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Abstract

Axolotls are unique in their ability to regenerate the spinal cord. However, the mechanisms that underlie this phenomenon remain poorly understood. Previously, we showed that regenerating stem cells in the axolotl spinal cord revert to a molecular state resembling embryonic neuroepithelial cells and functionally acquire rapid proliferative divisions (Rodrigo Albors et al., 2015). Here, we refine the analysis of cell proliferation in space and time and identify a high-proliferation zone in the regenerating spinal cord that shifts posteriorly over time. By tracking sparsely-labeled cells, we also quantify cell influx into the regenerate. Taking a mathematical modeling approach, we integrate these quantitative datasets of cell proliferation, neural stem cell activation and cell influx, to predict regenerative tissue outgrowth. Our model shows that while cell influx and neural stem cell activation play a minor role, the acceleration of the cell cycle is the major driver of regenerative spinal cord outgrowth in axolotls.

Introduction

Neural stem cells exist in the spinal cord of all vertebrates, but only in salamanders these cells are mobilized efficiently to resolve spinal cord injuries (Becker & Becker, 2015; Tanaka and Ferretti, 2009). In axolotls, this is best exemplified following tail amputation, when cells adjacent to the cut end regrow a fully functional spinal cord (Holtzer, 1956; Mchedlishvili *et al*., 2007). Despite the regenerative potential of axolotl neural stem cells, little was known about the molecular changes occurring upon them and the changes in cell behavior that lead to the rapid expansion of the stem cell pool during regeneration.

In our previous study, we looked at spinal cord regeneration at the molecular and cellular levels. There, we found that resident SOX2^{+} neural stem cells re-activate an embryonic-like gene expression program following tail amputation (Rodrigo Albors *et al*., 2015). Part of this program involves the re-establishment of planar cell polarity (PCP) signaling, the downregulation of pro-neural genes, and upregulation of proliferation-promoting genes. In line with these gene expression changes, we also found that regenerating neural stem cells speed up their cell cycle, and switch from neuron-generating to proliferative cell divisions. PCP turned out to be key for the efficient and orderly expansion of the regenerating spinal cord, at least in part by instructing cells to divide along the growing axis. However, besides oriented cell division, whether other cellular mechanisms such as convergence and extension, which leads to the narrowing and lengthening of tissues, are involved in the rapid expansion of the regenerating spinal cord remained unknown.

70   In this follow-up study we investigate the contribution of different cellular mechanisms to the

71   elongation of the regenerating spinal cord in the axolotl. To address this question, we apply a

72   quantitative modeling approach to causally link previous (Rodrigo Albors *et al*., 2015) and new

73   datasets to the time-course of spinal cord outgrowth. In particular, we calculate neural stem cell

74   density from previous measurements (Rodrigo Albors *et al*., 2015) to show that convergence and

75   extension is negligible. We make use of cell proliferation-related measurements along the anterior-

76   posterior axis (AP) of the spinal cord (Rodrigo Albors *et al*., 2015) to identify a high-proliferation zone,

77   which initially extends 800 μm anterior to the amputation plane, and calculate changes in cell cycle

78   kinetics within this zone. By tracing sparsely-labelled cells, we also determine cell influx into the

79   regenerating spinal cord. Finally, we set up a mathematical model of spinal cord outgrowth that

80   incorporates cell proliferation, neural stem cell activation, and cell influx. Using this model, we test the

81   contribution of each of these cellular mechanisms to the regenerative spinal cord outgrowth.

82   Comparing the model predictions with experimental data of tissue outgrowth we show that while cell

83   influx and activation of quiescent neural stem cells play a minor role, the acceleration of the cell cycle

84   in the high-proliferation zone is the major driver of the observed regenerative spinal cord outgrowth.

85   **Results**

86   **The regenerating spinal cord grows with increasing velocity**

87   To refine the outgrowth time-course of the regenerating spinal cord, we measured the spinal

88   cord outgrowth in individual axolotls, 2-3 cm snout to tail, during the first 8 days of regeneration (Figure

89   1A, Figure 1 – figure supplement 1 and Supplementary file 1). Initially, the regenerating spinal cord

90   extended slowly to a mean outgrowth of 0.45 ± 0.04 mm at day 4 (Figure 1B). Thereafter, the spinal

91   cord grew faster, reaching an outgrowth of 2.26 ± 0.07 mm by day 8.

92   **The density of neural stem cells stays constant along the AP axis of the regenerating**

93   **spinal cord**

94   To explain the outgrowth time-course of the regenerating spinal cord in terms of underlying

95   cellular mechanisms, we first set out to translate tissue outgrowth into cell numbers. To quantitatively

96   investigate neural stem cell arrangement in space and time, we revisited our previously published

97   dataset of the number of SOX2$^+$ cells per cross section in uninjured and regenerating spinal cords

98   (Figure 2A and see Materials and methods) (Rodrigo Albors *et al*., 2015). We found that the number of

99   SOX2$^+$ cells per spinal cord cross section is constant along the AP axis in both uninjured and

100   regenerating samples at any time (Figure 2B,B' and Figure 2 – figure supplement 1 and see Materials

101   and methods). We also found that the number of SOX2$^+$ cells per cross section spatially averaged

102   along the AP axis is constant during regeneration (Figure 2C and see Materials and methods). On

103   average, 30.4 ± 0.6 SOX2$^+$ cells make up the circumference of the axolotl spinal cord. Since the length

104   of SOX2$^+$ cells along the AP axis does not change during regeneration (l_c = 13.2 ± 0.1 μm) (Rodrigo

105   Albors *et al*., 2015), the density of cells along the AP axis is spatially homogeneous and equal to 2.3 ±

106   0.6 cells/μm (Figure 2A).

107   Taken together, these findings allow us to exclude mechanisms such as cell shape changes

108   and convergence and extension as driving forces of regenerative spinal cord outgrowth in the axolotl.

109   Instead, constant neural stem cell density implies an increasing neural stem cell number during

110   regeneration. This suggests that the expansion of the regenerating neural stem cell pool mostly relies

111   on proliferation-based mechanisms.
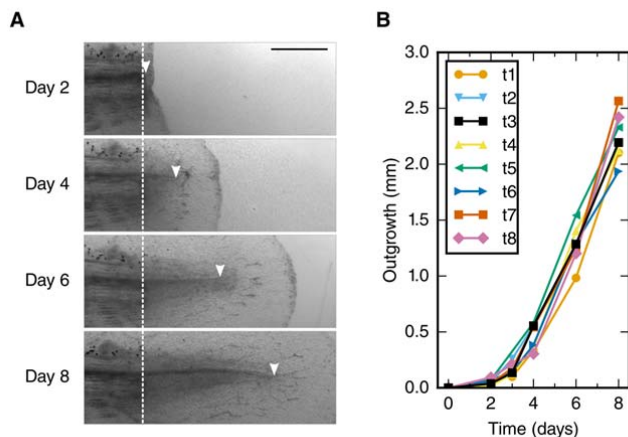
112

113   **Figure 1. Spinal cord outgrowth time-course during regeneration (A)** Representative images of a

114   regenerating spinal cord after tail amputation (individual time-lapse images are in Figure 1 – figure supplement 1).

