to globally

15   reverse the physiological effects of aging in order to extend healthspan is unknown. The

16   freshwater planarian *Schmidtea mediterranea* has been considered "immortal" due to its

17   exceptional tissue regeneration capabilities. Here, we report that a sexually reproducing lineage

18   of *S. mediterranea* exhibits age-associated physiological decline 12 months after birth. Age-

19   associated changes include alterations in sensory organs, loss of neurons and muscle, loss of

20   fertility, and impaired motility, but no reduction in stem cells at the age of 3 years. Differential gene

21   expression analysis, comparing young and old planarian cells, furthermore revealed cell-type-

22   specific changes in transcription as well as changes in classical aging pathways (e.g., insulin

23   signaling). Remarkably, amputation followed by regeneration of lost tissues led to a global

24   reversal of these age-associated changes. Older individuals that underwent regeneration showed

25   restored youthful patterns of gene expression, stem cell states, tissue composition and

26   rejuvenation of whole-animal physiology. Our work reveals a naturally evolved solution to age

27   reversal in planaria that may provide insights into anti-aging strategies in humans.

28

29

Introduction

Adult stem cells (ASCs) undergo self-renewal and differentiation to replace cells lost to wear and tear. In old age, these regenerative capacities of ASCs diminish[1-5], and this phenomenon is one of the major causes of frailty and disease[1]. Humans begin losing skeletal muscle mass in their 40s; by the age of 80, up to 50% of muscle mass may be lost, contributing to impaired function and disability[6,7]. Similarly, cell type specific neuronal loss occurs in the human hippocampus, already beginning at the age of 13 and continuing into the 80s[8]. Age-associated loss of neurons is even more devastating in the context of neurodegenerative diseases, such as Alzheimer's disease[9,10]. A method for rejuvenating the regenerative functions of aged ASCs would revolutionize medicine and significantly aid efforts to combat aging and age-related disease.

Several interventions have been shown to rejuvenate old cells in multiple tissues[2,4]. These include heterochronic parabiosis[11], exercise[12-14], caloric restriction[15], and reprogramming[2,4,16]. However, the aged hematopoietic stem cell system, which is critical to mammalian healthspan and lifespan, remains refractory to these interventions[2,17] and reprogramming reagents have to be delivered in a tissue-specific fashion[16]. Hence, while rejuvenation research has shown great promise in restoring stem cell or niche functions in multiple tissues, global rejuvenation of all tissues or age-reversal of whole-animal physiology remains elusive.

Long-lived species provide unique opportunities to uncover naturally-evolved mechanisms for the extension of health span and lifespan. For example, studies in the naked mole rat, a long-lived rodent, reported a large quiescent hematopoietic stem cell pool with prolonged cell cycles[18] relative to the short-lived house mouse, motivating extensive research on stem cell regulation in long-lived species and underscoring its potential to extend lifespan and healthspan.

55 Planarians constitute a popular long-lived model system. Freshwater planarians have been called

56 "immortal" due to their extremely long lifespan[19-23] and unique tissue regeneration capabilities

57 [24,25]. Regeneration is tightly regulated by division and differentiation of abundant adult stem cells

58 (neoblasts), and positional guiding signals expressed by muscle cells[26,27,28]. In the past two

59 decades, genomic[29,30] and molecular analyses[31-36] have provided a mechanistic understanding of

60 regeneration in several planarian species, including *Schmidtea mediterranea*. It was reported that

61 telomeres shorten[37], and viable progeny decline[38] in older planarians. Whether planarians

62 experience aging and show a typical age-dependent decline in physiological, cellular and

63 molecular functions has not been systematically examined, in part because of the challenges

64 inherent in measuring lifespan in a long-lived animal, or even defining age in asexual planarians

65 that undergo a vegetative mode of reproduction (i.e., fission[39]). Inbred lines of a sexual lineage of

66 *S. mediterranea* have been established to study genetic variations and chromosome biology[30,40].

67 This resource provides a unique opportunity to examine aging in this long-lived model system and

68 disentangle genetic control from environmental effects.

69

70 Here, we examine aging in the sexual lineage of *S. mediterranea.* To employ this model for aging

71 research, we define chronological age as time since fertilization, thus overcoming the challenges

72 involved in lineages that rely on vegetative reproduction. As in the more traditional short-lived

73 model systems (mice, fish, nematodes and fruit flies), *S. mediterranea* exhibits signs of aging at

74 multiple levels—molecular, cellular and physiological. However, unlike other model systems, we

75 show here that *S. mediterranea* is capable of reversing age-related changes. We show that

76 amputation and regeneration trigger global rejuvenation that completely reverses the multi-level

77 effects of aging.

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80

81 ***S. mediterranea* shows age-related eye phenotypes that can be reversed by the process of**

82 **regeneration**

83 To study aging in planaria, we turned to *S. mediterranea* strains that proliferate via sexual

84 reproduction, and defined zygotes as age zero. We found that most of these strains, including

85 both wild isolates of unknown age and lines bred in captivity, develop visible morphological

86 changes in the eyes between 6 months (6 MO) and 5 years of age. The most prominent phenotype

87 is the development of ectopic eyes or ectopic eye pigment cells (**Fig. 1A-D**). This eye phenotype

88 is also observed in isolates of a closely related species, *Schmidtea polychroa*, directly captured

89 in the wild (**Fig. 1E**).

90

91 To confirm that the development of ectopic eyes or pigment cells (EEP) is age-associated, we

92 performed two experiments. In the first experiment, we tracked a cohort of planarians from the

93 time of their birth from egg capsules[40]. At the age of 16 weeks, when all animals were sexually

94 mature, 2 out of 30 (6.7%) showed the EEP phenotype. At the age of 31 weeks, this fraction

95 increased to 8 out of 30 (26.7%) (**Fig. 1F**). Notably, the development of EEP did not correlate with

96 animal size (**Fig. 1F**). In a second experiment, we tracked clones of one individual (line D5D) that

97 were produced by repeated amputation and regeneration. After this cohort of clones (n=50)

98 reached sexual maturity, and at the first time point of observation, 10% of the animals showed

99 the EEP phenotype. Eight weeks later, the fraction of animals with EEP increased to 35%. In a

100 second cohort of clones (n=50) of a different individual (line A5G), we observed an increase of

101 EEP from 46% to 55% after eight weeks (**Fig. 1G**). Thus, in several different experimental

102 paradigms, the frequency of EEP phenotypes increases with age.

103

104 While generating clones for the above experiments, we noticed that amputating the heads of old

105 planarians exhibiting EEP led to the generation of new heads lacking this phenotype. We

106 wondered whether the amputation and regeneration process had somehow reset the age of the

107   head. We therefore examined directly whether tissue loss and regeneration could reverse aging

108   phenotypes. We removed the head from individuals with EEP phenotypes at the age of 32 MO

109   and examined the newly regenerated heads. In these experiments we either removed the whole

110   head (i.e., both eyes) to examine whether the remaining body would regenerate a head with two

111   young eyes (**Fig. 1H**), or removed half of the head to examine whether the newly regenerated

112   half of the head would have a young eye (**Fig. 1I**). In both experiments, amputation and

113   regeneration produced young eyes. These young eyes developed EEP again in ~6 months post-

114   regeneration.

115

116   To further characterize the rejuvenation effect and determine whether rejuvenation induced by

117   repeated rounds of amputation/regeneration led to delayed aging, we compared the eye aging

118   phenotypes of two cohorts of clones that were created from a single individual and were both

119   chronologically 36 MO. The ancestral individual was obtained by mating a pair of adults (**Fig. 2A**).