115   The white dashed line marks the amputation plane. The arrowheads mark the tip of the regenerating spinal cord.

116   Scale bar, 1mm. **(B)** Spinal cord outgrowth time-course during the first 8 days after amputation (n = 8 axolotls).


117   **Cell proliferation increases within an 800 μm zone anterior to the amputation plane in 4-day**

118   **regenerates**

119   To determine spatial and temporal changes in cell proliferation during regeneration, we

120   calculated different cell proliferation parameters along uninjured and regenerating spinal cords. In our

4

121 previous study, we quantified the number of proliferative cells, i.e. SOX2$^+$ cells that are positive for

122 proliferating cell nuclear antigen (PCNA) and the number of cells in mitosis, i.e. SOX2$^+$/PCNA$^+$ cells

123 with condensed chromosomes based on Hoechst DNA stain (Rodrigo Albors *et al.*, 2015). Here, we

124 used these datasets to estimate the growth fraction, i.e. the fraction of proliferative cells and the mitotic

125 index, i.e. the ratio of mitotic cells over proliferative cells. Although neither SOX2$^+$/PCNA$^+$ cells nor

126 mitotic cells showed any evident spatial pattern along the AP axis in uninjured animals (Figure 2D,

127 points), they showed a tendency to increase posteriorly from day 4 (Figure 2D', points). To elucidate

128 whether proliferation was patterned along the AP axis during regeneration, we tested the data with a

129 mathematical model of two spatially homogeneous zones characterized by their growth fraction and

130 mitotic index and separated by a border that we call the *switchpoint* (Figure 2E, E'). We reasoned that

131 in the absence of an AP pattern of cell proliferation the two zones would be indistinguishable; while if

132 cell proliferation would be locally increased, the model would allow us to determine the magnitude and

133 the location of the increased cell proliferation. For a given growth fraction and mitotic index, the model

134 predicts the expected number of proliferative cells and mitotic cells per cross section (Figure 2 – figure

135 supplement 2). Hence, we fitted the model to the cell number datasets of uninjured and regenerating

136 spinal cords at day 3, 4, 6 and 8 after amputation (Figure 2D,D', Figure 2 – figure supplement 3 and

137 Figure 2 – figure supplement 4) to determine the growth fraction, the mitotic index, and the switchpoint

138 for each time point (Figure 2F-F''). Not surprisingly, we found that in the uninjured spinal cord the

139 growth fraction and the mitotic index in the two modeled zones are not significantly different (Figure

140 2D,F,F' and Figure 2 – figure supplement 3). Similarly, at day 3 there are no significant differences

141 between the two zones (Figure 2F,F' and Figure 2 – figure supplement 3). In contrast, the growth

142 fraction and the mitotic index are higher in the posterior zone from day 4 onward (Figure 2D', F, F' and

143 Figure 2 – figure supplement 3). These findings reveal that a high-proliferation zone emerges in the

144 regenerating spinal cord at day 4. At this time point, the switchpoint between the two zones is located

145 800 ± 100 µm anterior to the amputation plane, but shows the tendency to shift posteriorly as the

146 regenerating spinal cord grows (Figure 2F'').

147   Next, we combined the mitotic index measurements with our previous cell cycle length

148 estimates (Rodrigo Albors *et al.*, 2015) to establish how the proliferation rate changes during

149 regeneration (Figure 2G and see Material and methods). We find that the proliferation rate is

150 0.06 ± 0.02 per day in the uninjured spinal cord which corresponds to a cell cycle length of 10 ± 4 days

151 (Figure 2 – figure supplement 5). The proliferation rate is similar at day 3. However, at day 4 the

152 proliferation rate increases to about 0.15 per day corresponding to a cell cycle length of about 5 days

153 and the proliferation rate remains that high until day 8.

154
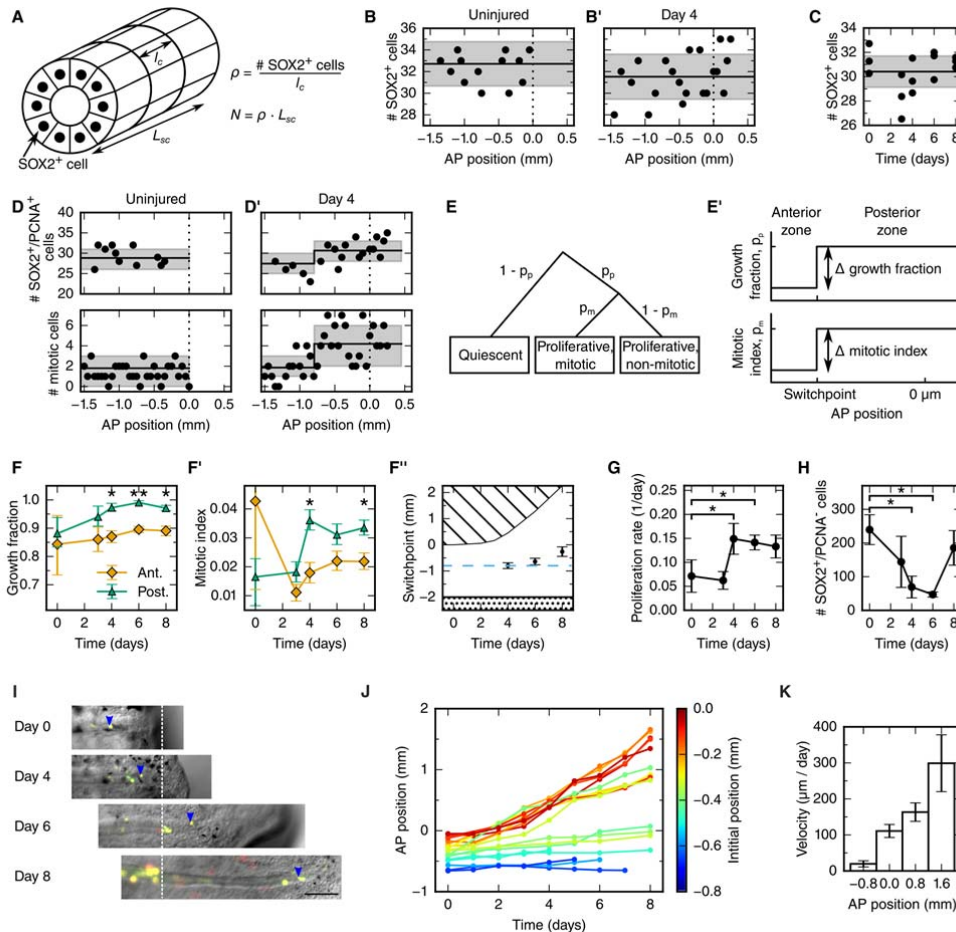
Figure 2. Cellular mechanisms underlying spinal cord outgrowth. (A) Sketch of measurements taken to

estimate the density and total number of neural stem cells (nuclei, black dots) in the axolotl spinal cord. The

density of SOX2$^+$ cells, ρ, is the ratio of the number of SOX2$^+$ cells per cross section (# stem cells) and the mean