120   We repeatedly amputated and regenerated this single individual into a cohort of 181 clones,

121   allowing three weeks for regeneration plus two weeks of feeding post-regeneration. We divided

122   this cohort into a normal aging group (group A), which contained 91 clones and was maintained

123   on a weekly feeding schedule for 30 more months, and a repeated-regeneration group (group B),

124   which started with 90 clones, but underwent three additional rounds of amputation and

125   regeneration over the same 30 months. During the expansion of group B, regenerated fragments

126   (e.g., heads, trunks, tails) were mixed and fed once a week until they reached sexual maturity, at

127   which point we initiated the next round of amputation and regeneration. At the end of the

128   experiment, group B contained 2000 clones (**Fig. 2A**). We observed that 14 of 91 individuals in

129   group A (15%) had developed EEP phenotypes, with sufficient heterogeneity in the phenotypes

130   to distinguish one individual from another (**Fig. 2B**). If the two groups had the same aging rates,

131   we would expect 15% of the clones in group B to develop EEP. Contrary to this expectation, a

132   random sampling of 217 individuals from group B did not find any individuals exhibiting EEP (0%,

133   the chi-square test, p<0.01) (**Fig. 2C**). All individuals in group B showed a uniform youthful eye

134   morphology, suggesting that repeated amputation and regeneration induced rejuvenation of the

135   eye and delayed the occurrence of the aging eye phenotypes.

136

137   **Regeneration reverses age-related physiological phenotypes**

138   To determine whether the rejuvenation effect was specific to the eye, or if it impacted other age-

139   related phenotypes more globally, we examined additional physiological traits. A common age-

140   related trait in many animals is reproductive senescence. We assessed planarian fertility by

141   counting both the number of egg capsules produced and the number of hatched egg capsules

142   every 100 days in cohorts of clones of an inbred line. We then measured fertility by tracking the

143   percentage of egg capsules hatched over two years. We found that fertility declined continuously

144   from ~40% at day 200 to ~10% at day 600 (**Fig. 3A, Supplementary Table 1**). Planarians older

145   than two years continued to produce egg capsules, but their total number was reduced and <10%

146   hatched, suggesting that reproductive senescence had occurred. We observed reproductive

147   senescence in all sexual lines/populations maintained in captivity.

148

149   We then asked whether reproductive senescence can also be reversed by regeneration. We

150   plotted fertility data for young, old and regenerated cohorts of animals from an inbred line. Fertility

151   in the old cohorts (12-24 MO) was ~6% (**Fig. 3B**). Regeneration of the 18-24MO cohorts restored

152   fertility to 58% (**Fig. 3B**), comparable to that of young (4-7MO) cohorts, which had an average

153   fertility of 59% (**Fig. 3B, Supplementary Table 1**). Hence, we conclude that planarian

154   regeneration also reverses age-related reproductive senescence.

155

156   Impaired motility is another characteristic of old age in mice and humans[41,42]. To test whether

157   aged planarians exhibit impaired motility and, if so, whether regeneration can reverse it, we

158   measured the total distance traveled by individual animals in 24 hours (**Fig. 3C-E**,

159   **Supplementary Table 2**). We found that young (5MO) planarians were more active than older

160   (17MO) planarians, traveling an average of 6 more meters in a day (**Fig. 3E**). The regenerated

161   cohort (32MO-Reg) traveled an average of 4 more meters in a day than the 17MO individuals

162   (**Fig. 3E**). We conclude that regeneration also reverses age-associated loss of motility.

163

164   **Single-cell transcriptomic analysis defines cell populations of the planarian head**

165   Most animals exhibit age-related decline in stem cell function, which reduces regenerative

166   abilities[2]. A decline in stem cell function could underlie the physiological effects we observed in

167   aging planaria, yet the continued ability to regenerate may reflect maintenance of stem cell

168   function. To understand the role of planarian stem cells during aging, we first characterized the

169   types of stem cells found in the sexual lineage of *S. mediterranea*.

170

171   We used single cell mRNA sequencing (scRNA-seq) to profile head cells. To improve consistency

172   of tissue sampling from different individuals, we used the posterior ends of the two auricles to

173   mark the amputation sites and profiled each head with scRNA-seq. Head removal by this

174   procedure leads to ~8.5% loss of body mass (**Fig. 4A**), minimizing the massive body remodeling

175   that occurs in traditional planarian amputation experiments[24,43,44]. New heads are regenerated

176   within 20 days, with no significant changes in whole body shape and size, allowing us to

177   consistently analyze tissue types from different chronological ages and from different animals that

178   underwent perturbation by regeneration.

179

180   We obtained a total of 53,136 high quality single cell transcriptomes from heads at different ages.

181   The heads were obtained from individuals that were 7 MO (2 heads), 12 MO (1 head), 18 MO (1

182   head), and 32 MO (1 head). In addition, regenerated heads were obtained from three individuals

183   which were amputated at the age of 32 MO and allowed to regenerate for 2 months (**Fig. 4A,**

184   **Extended Data Fig. 1a,b, Supplementary Table 3**). Unsupervised clustering of the data

185 identified cells from all eight major planarian tissue types[45,46], including neoblasts (planarian stem

186 cells), epidermis, secretory cells, intestine, protonephridia, cathepsin+ cells, neurons, and

187 muscles (**Fig. 4B, Extended Data Fig. 1c,d**). With unsupervised sub-clustering of these eight

188 tissue types, we identified a total of 97 sub-clusters (**Fig. 4C, Extended Data Fig. 2**), and

189 annotated those from neoblast, neurons, and muscles (**Supplementary Table 4**).

190

191 We then focused on the stem cell populations. In general, we found that the adult stem cell system

192 of sexual planarians is comparable to that of asexual planarians, which contains multiple stem

193 cell subtypes (**Fig. 4C**). Sexual adults harbored several stem cell subtypes that appeared

194 homologous, based on their transcriptomic signature, to stem cells in asexual animals, and hence

195 likely have similar differentiation potentials. These include sigma (soxP-1$^+$, soxP-2$^+$), zeta (soxP-

196 3$^+$, zfp-1$^+$, egr-1$^+$, p53$^+$) and gamma (hnf4$^+$, gata456-1$^+$, nkx2.2$^+$) neoblast populations[47,48] (**Fig.**

197 **4C, Supplementary Figure 1**). Indeed, an analysis of cell cycle states associated with these cells

198 was consistent with this idea. Previous work has shown that stem cell differentiation is associated

199 with cell cycle progression[49]. Consistent with these findings, we found that progressions from zeta

200 neoblasts to early epidermal progenitors (p53$^+$, prog-1$^+$)[47,48,50], and from gamma neoblasts to

201 intestinal cells (apob-1$^+$ or apob-2$^+$)[51,52], were both associated with a transition from the S/G2/M

202 phases to the G1 phase (**Supplementary Figure 2-3**). We confirmed cell cycle states by

203 examining additional markers of proliferation (mcm4, mcm5, mcm7) and post-mitotic cells (xbp-

204 1,p4hb)[49] (**Supplementary Figure 2-3**). Furthermore, we found that the pluripotent tgs-1$^+$ cells[26]

205 are sigma neoblasts. In the sexual planarians, ~58.8% (+/- 2.6%, SEM) of tgs-1$^+$ cells are soxP-

206 1$^+$ and soxP-2$^+$ (sigma neoblasts), while 75.0% (+/-2.7%, SEM) of sigma neoblasts are tgs-1$^+$.

207 Tetraspanin-1, the antibody of which was used to purify tgs-1$^+$ adult pluripotent stem cells in

208 asexual planarians[26], correspond to 26.2% (+/- 2.0%, SEM) of sigma neoblasts (38% of tspan-1$^+$

209 cells, +/-3.4%, SEM) and to 19.2% (+/- 2.1%, SEM) of muscle progenitors (37.2% of tspan-1$^+$

210 cells, +/-3.7%, SEM) (**Supplementary Figure 4, Supplementary Table 5**).

211

212   Sexual planarians also harbor four previously uncharacterized stem cell types, which we name

213   phi, beta, CPEB1+ and es-neo neoblasts. Among the phi and beta cells, which account for 1.7%

214   and 1% of the head mass, respectively, 89.7% (+/-1.6%, SEM) and 99.3% (+/-0.3%) of the cells,

215   respectively, are in the G1 phase (**Fig. 4D, Supplementary Table 5**), similar to the pattern in

216   early epidermal progenitors (prog1+ cells)[48]. Both phi and beta neoblasts express high levels of

217   foxF-1 (**Supplementary Figure 5**), a gene required for the differentiation of dorsal-ventral

218   muscles (DVM), intestinal muscles (IM), and cathepsin+ parenchymal cells in asexual planarians

219   (**Supplementary Figure 6**)[53]. These cells also express high levels of *F-spondin* [54], *LDLRR-3*[54],

220   and ETS-1[55] (**Supplementary Figure 9**). Phi neoblasts show somewhat higher expression of

221   *foxF-1*, *nf1c* (dd_v6_12312) and *ptprj* (dd_v6_4490), and lower expression of *F-spondin* and

222   *LDLRR-3* (**Supplementary Figure 10**), which suggest that they may be precursors to beta

223   neoblasts.

224

225   Several lines of evidence suggest that phi and beta cells are progenitors of cathepsin+

226   parenchymal cells, rather than muscle cells. First, phi and beta neoblasts do not express muscle-

227   differentiation-related transcription factors, such as myoD and nkx1-1[56], which are normally

228   expressed in muscle progenitors (**Supplementary Figure 7**). On the other hand, they do show

229   enriched expression of cathepsin+ cell markers[57] (**Supplementary Figure 8**). Furthermore, *F-*

230   *spondin* [54], *LDLRR-3*[54], and ETS-1[55] have all been associated with parenchymal cells: *F-spondin*

231   and/or *LDLRR-3* are secreted by groups of differentiated and irradiation resistant brain

232   parenchymal cells in asexual planarians[54], and loss of *ETS-1* function has a specific effect on

233   gene expression in cathepsin+ cells[55]. Finally, differentiation trajectory analyses by CytoTRACE[58],

234   based on mRNA quantity and diversity, CellRank[59], which uses transcript similarity and mRNA

235   splicing dynamics computed by RNA velocity[60], and SlingShot[61], which computes a minimum

236   spanning tree and simultaneous principal curves from clusters defined by transcript similarity,

237   further support this conclusion (**Extended Data Fig. 3e-g**).

238

239   CPEB1+ neoblasts only accounted for 0.08% (+/-0.03%, SEM) of the head mass

240   (**Supplementary Table 5**). The top genes enriched in this group (**Supplementary Figure 11**),

241   including tyrosinase, C-type lectin-like protein, capsule tanning factor 1 and surfactant B-

242   associated protein, are all expressed in yolk glands, and are required for tanning of the egg

243   capsule shells[62], suggesting that this group of neoblasts represents progenitors of yolk glands.

244   The high and specific expression of CPEB1 (**Supplementary Figure 11**) supports this conclusion,

245   given that knockdown of CPEB1 leads to loss of yolk glands and ovaries[62]. Due to the small

246   number of CPEB1+ neoblasts, their aging and rejuvenation process were not examined in depth.

247

248   Es-neo stem cells accounted for 1.9% of the head mass (+/-0.7%, SEM, **Supplementary Table**

249   **5**). This group of neoblasts had three unique features. First, es-neo is the only group of stem cells

250   with comparable proportions of S (17.9%, +/-2.2%, SEM), G2/M (23.1%, +/-1.5%, SEM), and G1

251   (59%, +/-3.4%, SEM) cells (**Fig. 4D, Supplementary Table 5**). The rest of the sub-types either

252   lack a G1 phase (i.e., actively dividing, sigma, zeta, gamma, and muscle) or are dominated by G1

253   (i.e., post-mitotic, phi and beta) (**Fig. 4D**). Second, es-neo cells show high expression of a range

254   of canonical markers, including general markers for stem cells (e.g., smedwi-1, 2, 3), for sub-

255   types of stem cells (e.g., sigma, zeta, gamma, **Fig. 4F**), and for specific differentiated lineages

256   (e.g., epidermis, muscle, intestine, protonephridia, nervous system) (**Supplementary Figure 12**).

257   Finally, the expression of these marker genes is highly stochastic (**Fig. 4E-F**); the cells do not

258   show consistent or deterministic marker expression, and no markers are expressed in 100% of

259   es-neo cells. As a result, no unique marker genes were identifiable for es-neo. The es-neo cluster

260   is defined by the stochastic state of gene expression, which is not affected by cell cycle transition.

261   These gene expression patterns are reminiscent of the stochastic expression of pluripotency

262 factors in embryonic stem cells (e.g., Nanog, Oct4) (reviewed by MacArthur and Lemischka[63]), a

263 likely signature of pluripotency. Trajectory analysis with CellRank[59] (mRNA splicing), and

264 Slingshot[61] (transcript similarity) both support the idea that es-neo may be a totipotent stem cell

265 population that gives rise to zeta, sigma, phi, muscle, gamma and CPEB1+ cells (**Extended Data**

266 **Fig. 4**).

267

268 **Age-dependent gain and loss of specific cellular populations can be reversed by**

269 **regeneration**

270 We next asked how aging affects stem cells and other cell types of the head. In mammals, most

271 tissues, with the exception of the hematopoietic system[2], exhibit an age-dependent decline in the

272 total number of stem cells. In our experiments in sexual planaria, however, we did not observe a

273 significant loss of stem cells with age (**Fig. 5A, Extended Data Fig. 5**). Instead, there was a

274 significant increase in the number of beta stem cells in older animals, and we also observed an

275 upward trend in the number of zeta stem cells as well between 7 MO and 32 MO. The lack of

276 statistical significance for the latter observation is likely due to the smaller sample size, or the fact

277 that the increase becomes extensive only in much older individuals (>18 MO) (**Extended Data**

278 **Fig. 5b**).

279

280 By contrast, and consistent with aging in mice and humans, planarian aging is also accompanied

281 by a drastic loss of muscle cells and neurons (**Fig. 5B-C**), which may contribute to the loss of

282 fertility and motility (**Fig. 3**). Compared to young (7 MO) planarians, old (12MO, 18MO and 32MO)

283 planarians, lost 67.9% of the neurons in 9 out of 15 neuronal cell types, which dropped from 11.1%

284 (+/-0.3%, SEM) to 3.6% (+/-2.0%, SEM) of the head mass (**Fig. 5B, Supplementary Table 5**).

285 The other 6 neuronal types remained unchanged. In the muscle, 6 out of 17 cell types lost 65.3%

286 of the cells, dropping from 4.4% (+/-0.16%, SEM) to 1.5% (+/-0.9%, SEM) of the head mass,

287 although one dorsal-ventral muscle population (mus-2) increased by 2.08-fold, from 0.8% (+/-

288   0.2%, SEM) to 1.8% (+/-1%, SEM) of the head mass. The other muscle cell types did not show a

289   significant change. Overall, there was a net 2% loss of muscle mass in the head (**Fig. 5C,**

290   **Supplementary Table 5**).

291

292   Other major tissue types (i.e., epidermis, cathepsin+ parenchymal cells, protonephridia, secretory

293   cells), showed a mixed pattern, in which planarian aging was accompanied by both gain and loss

294   of specific cell types[4]. In the protonephridia system[64-66], aging led to loss of 66.7% of tubule cells

295   (pro-1) and 58.8% of flame cells (pro-6), but a two-fold expansion of the collecting duct cells (pro-

296   3) (**Extended Data Fig. 3a-c, Supplementary Table 5**). In the cathepsin+ cell system, trajectory

297   analysis with CytoTRACE, CellRank and SlingShot all support the idea that ldlrr-1+ cells (cat-

298   1/cat-3 and cat-2) are differentiated progeny of progenitor cells (cat-0) (**Extended Data Fig. 3d-**

299   **g**). Aging led to a ~2.5-fold expansion of cat-1 and cat-3 cells, but a 44.8% loss of cat-2 cells

300   (**Extended Data Fig. 3d, Supplementary Table 5**), suggesting that the differentiation potential

301   of cat-0 is likely shifted towards cat-1/cat-3 with age, a phenomenon similar to aging in human

302   hematopoietic stem cells[67]. In the epidermal system, aging led to an expansion of early epidermal

303   progenitors (epi-0, epi-2), but a loss of late epidermal progenitors (epi-5) and mature epidermis

304   (epi-8) (**Extended Data Fig. 3h-k, Supplementary Figure 13**).