AP cell length, l_c. The density of SOX2$^+$ cells is the proportionality constant between the total number of stem cells

in a zone along the spinal cord with zone length, L_{SC}. **(B,B')** Number of SOX2$^+$ cells per cross section along the

AP axis of a selected uninjured (B) and a selected day 4-regenerating spinal cord (B'). Black line and gray region

indicate the mean number of SOX2$^+$ cells and the standard deviation, respectively. Plots for all individual axolotls

in Figure 2 – figure supplement 1. **(C)** Spatial average of the number of SOX2$^+$ cells per cross section of individual

axolotls against time (black dots). Black line and gray region indicate the mean number of SOX2$^+$ cells and the

standard deviation of all animals, respectively. **(D,D')** Number of SOX2$^+$/PCNA$^+$ cells per cross section (upper

panel) and mitotic cells per section (lower panel) along the AP axis in a selected uninjured (D) and a selected day

4-regenerating spinal cord (D'). Black line and the gray region show the expected number and the 68%

confidence belt for the best fit of the model with two spatial proliferation zones, respectively. Plots for all animals

in Figure 2 – figure supplement 3. **(E)** Possible cell states in the two spatial proliferation zones model used to

analyze the spatial cell proliferation dataset (D,D'). p_p, probability that a cell is proliferative, otherwise quiescent.

6

170   p_m, probability that a proliferative cell undergoes mitosis at the time of analysis. **(E')** The model assumes two

171   proliferation zones. The location of the border between zones is called *switchpoint*. **(F-F')** Results of model fitting

172   for growth fraction (F) and mitotic index time-course (F') in the anterior (orange diamonds) and posterior (green

173   triangles) zone. Error bars indicate the 68% credibility interval. **(F'')** Black dots mark the switchpoint. Blue dashed

174   line marks 800 μm anterior to the amputation plane. The dashed region marks the space outside of the embryo,

175   the dotted region marks the unaffected part of the embryo. **(G)** Proliferation rate time-course in the high-

176   proliferation zone. **(H)** Total number of SOX2$^+$/PCNA$^-$ cells in the high-proliferation zone (mean ± linearly

177   propagated 1-σ error). **(I)** Selected time-lapse images of clone (blue arrowhead) tracking during spinal cord

178   regeneration. Dashed line marks the amputation plane. **(J)** Tracking of 19 clones along the AP axis during

179   regeneration. Clone trajectories are color coded by their initial position. **(K)** Clone velocities at different positions

180   along the AP axis.

181   **Quiescent neural stem cells re-enter the cell cycle during regeneration**

182   　　　　Two possible scenarios could lead to the observed increased growth fraction in the high-

183   proliferation zone (Fig. 2F): the activation of quiescent neural stem cells, or the dilution of quiescent

184   cells by the expansion of the proliferating cell population. If quiescent cells were activated, the total

185   number of quiescent cells in the high-proliferation zone would decrease. We estimated the total

186   number of quiescent cells in the high-proliferation zone from the mean number of SOX2$^+$/PCNA$^-$ cells

187   per cross section, the mean AP cell length, and the outgrowth time-course (see Materials and

188   methods). The number of SOX2$^+$/PCNA$^-$ cells drops from 180 ± 30 at day 0 to 23 ± 13 at day 6 (Figure

189   2H) which suggests that quiescent SOX2$^+$ cells get activated and re-enter the cell cycle upon injury.

190   The number of quiescent SOX2$^+$ cells appears to increase again at day 8, when cells resume

191   neurogenesis (Rodrigo Albors *et al*., 2015).

192   **Cells translocate faster the closer they are to the tip of the regenerate**

193   　　　　Cell movement could also contribute new cells to the regenerative spinal cord outgrowth. To

194   investigate whether anterior spinal cord cells move into the high-proliferation zone, we followed

195   individual cells during regeneration. For that, we co-electroporated cytoplasmic GFP and nuclear

196   mCherry plasmids at very low concentration to achieve sparse labelling of cells and tracked them daily

197   during the first 8 days of regeneration (Figure 2I). We found that labelled cells preserve their original

198   spatial order: cells located close to the amputation plane end up at the posterior end of the

199   regenerated spinal cord (Figure 2J). Most-anterior cells, however, almost do not change their position.

200   From the clone trajectories, we calculated the mean clone velocity at different positions along the AP

201   axis (Figure 2K and see Materials and methods). Clones initially located 800 μm anterior to the

202 amputation plane translocate slowly, with a velocity of 20 ± 9 μm/day. In contrast, the more posterior a

203 clone is, the faster it translocates.

204 **Cell proliferation drives the outgrowth of the regenerating spinal cord**

205 The fact that cell density along the AP axis is constant in space and time (Figure 2B-C), made

206 us reason that the spinal cord must grow as a result of increasing cell numbers. In line with this, we

207 found a high-proliferation zone, first spanning from 800 μm anterior to the amputation plane, and

208 showed that the increase in cell proliferation is due to both (i) the acceleration of the cell cycle and (ii)

209 the activation of quiescent stem cells (Figure 2D-H). The influx of cells that we identified could also

210 contribute to increasing cell numbers in the regenerating spinal cord (Figure 2I-K). To assess the

211 contribution of these cellular mechanisms to the outgrowth time-course, we used a quantitative

212 mathematical modeling framework (Greulich & Simons 2016; Rué & Martinez Arias, 2015; Oates *et al.*,

213 2009). We formalized the influence of each cellular mechanism on the total number of proliferative and

214 quiescent SOX2$^+$ cells in the high-proliferation zone in a mathematical model of cell numbers (Figure

215 3A, see Materials and methods, equations (3) and (4)). As cell density along the AP axis is constant,

216 the cell number is proportional to the AP length of the growing high-proliferation zone. Hence, we can

217 transform the model of cell numbers into an equivalent model for the tissue geometry that predicts the

218 spinal cord outgrowth, *L*, and growth fraction, *GF*:

$$\frac{dL}{dt} = \overbrace{r(t)\,(L + L_0)\,GF}^{\text{divisions of proliferative cells}} + \overbrace{\vec{v}}^{\substack{\text{influx of cells} \\ \text{into the high-proliferation zone}}} , \qquad L(t = 0) = 0, \qquad (1)$$

$$\frac{dGF}{dt} = \underbrace{(1 - GF)\,k}_{\text{activation of quiescent cells}} + \underbrace{(1 - GF)\,r(t)\,GF}_{\substack{\text{dilution of quiescent cells} \\ \text{in the expanding pool} \\ \text{of proliferative cells}}} , \qquad GF(t = 0) = GF_0, \qquad (2)$$

219 where L_0 = 800 μm is the length of the high-proliferation zone, GF_0 is the growth fraction in

220 uninjured tails, $r(t)$ is the proliferation rate at time *t*, *v* is the velocity of cells 800 μm anterior to the

221 amputation plane, and *k* is the cell cycle entry rate. As we determined the proliferation rate time-

222 course $r(t)$ (Figure 2G), the initial growth fraction GF_0 (Figure 2F) and the influx velocity *v* (Figure 2K),

223 only the cell cycle entry rate *k* is unknown. By fitting the model to the experimental growth fraction data

224 from day 0 to day 6 (Figure 3B), we determined this parameter as k = 0.2 ± 0.1 day^{-1}. Strikingly, the

225 model predicts a spinal cord outgrowth time-course that recapitulates the observed experimental data

226 (Figure 3C). This fit-free agreement shows that the acceleration of the cell cycle, the activation of

227   quiescent neural stem cells, and an influx of cells into the regenerate quantitatively explain the

228   observed spinal cord outgrowth.

229   To quantitatively determine the contribution of each cellular mechanism to the regenerative

230   spinal cord outgrowth, we switched them off one by one *in silico*. First, we switched off the

231   acceleration of the cell cycle, modeling growth only with basal cell proliferation, the influx of cells, and

232   the activation of quiescent neural stem cells (Figure 3D). This predicted a maximum outgrowth of

233   1.7 mm (p = 0.003) which is 0.6 mm shorter than the observed outgrowth at day 8. This result shows

234   that the acceleration of the cell cycle is a key driver of regenerative spinal cord outgrowth. In contrast,

235   switching off cell influx (Figure 3E) or the activation of quiescent neural stem cells (Figure 3F) has

236   almost no effect on the predicted outgrowth, which suggests that these cellular mechanisms are not

237   major drivers of regenerative spinal cord outgrowth. Indeed, even when we switched off both cell influx

238   and cell activation the observed outgrowth time-course is in agreement with the model prediction

239   (Figure 3G). Together, these results show that the acceleration of the cell cycle in cells that were

240   already proliferating in the uninjured spinal cord can explain the observed spinal cord outgrowth during

241   regeneration.

242   To test the prediction of our model against an independent experimental dataset, we revisited

243   data of *Sox2*-knockout spinal cords (Fei *et al.,* 2014). Fei and colleagues found evidences that *Sox2*-

244   knockout prevented the acceleration of the cell cycle during regeneration and lead to shorter spinal

245   cord outgrowth. In agreement with these findings, running our model with the acceleration of the cell

246   cycle switched off recapitulated the shorter outgrowth in the *Sox2*-knockout condition (Figure 3 – figure

247   supplement 2 and see Materials and methods).
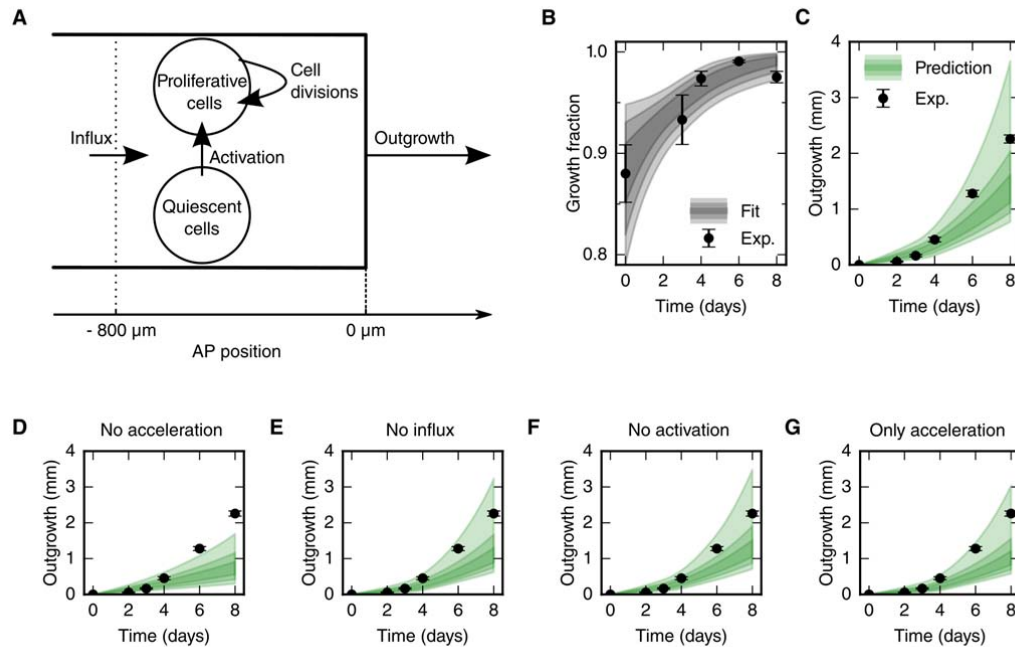
Figure 3. Mechanistic model of spinal cord outgrowth. (A) Sketch of cellular mechanisms included in the

model: cell proliferation, quiescent cell activation, and cell influx into the 800 μm high-proliferation zone. **(B)**

Growth fraction time-course of the SOX2$^+$ cell population in the high-proliferation zone as observed (black dots)

and fitted by the model (gray shaded areas, from darker to lighter, 68%, 95% and 99.7% confidence intervals of

the model prediction). **(C)** Spinal cord outgrowth during the first eight days of regeneration as observed (black

dots, n = 8 axolotls) and predicted by the model (equations (1) and (2)) (green shaded areas, from darker to

lighter, 68%, 95% and 99.7% credibility intervals). The model prediction is in agreement with the experimental

data. **(D-G)** Prediction of spinal cord outgrowth for four model scenarios based on equations (1) and (2) with

selected mechanisms switched off (green shaded areas). Black dots show the same experimental data as in

panel (C). (D) The acceleration of the cell cycle is switched off. Hence, the proliferation rate is fixed to the basal

proliferation rate of uninjured animals. (E) Cell influx is switched off (v = 0). (F) Quiescent cell activation is

switched off (k = 0). (G) Cell influx and quiescent cell activation are switched off (k = 0, v = 0). Corresponding

predictions for growth fraction in Figure 3 – figure supplement 1.