305

306   For es-neo stem cells, there was no change in the total numbers, but we observed a shift from

307   the S/M cell cycle phases to G in 32 MO planarians (**Fig. 5D-G**). Unsupervised sub-clustering of

308   the heterogeneous es-neo populations showed four distinct sub-populations, es-neo-0 to es-neo-

309   3 (**Fig. 5D**). As planarians aged, the proportion of the es-neo-0 population decreased,

310   accompanied by a significant increase in the es-neo-1 population (**Fig. 5E**). Most es-neo-1 cells

311   are not in the S or M phases (>77%, **Supplementary Table 6, Fig. 5G**), and the proportion of

312   these cells in aged animals is significantly higher compared to the other three subclusters (**Fig.**

313   **5F**), which may explain the shift in cell cycle states.

314

315   We then asked whether these cell-type specific changes could be reversed by regeneration.

316   Indeed, single cell transcriptomic analysis showed that regeneration reversed the age-dependent

317   changes in beta/zeta stem cell, muscle and neuron cell numbers, as well as shifts in es-neo stem

318   cell cell cycle states, restoring the youthful composition and character of all the cell types (**Fig 5A-**

319   **F, Extended Data Fig. 3 and Supplementary Table 5**).

320

321   **Regeneration reverses aging-dependent molecular changes**

322   Finally, we sought to understand the molecular changes that underlie rejuvenation. To do so, we

323   examined changes in gene expression between young, old and regenerated planarian heads. All

324   tissues showed changes under these different conditions, but the degree and pattern of gene

325   expression changes varied. Comparing old and young planarians, for example, the neoblast

326   compartment, had fewer differentially expressed genes (DEGs) relative to differentiated tissues

327   (**Fig. 5H-I**). Furthermore, neoblasts showed a balance between genes that were down- and up-

328   regulated (50 downregulated genes versus 51 upregulated, in aged vs young planarians). In

329   protonephridia and epidermis, on the other hand, many more genes were up-regulated in aged

330   planarians, whereas in secretory cells, the nervous system, muscles, intestines and cathepsin+

331   cells, many more genes were down-regulated in aged planarians (**Fig. 5H**). The tissue specific

332   pattern of DEGs suggests that aging has tissue-specific impacts. Notably, the pattern of gene

333   expression changes between aged and young planarians was fully recapitulated when we

334   identified DEGs in aged v.s. regenerated planarians (**Fig. 5H, right**). These results suggest that

335   regeneration led to a global rejuvenation of gene expression in all tissues.

336

337   In further support of this conclusion, comparing young and regenerated planarians revealed only

338   a small number of DEGs (**Fig. 5H, middle**). In fact, when looking at individual genes showing

339   either an increase or a decrease in expression, we find that regeneration led to upregulation of

340 34-81% of the genes that were downregulated in aged individuals relative to young ones in

341 different tissues (**Fig. 5I, left**); conversely, regeneration led to downregulation of 45-100% of

342 genes showing upregulation in aged individuals in different tissues (**Fig. 5I, right**). Thus,

343 regeneration reverses most age-dependent changes in gene expression.

344

345 Beyond the general patterns of gene expression shifts, the specific molecular changes revealed

346 by our gene expression analysis indicate parallels in the effects of aging between sexual

347 planarians and traditional animal models[1], and here, too, there are tissue specific effects[68]

348 (**Extended Data Fig. 6 and 7**). For example, we observed downregulation of insulin and target of

349 rapamycin complex-1/2 signaling in the nervous system (neu-2, npp-2+ neurons), in muscle (mus-

350 0, ETS-1+ cells), and in the cathepsin+ parenchymal cells (cat-4, psap+ cells). By contrast, we

351 found alterations in the expression of genes regulating mitochondrial functions in all cell types.

352 These results highlight conserved aspects of the aging process, yet underline cell-specific

353 patterns.

354

355 The fact that some cell types show many age-dependent gene expression changes, while other

356 cell types show only a few, suggests that the tissues may exhibit differential vulnerability to age—

357 i.e., that cell types showing a larger number of changes may be more vulnerable to the effects of

358 aging (**Extended Data Fig. 7, Supplementary Table 7**). Our results suggest that the number of

359 changes—and hence vulnerability to aging—may correlate with the extent of differentiation. For

360 example, in the nervous system, npp2+ neurons (neu-2) were most sensitive to age-induced

361 transcriptional alteration, showing changes in the expression of 473 genes. Ciliated cholinergic

362 neurons (neu-3) and neuronal progenitors (neu-1), which were similarly abundant to neu-2

363 neurons in the heads (~2%, **Fig. 5B**), showed changes in only 91 and 25 genes (**Extended Data**

364 **Fig. 7**). We propose that cells that are less differentiated (medium CytoTRACE values) are more

365   sensitive to age-induced transcriptional alterations than highly pluripotent (high CytoTRACE

366   values) or highly differentiated cells (low CytoTRACE values) (**Supplementary Table 7**).

367

368   Our studies reveal a naturally evolved solution to age reversal in planarians. We show that

369   rejuvenation reverses the effects of aging on multiple tissues, hundreds of genes, and several

370   physiological traits. Considering the life history of these animals is near "immortal", the extent of

371   rejuvenation is likely more comprehensive and happens on the whole-body scale. Understanding

372   the regulatory mechanism of global rejuvenation has the potential to point the way toward new

373   classes of anti-aging interventions.

374

375 **Methods**

376 **Planarian strains, lab culture and fertility**

377   All planarians were maintained in 1x Montjuïch salts, in plastic containers, at 18°C, with once

378 a week feeding of organic cow liver as previously described[69]. All animals used in the current

379 study were sexually mature and starved for a week before experimentation. Fertility data was

380 collected by documenting the number of egg capsules produced every week by the examined

381 cohorts. The number of hatchlings and the number of hatched egg capsules were documented

382 for each batch of egg capsules 3 weeks after collection. Fertility value was calculated as the

383 percentage of egg capsules that hatched divided by the total number of egg capsules collected

384 over a defined period.

385   Inbred strains used in current study include A5G, D5D, D2E, S2F8b, S2F12 and LAF. Each

386 assay reported has been repeated in multiple inbred strains, instead of using one single strain or

387 a mixed population with drifting genetic backgrounds.

388

389 **Single cell RNA Sequencing Sample Preparation**

390 Heads of the animals were surgically removed under a dissection microscope, along the posterior

391 end of the auricle. Tissues were macerated with a surgical knife in CMF buffer supplemented

392 with 0.5% BSA (CMFB) as previously described[26]. Macerated tissues were kept on ice-cold CMFB

393 in a 6-cm petri dish with gentle horizontal shaking for 20min. Tissues were pipetted up and down

394 every 5 minutes with a transfer pipette for 5 to7 times to ensure sufficient disassociation and

395 optimal viability. Cells were filtered through a 40 μm strainer before collection by spinning at 400rcf

396 for 5min at 4°C. Cells were counted manually. Cell viability was assessed by labeling with trypan

397 blue (0.4%). A general viability reading of more than 70% is required before library preparation

398 with 10x single cell 3'GEM kit. To compare the young, aged, and rejuvenated heads, single cells

399 were prepared on the same day for one head from each condition. Libraries were prepared and

400   sequenced in the same batch. In general, each cell was sequenced to 35k to 80k reads on

401   NovaSeq S4 platform.

402

403   **Single-cell gene expression quantification and data quality control**

404   Using CellRanger software V6.0.1 (10x Genomics), the sequencing data were pre-processed and

405   mapped to the reference genome[29,30], and the count pipeline was run to generate the gene

406   expression matrix. Low-quality cells were filtered to remove signals from empty droplets or

407   degraded RNA by discarding cells with less than 500 detected genes, and cells with high

408   expression levels of mitochondrial genes (**Supplementary Table 3**). Doublets were detected by

409   identifying outliers with extremely high detected UMI numbers (**Supplementary Table 3**) and by

410   employing DoubletFinder[70] algorithm and were removed from each sample (**Supplementary**

411   **Table 3**).