Discussion

The spinal cord tissue size and architecture is faithfully restored after tail amputation in

axolotls. This unique regenerative capability relies on neural stem cells surrounding the central canal

of the spinal cord. These cells re-activate an embryonic-like gene expression program that implements

PCP signaling to make possible the increase in cell proliferation while maintaining a tube-like structure

268   (Rodrigo Albors *et al*., 2015). However, the precise contribution of proliferation-based mechanisms to

269   the outgrowth of the regenerated spinal cord and whether other cellular mechanisms are involved

270   remained unknown.

271   Here, we combined detailed quantitative datasets with mathematical modeling to dissect the

272   cellular mechanisms that underlie regenerative spinal cord outgrowth in axolotls. We found that the

273   response to injury involves (i) changes in the cell proliferation rate, (ii) activation of quiescent neural

274   stem cells, and (iii) cell influx into the regenerating spinal cord, while maintaining a surprisingly

275   organized neural stem cell-scaffold. Modeling the contribution of each of these mechanisms to tissue

276   outgrowth upon regeneration, we uncovered that the acceleration of the cell cycle is the main driver of

277   regenerative spinal cord outgrowth in axolotls.

278   Increased proliferation of SOX2$^+$ cells upon spinal cord injury is a common feature among

279   vertebrates (Becker & Becker, 2015). In zebrafish (Hui *et al*., 2010; Hui *et al*., 2015), *Xenopus* (Gaete

280   *et al*., 2012), mouse (Lacroix *et al*., 2014) and axolotl (this work, Rodrigo Albors *et al*. 2015, Holtzer,

281   1956) traumatic spinal cord injury triggers a long-range wave of increased cell proliferation. It is

282   however clear that although the potential to replace lost cells or tissue exists in other species, they are

283   not as efficient as axolotls at resolving spinal cord injuries. A more comprehensive characterization of

284   cell proliferation responses is thus needed to understand fundamental differences between species

285   with different regenerative capabilities. In our previous study, we uncovered that spinal cord stem cells

286   in the axolotl speed up their cell cycle during regeneration (Rodrigo Albors *et al*., 2015). Performing

287   detailed quantifications, we were now able to delineate a high-proliferation zone that initially spans

288   from the 800 μm adjacent to the amputation plane to the regenerating tip, and later shifts posteriorly

289   as the spinal cord regrows. Although some quiescent neural stem cells enter the cell cycle during

290   regeneration, we demonstrate that the observed increase in proliferation is primarily due to the

291   acceleration of the cell cycle within the regenerating neural stem cell pool. By performing experiments

292   *in silico* using our mechanistic model of spinal cord regeneration, we demonstrate that the acceleration

293   of the cell cycle can explain the observed spinal cord outgrowth.

294   We further applied our model to an independent experimental dataset in which *Sox2*-knockout

295   spinal cords do not regrow properly upon amputation, due to the inability of *Sox2*-knockout cells to

296   'change gears' in response to injury (Fei *et al*., 2014). Indeed, *Sox2*-knockout cells express PCNA and

297   are in theory able to proliferate, but their lower incorporation of the thymidine analog 5-ethynyl-2'-

298   deoxyuridine (EdU) suggests that they cannot speed up the cell cycle (Fei *et al.*, 2014). We were able

299   to show that the reduced outgrowth in *Sox2*-knockout spinal cords can be quantitatively explained by

300   the lack of cell cycle acceleration (Figure 3 – figure supplement 2). However, it is important to point out

301   that our model does not include the regulation of individual cellular mechanisms and thus it does not

302   consider compensatory mechanisms that may operate under perturbed conditions. To apply our model

303   to the *Sox2*-knockout dataset, we assumed that knocking out *Sox2* only affects the acceleration of the

304   cell cycle. The fact that the model successfully recapitulated the experimental outgrowth in the *Sox2*-

305   knockout scenario suggests that compensatory mechanisms might have a small contribution in this

306   condition. Nevertheless, the validity of this assumption remains to be further investigated.

307   Our approach and findings highlight the importance of mathematical modeling and careful

308   quantification of cellular mechanisms to understand the mechanisms of regeneration. Moreover, our

309   detailed spatial and temporal characterization of cell proliferation may help to focus the search for key

310   signals that might be operating in the high-proliferation zone to speed up the cell cycle of regenerative

311   neural stem cells. It will be interesting to see whether the expression of AxMLP, the recently identified

312   regeneration-initiating factor in axolotls (Sugiura *et al.*, 2016), correlates in time and space with the

313   high-proliferation zone. This work thus provides a deeper understanding of spinal cord regeneration in

314   axolotls and new insights to help elucidating the molecular mechanisms that drive spontaneous spinal

315   cord regeneration *in vivo*.

316   Besides the increase in cell proliferation, we uncovered an influx of cells into the regenerating

317   spinal cord. Cells move along the AP axis of the spinal cord but maintain their relative position: cells

318   translocate faster the closer they are to the amputation plane (Figure 2J,K). In line with earlier work

319   (Mchedlishvili *et al.*, 2007), we found that cells initially located within the 500 μm anterior to the

320   amputation plane contribute to the regenerated spinal cord; while cells outside this zone translocate

321   slower, and cells at 800 μm, the border of the high-proliferation zone, almost do not move. This would

322   be consistent with a model in which cells are passively displaced, pushed by more anterior dividing

323   cells. In this model, the more posterior a cell is the more cells anterior to that cell divide and the

324   stronger is the push, making the cell translocate faster (Figure 4). Importantly, the proliferative

325   response extends beyond the 500 μm anterior to the amputation plane that gives rise to the

326   regenerated spinal cord (Mchedlishvili *et al.*, 2007). In the light of this model, it is plausible that cells in

327   the posterior 500 μm of the high-proliferation zone regenerate the spinal cord while cells from the

328   anterior 300 µm of the high-proliferation zone replenish and push out the 500 µm regeneration source

329   zone.
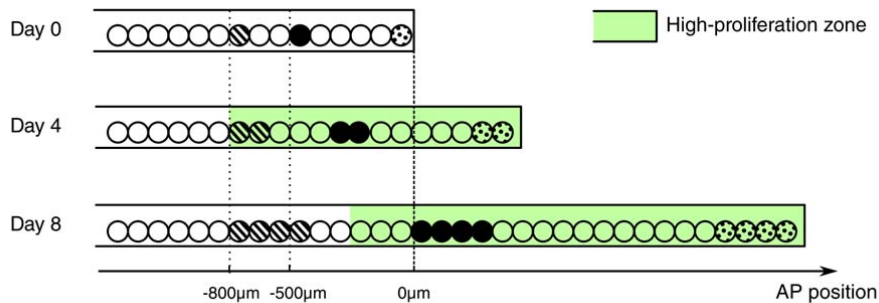
330

331   **Figure 4. Conceptual model of spinal cord growth during regeneration.** Only one row of stem cells is shown
332   as circles and three cell clones are marked with different patterns (striped, black and dotted). In the uninjured
333   spinal cord (Day 0), cells divide at a slow, basal proliferation rate (white background). From day 4 after
334   amputation, cells speed up their cell cycle and the growth fraction increases, within a high-proliferation zone that
335   initially extends 800 µm anterior to the amputation plane (green background). The density of neural stem cells
336   along the spinal cord stays constant and spinal cord outgrowth is achieved by an increase in the total number of
337   neural stem cells. Acceleration of the cell cycle in the high-proliferation zone is the major driver of this increase in
338   cell numbers. Dividing cells might push cells posteriorly. The more posterior a cell is the more cells anterior to that
339   cell divide and push the cell making it move faster: While an anterior clone (striped) hardly moves, clones in the
340   center of the high proliferation zone (black) move faster. Clones that start at the amputation plane (dotted) stay at
341   the tip of the regenerating spinal cord and move fastest.

342

343   A notable finding of this study is that the increase in cell numbers during regeneration is tightly

344   regulated so that the regenerating spinal cord extends while maintaining constant cell density and

345   proper tube-like structure. This tube-like structure made up almost entirely of neural stem cells might

346   be essential to act as a scaffold for rebuilding the spinal cord tissue architecture. Previously, we

347   showed that the activation of PCP signaling within the source zone instructs cells to divide along the

348   growing axis of the spinal cord and is key for effective spinal cord regeneration. This work highlights

349   the importance of orderly and directed expansion of the neural stem cell pool for efficient spinal cord

350   regeneration.

351   Together, our findings provide a quantitative mechanistic understanding of the cellular

352   mechanisms that drive complete spinal cord regeneration in axolotls. By performing a quantitative

353   modeling approach combined with quantitative experimental data, we found that axolotl spinal cord

354   outgrowth is driven by the acceleration of the cell cycle in a pool of SOX2$^+$ neural stem cells restricted

355   in space and time. Whether this peculiar spatiotemporal proliferative pattern is unique to the axolotl

356   and how this correlates with injury-induced signals remain to be elucidated.

Materials and methods

Axolotls

Axolotls, *Ambystoma mexicanum*, from 2 to 3 cm in length snout-to-tail were used for experiments. Axolotls were kept in tap water in individual cups and fed daily with Artemia. Before any manipulation or imaging, axolotls were anaesthetized in 0.01% benzocaine. The axolotl animal work was performed under permission granted in animal license number DD24-9168.11-1/2012-13 conferred by the Animal Welfare Commission of the State of Saxony, Germany (Landesdirektion Sachsen).

Measurement of spinal cord outgrowth

Images of regenerating tails were acquired on an Olympus SZX16 stereomicroscope using the Cell^F software by Olympus. Spinal cord outgrowth was measured from bright field images in Fiji (RRID:SCR_002285). First, the amputation plane which is clearly visible in the myotome was marked with a line. Then, the length between the intersection of the amputation plane with the spinal cord and the spinal cord tip was measured with Fiji's line tool.

Cell count data

The cell count data of SOX2$^+$ and SOX2$^+$/PCNA$^+$ cells per cross section and mitotic cells in 50 μm sections were taken from Rodrigo Albors *et al*., 2015.

Analysis of SOX2$^+$ cell count data

To test whether the SOX2$^+$ cells per cross section showed a spatial pattern along the AP axis or not, we used three different methods (Figure 2B,B', Figure 2 – figure supplement 1). First, it was tested if the cell count data linearly depends on spatial position along the AP axis using Bayesian inference (see Supplementary notebook "Constant density"). The slope was always smaller than 0.13 cells / mm and only significantly different from 0 ($p < 0.05$) for 4 of the 15 replicates. Second, a model of two spatially homogeneous zones was fitted to the data using Bayesian inference (see Supplementary notebook "Constant density"). Here, only 4 of the 15 replicates showed a significant difference in density between the two zones ($p < 0.05$). These first two methods indicated that, for an average animal, there is no significant change of the number of SOX2$^+$ cells per cross section along the AP axis. Third, the data was collapsed ignoring the spatial position, and the resulting cell count

14

385   histogram was tested for being a normal distribution using the SciPy function scipy.stats.normaltest

386   (D'agostino, 1971; D'agostino and Pearson, 1973). Only for one of the replicates the null hypothesis

387   could be rejected (p < 0.05), hence SOX2$^+$ cell density in an average animal was considered spatially

388   homogeneous with Gaussian noise in this study.

389   For each replicate the mean number of SOX2$^+$ cells per cross section averaged over all

390   measurements along the AP axis was calculated. To access whether there was a significant change in

391   this mean number, the replicates were grouped according to their time post amputation. A one-way

392   ANOVA-test showed no significant differences among the groups (*p* = 0.08, see Supplementary

393   Notebook "Constant density").

394   **Analysis of proliferation count data**

395   The counts of SOX2$^+$ cells, SOX2$^+$/PCNA$^+$ cells and mitotic cells were analyzed by fitting a

396   mathematical model of two adjacent spatial proliferation zones to the data of each time point (Figure

397   2D,D', Figure 2 – figure supplement 3).

398   The model that predicts the number of SOX2$^+$/PCNA$^+$ cells per cross section and the number

399   of mitotic cells in three-dimensional (3D) 50 µm sections based on the growth fraction and mitotic

400   index was defined as follows: If the number of SOX2$^+$ cells for a specific cross section along the AP

401   axis, N_S, had been measured, it was used for this section. If the data for the specific section was

402   missing, N_S was computed by assuming that there is a constant expected number of SOX2$^+$ cells per

403   cross section and that the deviations from the expected value follow a normal distribution. The mean

404   and standard deviation of this normal distribution were estimated by the sample mean and standard

405   deviation of the sample of the measured numbers of SOX2$^+$ cells per cross section for each replicate.