412

413   **Data integration, clustering, and visualization**

414   Totally 53,136 cells from 8 samples were kept for subsequent analysis. The UMI counts of each

415   cell were normalized by Seurat[71], and the normalized data were log-transformed for further

416   analyses and visualization. The top 2,000 genes with the highest standard deviations were

417   obtained as highly variable genes (HVGs). For the integration of different condition samples,

418   Seurat anchor-based method[72] was performed to identify shared sources of variation across

419   datasets, allowing for robust identification of cell types and differential gene expression analysis.

420   Dimension reduction using the PCA method was performed, and the top 30 PCs were used for

421   downstream clustering analysis. The graph-based clustering approach was used for cell

422   clustering with the resolution parameter set to 0.4, resulting in 32 distinct cell clusters. Uniform

423   Manifold Approximation and Projection (UMAP) was used for visualizing cell distance in reduced

424   two-dimensional space.

425

Cell cluster annotation and subclustering

To annotate the clustering results of the whole dataset to specific tissue types, two steps were followed. (1) The cluster markers of each cluster were calculated, and clusters expressed classic markers of known tissue type were assigned to the same tissue type. (2) The expression patterns of clusters were compared to the comprehensive planarian cell atlas[45,46], clusters that shared high similarity with certain cell types were considered to be the same tissue type. Cells of each tissue type were subset from the whole dataset and integrated following the similar processes mentioned above. After significant PCs were identified (1-20 PCs were used for subclustering of cells in each tissue type), graph-based clustering was performed with variable resolution parameters according to the tissue heterogeneity. For some tissues, for example, neoblast, the sub-clusters were annotated as specific cell types according to the expression of cell-type specific markers. UMAP was used for sub-cluster visualization.

Cell cycle analysis and CytoTRACE analysis

For each tissue type, the cell-cycle state (G1, G2/M, S) of cells in the data was computationally predicted by the expression of cell-cycle-associated markers[73] (S and G2M, **Supplementary Table 9**). The CellCycleScoring function in Seurat was performed to assign the S and G2M cell cycle score to each cell, along with the phase assignment in either G1, G2M, or S phase based on the cell cycle score. CytoTRACE[58] was applied to order cells by their differentiation status using default parameters for each single tissue type. Cells with higher CytoTRACE scores are less differentiated and with higher developmental potential, and the verse visa.

Differential gene expression analysis and gene ontology enrichment analysis

Differential gene expression analysis was conducted to characterize gene **expression** changes during aging and after regeneration. For each tissue type and sub-clusters, differential expression genes (DEGs) between young and aged, aged and regenerated, and young and regenerated

452   pairs were identified using FindMarkers function[71] which employs Wilcoxon rank sum test, with p-

453   values adjusted by the Benjamini-Hochberg method for multiple testing correction. Significant

454   DEGs were identified with an average $|log2FC| > 0.25$, and an adjusted p-value < 0.05.

455   Gene ontology (GO) enrichment analysis of significant DEGs was performed using the

456   EnrichPipeline[74], and significantly enriched GO terms were identified with a false discovery rate

457   cut-off of 0.05.

458

459   **Lineage and trajectory inference**

460   The specialized neoblast subtype was combined with a specific tissue type to characterize the

461   differentiation relationships. Slingshot[61] algorithm (Version 2.6.0) was used to compute the single-

462   cell pseudotime trajectory. Specifically, after dimension reduction and clustering steps in the

463   Seurat analysis pipeline, the slingshot function was called to determine the lineage structure by

464   constructing a minimum spanning tree (MST) between the clusters. Cell fate prediction was

465   performed utilizing the following computation tools: velocyto[60] (version 0.17.17) was used to

466   quantify spliced and unspliced mRNA counts, scVelo[75] (version 0.2.5) was used to compute RNA

467   velocity vectors based on splicing ratios using the steady-state model on a per cell basis, and

468   CellRank[59] (version 1.5.1) was used to compute the initial and terminal states, which combines

469   the robustness of trajectory inference with directional information from RNA velocity.

470

471   **Motility measurements**

472   An automated video tracking setup was used to record the movements of animals[76,77]. Video

473   recordings were taken for 24 hours and analyzed with the video analysis software DDrop[76,77].

474   Based on the tracking data, the cumulative distances, and total distances of each animal in 24

475   hours were calculated.

476

477   ***In situ* Hybridization Chain Reaction (HCR)**

478 HCR probes for target genes were synthesized by Molecular Instruments. HCR RNA fluorescence

479 *in situ* hybridization was carried out as previously described[30,78]. Briefly, the animals were treated

480 by 10% NAC for 10-15min and fixed in 4% PFA for 20min. After washes with PBS + 0.5% Triton

481 X, the animals were treated by Proteinase K for 20min at room temperature. Prehybridization,

482 hybridization and amplification of HCR probes were carried out following standards of HCR

483 protocol. Probes were used at 16nM. Hairpins were used at 60nM. DAPI was used to stain cell

484 nuclei at the second last wash step. Samples were mounted with Prolong Diamond (Thermo

485 Fisher) and procured for 24 hours before imaging.

486

487 **Microscope and Image process**

488 Images were taken with Nikon A1 confocal using 10x, 20x or 40x lens. Images were read into Fiji

489 (version 2.9.0/1.54d)[79] or Imaris (version 9.9.1) and converted into RGB format. Quantification of

490 cells were carried out manually in a double-blinded fashion.

491

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502

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509

510   **Competing Interests**

511   The authors have declared that no competing interests exist.

512

513   **Contributions**

514   LG: funding, conceptualization, experimental design, data acquisition and interpretation, draft,

515   revision. XD: experimental design, data acquisition and interpretation, revision. XL, DP, JS: data

516   acquisition. LK: funding, experimental design, revision. SP: data acquisition, revision.

517

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Figure legends

Figure 1 Aging and rejuvenation of planarian heads

(A) Young planarian heads, normal eyes. (B-D) Abnormal eyes in old planarians. Red arrow: eye pigment cells. (E) Abnormal eyes of wild-caught *S. polychroa.* Young *S. polychora* were born and bred in the laboratory as a control. (F) The same cohort of hatchlings were tracked and examined at the age of 16-week-old and 31-week-old. Each square and triangle represented one animal of the cohort. (G) Cohort sizes for line A5G and line D5D were both 50 animals each. (H-I) Original heads with two abnormal eyes, and regeneration after head removal (H) or half-head removal(I). Dashed line: amputation plane. Red arrows: old eyes. Blue arrowhead: new eyes. Scale bar = 0.5mm.

Figure 2 Repeated amputation and regeneration maintains youthful heads

(A) Theme of amputation, regeneration, feeding and experimental design. Scale bar = 1mm. (B) Representative images of 6 clones with heterogeneous EEP phenotypes from group A. (C) Representative images of 6 clones with homogenous young eyes from group B.

Figure 3 Aging and rejuvenation of fertility and motility

(A) Four biological replicates. Day 200 v.s. day 500, and day 200 v.s. day 600 were statistically significant, with p.values <0.05 and <0.01. One-Way ANOVA, with Tukey's 'Honest Significant Difference' method. (B) Young cohorts: 4MO, n=1; 6MO, n=3, 7MO, n=1. Old cohorts: 12MO, n=1; 13MO, n=1; 14MO, n=1; 24MO, n=1. Old_reg cohorts: cutting old animals into multiple random fragments, n=1, 5 months post amputation of 24MO animals; cutting old animals into 9 fragments, n=1, 3 months post amputation of 18MO animals; removing the heads only, n=1, 3 months post amputation of 21MO animals. Old v.s. old_reg, and old v.s. young were statistically significant, with p.values < 0.05. One-Way ANOVA, with Tukey's 'Honest Significant Difference' method. (C) Camera and chamber set up for video recording of planarian motility. (D) Distance traveled (in meters) over time (in hours) plot for one set of tested individuals. (E) Eight replicates. Each replicate measures distance traveled by one 5MO, one 17MO, and one 32MO-Reg individual.