406   The number of SOX2$^+$ in a cross section is independent from other cross sections. The state

407   'Proliferative', i.e. SOX2$^+$/PCNA$^+$, is independently assigned to each SOX2$^+$ cell with probability p_p or

408   'Quiescent' with probability 1 – p_p (Figure 2E). Hence, for a given number of SOX2$^+$ cells in a cross

409   section, N_S, the number of SOX2$^+$/PCNA$^+$ cells per cross section, N_P, follows a binomial distribution

410   with N_s experiments and success probability p_p. Consequently, the expected growth fraction equals p_p.

411   As the number of mitotic cells, N_M, in 3D 50 µm sections was measured previously, we estimated the

412   number of SOX2$^+$/PCNA$^+$ cells also in a 3D 50 µm section, $N_{PS} = 50\ \mu m/l_{cell} \cdot N_P$, where $l_{cell} = 13.2 \pm$

413    $0.1\ \mu m$ is the mean AP length of SOX2$^+$ cells (Rodrigo Albors *et al*., 2015). Assuming that the cell

414   cycle position and hence the cell cycle phase of each cell is independent of all other cells, the state

415   'Proliferative, mitotic' is independently assigned to each SOX2$^+$/PCNA$^+$ cell with probability p_m or

416   'Proliferative, non-mitotic' with probability $1 - p_m$. Hence, the number of mitotic cells per section, N_M,

417   follows a binomial distribution with N_{PS} experiments and success probability p_m. Consequently, the

418   expected mitotic index equals p_m. For given values of p_p and p_m the model gives a likelihood for the

419   observed number of SOX2$^+$/PCNA$^+$ cells per cross section and mitotic cells per 3D section that can be

420   used to fit the model parameters. To reflect the assumption of two spatial proliferation zones, p_p and

421   p_m have spatial dependencies in the form of step functions (Figure 2E'). Hence, there can be different

422   growth fractions and mitotic indices for the anterior and the posterior zone, respectively. The spatial

423   position of the border between the zones is another model parameter termed *switchpoint*.

424   Furthermore, variability between replicates in the switchpoint is modeled as a normal distribution with

425   standard deviation σ_{switch}. Likewise, variability in growth fraction and mitotic index between replicates

426   is modeled with a normal distribution with spatially homogeneous standard deviations σ_{GF} and σ_{mi},

427   respectively. Hence, the resulting model to describe the cell count data of all replicates at a given time

428   point has 8 parameters: the switchpoint, growth fraction and mitotic index in the anterior zone and in

429   the posterior zone, respectively, and the inter-replicate variabilities σ_{switch}, σ_{GF} and σ_{mi}. Those

430   parameters were estimated with Bayesian inference using uniform priors for uninjured animals and at

431   3, 4, 6 and 8 days. Fitting was performed using a Markov chain Monte Carlo algorithm implemented in

432   pymc (Figure 2F-F'', Figure 2 – figure supplement 4, see also Supplementary notebook

433   "step_model_fixed_density_fit_per_timepoint"). To verify the fitting procedure, test data were created

434   by simulating our model with picked parameter values. These ''true'' parameter values were then

435   found to be included in the 95% credibility intervals of the parameter values inferred from the test data

436   with our fitting procedure.

437   **Proliferation rate time-course**

438   The cell cycle length at day 6 was estimated previously using a cumulative 5-bromo-2'-

439   deoxyuridine (BrdU) labelling approach (Rodrigo Albors *et al*., 2015). For the sake of consistent

440   methodology within the present study, the data were reanalyzed with bootstrapping using case

441   resampling (see Supplementary Notebook "brdu_bootstrapping_day6"). In agreement with the

442   previous analysis the cell cycle length was estimated as 117 ± 12 h corresponding to a proliferation

443   rate of 0.21 ± 0.02 per day at 6 days after amputation.

444   As the mitotic index is proportional to the proliferation rate (Smith & Dendy, 1962), the mitotic

445 index time-course in the high-proliferation zone was rescaled with the proliferation rate at day 6 to

446 obtain the proliferation rate time-course:

$$r(t) = \frac{mi(t)}{mi(day\ 6)} r(day\ 6),$$

447   where $r(t)$ is the proliferation rate at time t, and mi is the mitotic index. The mitotic index in the

448 high-proliferation zone was estimated as described in (Rodrigo Albors *et al*., 2015).

449 **Axolotl spinal cord electroporation**

450   Axolotl larvae (2 cm snout-to-tail) were electroporated with a dual fluorescent reporter plasmid

451 (cytoplasmic eGFP and nuclear Cherry). Cells were electroporated by cutting the tail of 2 cm-long

452 larval axolotls and inserting a DNA-filled electrode into the spinal cord (Echeverri & Tanaka 2003). To

453 transfect DNA into only a few cells, optimum electroporation conditions were three pulses of 50 V, 200

454 Hz and a length of 100 ms, applied using an SD9 Stimulator (Grass Telefactor, West Warwick, RI).

455 ***In vivo* imaging of labeled cells in the spinal cord**

456   Axolotls with sparsely labelled cells in the spinal cord were amputated, leaving cells at

457 different distances from the amputation plane. Regenerating axolotls were anaesthetized and imaged

458 every 1-2 days by placing them on a cover slip. Labelled cells were imaged using a Zeiss Apotome A1

459 microscope.

460 **Clone tracking**

461   The distance between the amputation plane and the anterior border of a clone was measured

462 manually in each image using AxioVision microscopy software (RRID:SCR_002677). Representative

463 images of one axolotl showing a clone at different distances from the amputation plane during

464 regeneration time are shown in Figure 2I. All the individual images are in Supplementary file 2.

465 **Clone velocity**

466   To estimate the mean velocity of clones at different spatial positions, the space along the AP

467 axis was subdivided into 800 μm bins. For each clone trajectory, the position measurements were

468 grouped according to these bins. Groups containing less than 2 measurements were excluded. The

469 average clone velocity for each group was estimated with linear regression. Then, the mean and

470 standard deviation of the velocity of all the clones in a bin was calculated (see Supplementary

471 Notebook "clone_velocities").

472 **Estimation of the total number of quiescent cells in the high-proliferation zone**

473   The total number of quiescent cells in the high-proliferation zone, $N_q(t)$, was estimated by

474    $N_q(t) = N_q^s \cdot L(t)/l_{cell}$, where N_q^s is the mean number of SOX2$^+$/PCNA$^-$ cells per cross section, l_{cell} is

475   the mean AP cell length, and $L(t)$ is the outgrowth time-course.