751   32MO-Reg: A 32MO population was amputated at the age of 28MO. After regeneration, it was

752   maintained for an additional 4 months before the experiment. Welch Two Sample t-test. p.values

753   < 0.01 for 5MO v.s. 17MO and 17MO v.s. 32MO-Reg.

754   **Figure 4 Single cell profiling of the heads and the sexual adult stem cell system**

755   (A) Head amputation and chronological ages of the samples. Young: 7 MO (n=2), Aged 12 MO,

756   18 MO, 32 MO (n=1 each), Reg: 32 MO-regenerated (n=3). (B) Major cell types identified from

757   the heads. (C) Subclusters of sexual adult stem cells (neoblasts). (D) Cell cycle distribution for

758   each type of adult stem cells. (E) Gene expression heatmap for each type of adult stem cells.

759   Gene expression in es-neo is most stochastic. (F) A significant number of es-neo cells express

760   high levels of markers representing every other stem cell type. Dots represent individual cells. y-

761   axis represents expression levels. Gene names were from previous studies (tgs-1[26], soxp-3[47]) or

762   dd_Smed_v6[80] (v6_numbers).

763   **Figure 5 Cellular and molecular rejuvenation of aged planarians**

764   (A-C) Proportion of each cell type to the cells in the whole head. (A) stem cells. (B) neurons. (C)

765   muscles. Young: 7MO. Aged:12MO, 18MO, 32MO. Reg:32MO-regenerated. Red rectangle: cell

766   types that are significantly reduced/increased in older planarians, and significantly reversed to

767   young after regeneration. p.values < 0.05. One-Way ANOVA, with Tukey's 'Honest Significant

768   Difference' method. (D) Four distinct subclusters of es-neo stem cells. (E) Within es-neo

769   population, es-neo-0 is significantly reduced in older planarians, while es-neo-1 is significantly

770   increased. (F-G) Cell cycle distribution in four es-neo subclusters (G). G1 cells of es-neo-1 is

771   significantly more than G1 cells of three other subclusters, p.values < 0.01, One-Way ANOVA,

772   with Tukey's 'Honest Significant Difference' method (F). (H) Number of differentially expressed

773   genes between aged and young (left), regeneration and young (middle), and aged and

774   regeneration (right), in eight tissue types. Blue=down regulated genes. Orange=up regulated

775   genes. (I) The exact same genes shared by aged vs reg, and aged vs young, and relative

776   percentage of such number of genes to aged vs young. Down regulated genes (left). Up regulated

777   genes (right). Each row corresponds to the tissue type in H.

778

779   **Extended data Fig.1 Single-cell RNA sequencing data quality and cluster characterization**

780   **a**, UMAP plot depicting the distribution of cells from different replicates. **b**, Violin plots of the

781   number of UMIs (top) and genes (bottom) of each sample. **c**, UMAP plot of the entire dataset (n

782   = 53,136) with cells labeled and colored by clusters (n = 32). **d**, Heatmap of the scaled expression

783   of tissue markers, which assigned the clusters to specific tissues.

784   **Extended data Fig.2 Subclustering of eight planarian tissues**

785   UMAP plots of different tissues colored by their sub-clusters. **a**, neoblast (8 clusters, 5,751 cells).

786   **b**, muscle (17 clusters, 5,571 cells). **c**, neurons (15 clusters, 7,092 cells). **d**, cathepsin+

787   parenchymal cell (11 clusters, 13,535 cells). **e**, epidermis (11 clusters, 7,183 cells). **f**, intestine

788   (10 clusters, 8,226 cells). **g**, protonephridia (10 clusters, 2,497 cells). **h**, secretory (15 clusters,

789   3,281 cells).

790   **Extended data Fig.3 Aging and Rejuvenation in differentiated tissues**

791   **a-c**, Protonephridia. (**a**) Cell proportion of each sub-cluster in samples of different ages. (**b**)

792   Lineage structure estimated by Slingshot. (**c**) UMAP plot showing piwi1$^+$/piwi1$^-$ protonephridia

793   cells. **d-g**, Cathepsin$^+$ parenchymal cell. (**d**) Cell proportion of sub-clusters. (**e**) Trajectory inferred

794   by CellRank/RNA-velocity analysis with subpopulations of phi and beta included. (**f**) lineage

795   structure predicted by Slingshot with phi and beta included. (**g**) UMAP plot showing CytoTRACE

796   score of cells in different sub-clusters, with phi and beta included. The color indicates the degree

797   of differentiation from low (red) to high (blue). **h-k**, Epidermis. (**h**) Trajectory inferred by

798   CellRank/RNA-velocity analysis with subpopulations of zeta included. (**i**) Cell proportion of sub-

799   clusters. Trajectory inferred by Slingshot (**j**) and CytoTRACE (**k**) of epidermis cells, with zeta

800   included. Cell proportion of intestine (**l**) and secretory (**m**) sub-clusters.

801   **Extended data Fig.4 Cell differentiation trajectory of neoblast subclusters**

802   **a**, Differentiation trajectory by CellRank/RNA-velocity. UMAP of neoblasts with scVelo-projected

803   velocities shown as streamlines. Clusters are colored and labeled according to original cluster

804   annotations. **b**, Differentiation trajectory by Slingshot. A minimum spanning tree (MST) between

805   neoblast cell types (nodes) was constructed by performing Slingshot algorithm to infer lineage

806   trajectories.

807   **Extended data Fig.5 Estimation statistics of stem cell loss in old planarians**

808   Error bars on estimation plots show 95% Confidence Intervals for the differences between the

809   means of the two tested age conditions (x-axis). There is 95% confidence that differences

810   between regenerated and aged, between young and aged are in a tight range below 0 for beta

811   stem cells (a). In other words, the significant increase of beta stem cells in older age (Fig 5A) is

812   unlikely going to be wrong even if sample sizes are increased. The likelihood is less than 5%. On

813   the other hand, the mean differences between age groups can be 0 in zeta (b), or most likely 0 in

814   es-neo (c) and sigma (d) stem cells. For es-neo and sigma, increasing sample sizes is unlikely to

815   change the conclusion that these stem cell types did not change between different age groups.

816   **Extended data Fig.6 Gene ontology of age-related transcriptional changes**

817   Bar graph (left) depicting number of differentially expressed genes between aged and young

818   heads, and box plot (right) displaying the enriched Gene Ontology (GO) terms in different cell

819   types. Orange: up regulated genes. Blue: down regulated genes.

820   **Extended data Fig.7 Molecular change of sub-clusters in all tissues with age**

821   Bar plot showing the number of differentially expressed genes in each sub-cluster between young

822   (7 MO x2) and aged (12 MO, 18 MO, and 32 MO) heads. Orange bars represent up-regulated

823   genes of aged heads to young heads, and blue bars represent down-regulated genes.

824   **Extended data Fig.8 Self-injury and fragmentation events**

825   **a**, A normal planarian. **b-d**, half-head injured worms. **e-h**, whole-head injured worms. **i-l**,

826   Worms recovering from head-injury. The white regions indicate the blastema. **m**, pharynx-injured

827   worm. **n-q**, head-fragments from pharynx-injury. **r-u**, tail-fragments from pharynx-injury. **v-w**,

828 Worms with nearly complete regeneration. **x-y**, worms injured at copulatory apparatus. **z**, anterior

829 and posterior-fragment from a copulatory apparatus-injury event.

830

Supplementary Table 1 Aging and rejuvenation of sexual reproduction

832 Table 1.1, Fertility rates at different ages in strain S2F12. Four independent repeats. Fertility =

833 hatched eggs/total eggs. Population size for each repeat > 20. For each time point, fertility was

834 calculated with accumulated egg data over 100 days.

835 Table 1.2, Fertility rates in young, aged and regenerated populations.

Supplementary Table 2 Aging and rejuvenation of motility

837 Total distances traveled by nine sets of different animals recorded in 24 hours. Young = 5MO.

838 Aged = 17MO. Regenerated = 4 months after amputation of 28MO animals. Distance unit = meter.

Supplementary Table 3 scRNA-Seq data summary

840 Sample information, sequencing information, and quality control thresholds of each sample.

841 you1/you2 = 7MO heads, mid1 = 12MO head, mid2 = 18MO head, aged = 32MO head,

842 amp1/amp2/amp3 = heads regenerated from 32MO animals.

Supplementary Table 4 Cell type annotation with published markers

844 Table4.1 Sub-clusters of neoblast, neurons, muscles, and cathepsin+ cells were assigned to cell

845 types according to specific expressions of certain markers.

846 Table4.2 References from which the cell type markers were collected.

Supplementary Table 5 Cell proportions and Cell cycle proportions

848 Table5.1 Cell counts and proportions of neoblast cell types in each sample at different cell cycle

849 phases.

850 Table5.2-9 Cell counts and proportions of neoblasts, neurons, muscle, protonephridia, cathepsin+

851 cells, epidermis, intestine, and secretory cell types in each sample and at different cell cycle

852 phases.

853 Table5.10 Cell counts and proportions of each tissue type in each sample.

854 **Supplementary Table 6 es-neo stem cell cell cycle proportions**

855 Cell proportions of sub-cluster of es-neo at different cell cycle phases.

856 **Supplementary Table   7 Cell clusters with high vulnerability to age-associated**

857 **transcriptional changes**

858 Summary information of vulnerable sub-clusters and control sub-clusters, including DEG number,

859 percentage of piwi1+ cells, mean CytoTRACE score, abundance in the tissue, and cell proportion

860 at each cell cycle phase in different conditions.

861 **Supplementary Table 9 Cell cycle markers used in cell cycle analysis**

862 Gene markers of S phase and G2M phase with planarian homologs.

863

864 **Supplementary Figure 1** Characterization of three neoblast subtypes, sigma, zeta, and gamma,

865 based on their cluster-enriched expression of soxp-1, soxp-2, soxp-3, zfp-1, egr-1, p53, hnf4,

866 gata456-1, and nkx2.2.

867 **Supplementary Figure 2** Characterization of the epidermal lineage. **a**, UMAP plot of epidermis

868 sub-clusters and zeta neoblasts. **b**, Cluster-enriched gene expression of epidermal lineage

869 markers, zeta (zfp-1) and early epidermal progenitors (p53 and prog-1). **c**, UMAP plot colored by

870 cell cycle phase of epidermal lineage cells.

871 **Supplementary Figure 3** Characterization of the intestine lineage. **a**, UMAP plot of intestine sub-

872 clusters and gamma neoblasts. **b**, Cluster-enriched gene expression of intestine markers (apob-

873 1 and apob-2), proliferating markers (mcm4, mcm5, and mcm7), and post meiotic markers (xbp1

874 and p4hb). **c**, UMAP plot colored by cell cycle phase of intestine cells.

875 **Supplementary Figure 4** Gene expression of tgs-1 and tspan-1 in neoblasts.

876 **Supplementary Figure 5** foxF-1 expression pattern in the neoblast sub-populations.

877 **Supplementary Figure 6** Violin plots showing expression level of the muscle markers in muscle

878 cell types.

879   **Supplementary Figure 7** Cluster-enriched gene expression of muscle differentiation-related TFs

880   in neoblast population.

881   **Supplementary Figure 8** Specific expression of cathepsin+ cell markers in neoblasts (**a**) and

882   cathepsin+ cells (**b**).

883   **Supplementary Figure 9** Specific expression of F-spondin, LDLRR-3, and ETS-1 in phi and beta
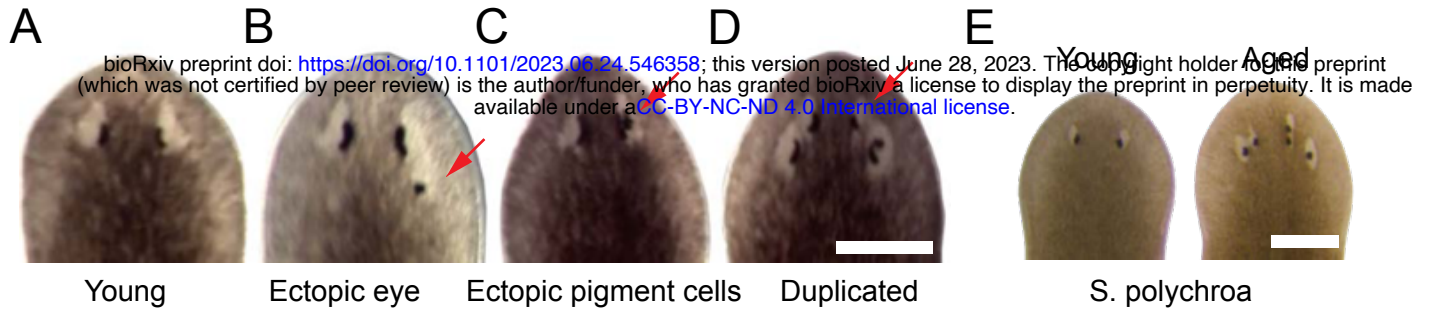
884   neoblasts.

885   **Supplementary Figure 10** Violin plots showing differential expression level of foxF-1, nf1c, ptprj,

886   F-spondin, and LDLRR-3 between phi and beta neoblast.

887   **Supplementary Figure 11** Restricted expression of yolk gland-related genes in CPEB1+

888   neoblasts.

889   **Supplementary Figure 12** Violin plots exhibiting extensive expression of different differentiated

890   lineage markers in es-neo.

891   **Supplementary Figure 13** Cluster-enriched expression of epidermal markers in epidermal

892   lineage cells with zeta included.

893
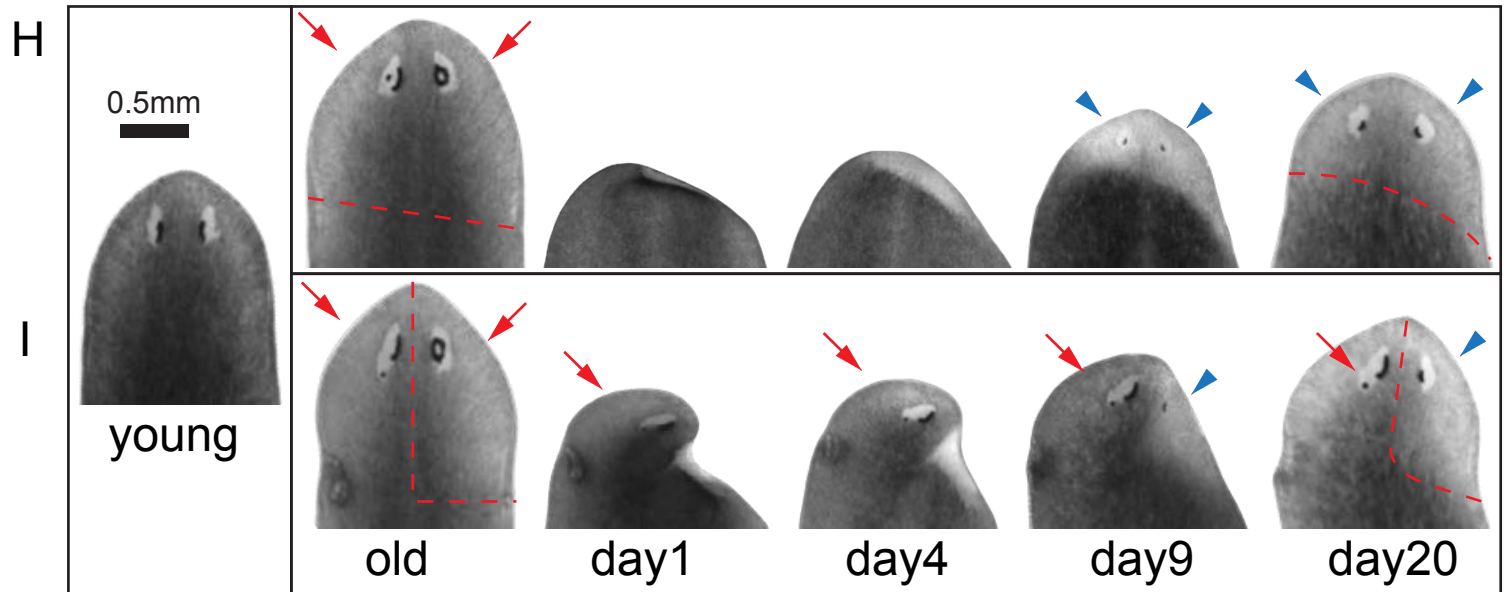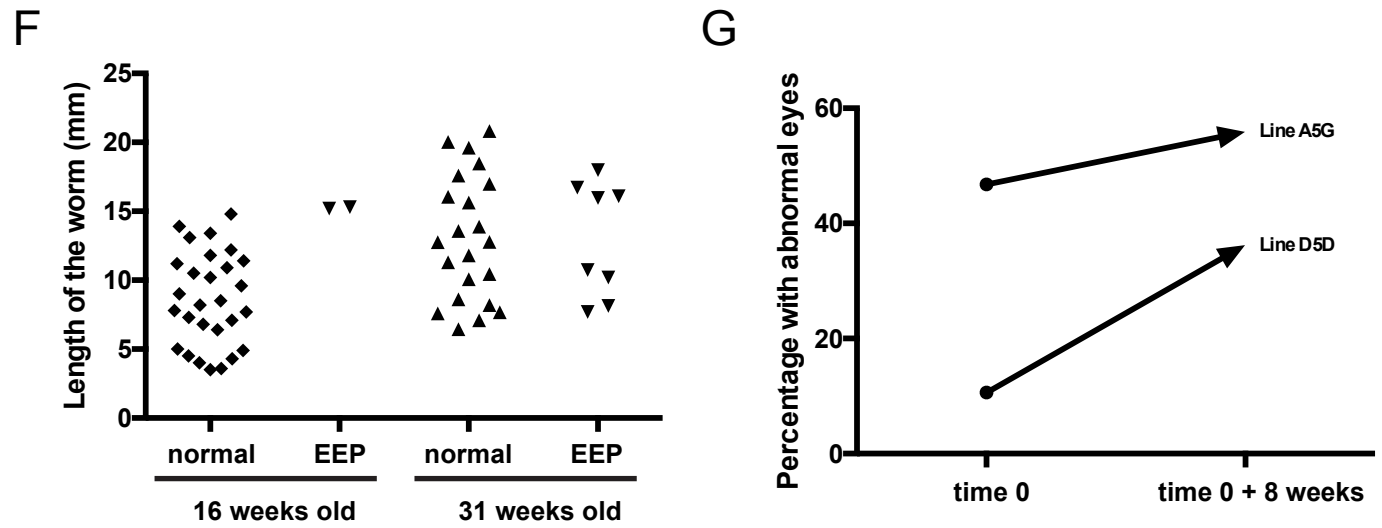
A

Sexual reproduction

n=1

Cut one animal into 2~3 pieces;
Regenerate; Repeat;
total=181 clones in 6 months

n = 91   **Split into two groups**   n = 90

Group A

Regular feeding
for 30 more months

total = 91

Group B

30 more months:
Amputation; Regeneration;
Feeding; Repeat
for 3 more rounds

total = 2,000
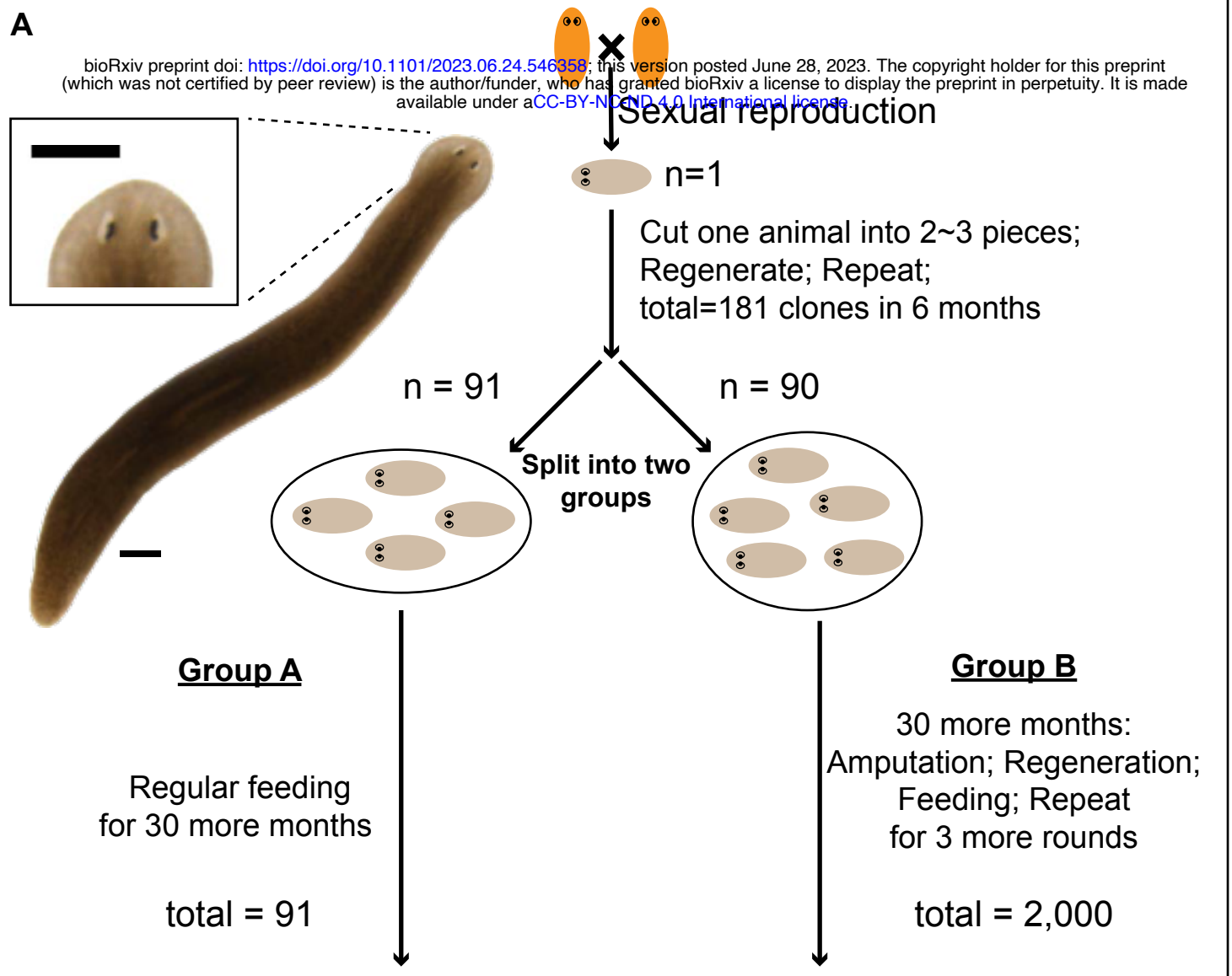
B

n = 14 (15% of total)
Ectopic pigment cells;
morphologies different in all individuals

C
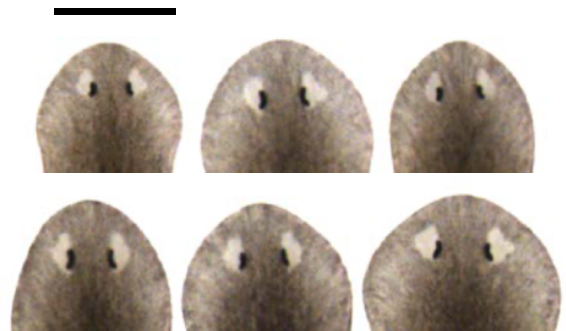
n = 0 (217 individuals sampled)
NO ectopic pigment cells;
morphologies similar in all individuals