476   **Mechanistic model of spinal cord outgrowth**

477   To simultaneously evaluate the importance of cell proliferation, cell influx and activation of

478   quiescent cells in the outgrowth of the spinal cord we performed a data-driven modeling approach

479   (Greulich & Simons 2016; Rué & Martinez Arias, 2015; Oates *et al*., 2009). This approach allows to

480   establish causal relationship between the individually quantified cellular processes and it has been

481   previously employed to unravel the stem cell dynamics during spinal cord development in chick and

482   mouse (Kicheva *et al*., 2014). Although less frequent so far, modeling is more and more being used in

483   the regeneration arena (Durant et al., 2016; for an overview see Chara *et al*., 2014). In this study we

484   model the number of proliferative and quiescent cells in the high-proliferation zone by the following

485   ordinary differential equations (Figure 3A):

$$\frac{dN_p}{dt} = \overbrace{r(t)\,N_p}^{\text{cell divisions}} + \overbrace{k\,N_q}^{\text{activation}} + \overbrace{\frac{N_p}{N_p+N_q}v\,\rho}^{\text{influx}}, \quad N_p(t=0) = N_p^0 \qquad (3)$$

$$\frac{dN_q}{dt} = \qquad\quad - k\,N_q + \frac{N_q}{N_p+N_q}v\,\rho, \quad N_q(t=0) = N_q^0 \qquad (4)$$

486   where N_p^0 and N_q^0 are the initial cell numbers in this zone, $r(t)$ is the proliferation rate at time t,

487   v is the velocity of cells 800 µm anterior to the amputation plane, ρ is the density of neural stem cells

488   along the AP axis and k is the quiescent cell activation rate. The factors $N_{p/q} / (N_p + N_q)$ ensure that the

489   influx of cells into the high-proliferation zone does not alter the growth fraction. As the density is

490   constant one can write

$$\rho \cdot (L + L_0) = N_p + N_q, \qquad (5)$$

491   where L is the outgrowth posterior to the amputation plane and L_0 = 800 µm is the high-

492   proliferation zone length at t = 0. Using this relation and the definition of the growth fraction GF,

$$GF = \frac{N_p}{N_p+N_q}, \qquad (6)$$

493   the cell number model was reformulated as a model for outgrowth and growth fraction (see

494   Results, equations (1) and (2)).

18

495   The assumption that the population mean model parameters can be used to estimate the

496   population mean outgrowth time-course was used when simulating the model and interpreting results.

497   The confidence intervals of the model prediction were estimated with a Monte Carlo approach using

498   bootstrapping with a case resampling scheme (100,000 iterations). In each iteration we case-

499   resampled the cell count data, the BrdU incorporation data and the clone trajectory data, and

500   calculated the proliferation rate time-course, clone velocity at -800 μm and initial growth fraction from

501   this resampled data as described above. Then, in each iteration, these bootstrapped parameter values

502   were used to estimate the activation rate k by fitting the model prediction of the growth fraction to the

503   data (Figure 3B). The growth fraction measurement of day 8 was excluded from the fit because its

504   precise value would only affect the model prediction after this day. Now, as all parameters were

505   estimated, an outgrowth trajectory was calculated for each iteration. This ensemble of trajectories was

506   used to calculate the confidence intervals of the model prediction (Figure 3C). The same approach

507   was used for the model scenarios with individual cellular mechanisms turned off (Figure 3D-G). The

508   source code is available in the Supplementary notebook "lg_model".

509   **Validation of a model prediction against an experimental dataset**

510   Control animals by Fei et al., 2014 showed less regenerative outgrowth than our 'normally'

511   regenerating animals. This could be either due to their control CRISPR treatment or due to their

512   reduced feeding. To account for the reduced growth, we assumed that all cellular mechanisms

513   maintain the same relative contribution in Fei and colleagues' control as they have in normal

514   regeneration. This assumption allowed linear rescaling of the outgrowth dataset from Fei and

515   colleagues to match our 'normal' outgrowth dataset (Figure 3 – figure supplement 2A, Supplementary

516   notebook "lg_model"). We also assumed that *Sox2*-knockout only affects the acceleration of the cell

517   cycle but that all other cellular mechanisms remain unaffected (i.e. compensatory mechanisms are not

518   considered). Fewer neural stem cells make up the circumference of *Sox2*-knockout spinal cords (Fei

519   et al., 2014). Assuming that AP cell length is unchanged this means that cell density is decreased in

520   this condition. Therefore, we corrected the outgrowth for the *Sox2*-knockout dataset to a density

521   corrected outgrowth by $L_{corr} = N_S^{Sox2^{ko}}/N_S^{control} \cdot L$, where L_{corr} is the density corrected outgrowth, L is

522   the measured outgrowth in the *Sox2*-knockout dataset and $N_S^{Sox2^{ko}}$ and $N_S^{control}$ are the mean number

523   of neural stem cells per cross section in the *Sox2*-knockout and control condition, respectively (Figure

524   3 – figure supplement 2B, Supplementary notebook "lg_model").

525 **Coordinate system**

526   Time starts with the event of amputation. For spatial positions along the AP axis of the spinal

527 cord, the amputation plane defines 0; positive values refer to positions posterior to the amputation

528 plane, in regenerated tissue; negative values refer to positions anterior to the amputation plane. In all

529 images, anterior is to the left.

530 **Statistics and computational tools**

531   If not stated otherwise, measurements are reported as mean ± standard error of the mean. In

532 the figures * denotes $p < 0.05$ and ** denotes $p < 0.01$ for the respective test as indicated in the figure

533 caption.

534   Image analysis was performed with Fiji (Schindelin *et al.*, 2012) and AxioVision Microscopy

535 software (Zeiss). Data analysis was performed using the python modules bokeh

536 (http://bokeh.pydata.org), iminuit (http://github.com/iminuit/iminuit), ipycache

537 (http://github.com/rossant/ipycache), Jupyter Notebook (http://jupyter.org/), matplotlib (Hunter, 2007),

538 numba (http://numba.pydata.org/), pandas (McKinney, 2010), probfit (http://github.com/iminuit/probfit),

539 pymc (Patil *et al.*, 2010), SciPy (Jones *et al.*, 2001) and uncertainties

540 (http://pythonhosted.org/uncertainties/).

541 **Supplementary notebooks**

542   Jupyter Notebooks containing the source code for all computations performed together with

543 the data and referred to as individually named Supplementary notebooks in this work can be found

544 under https://doi.org/10.5281/zenodo.160333.

549 **Competing interests**

550   The authors declare no competing interests.

Author contributions

FR, Conception and design, Analysis and interpretation of data, Drafting or revising the article.

ARA, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article.

VM, Acquisition of data, Drafting or revising the article.

LB, Analysis and interpretation of data, Drafting or revising the article.

AD, Analysis and interpretation of data, Drafting or revising the article.

EMT, Conception and design, Analysis and interpretation of data, Drafting or revising the article.

OC, Conception and design, Analysis and interpretation of data, Drafting or revising the article.

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633 **Figure supplements**

634 **Figure 1 – figure supplement 1**
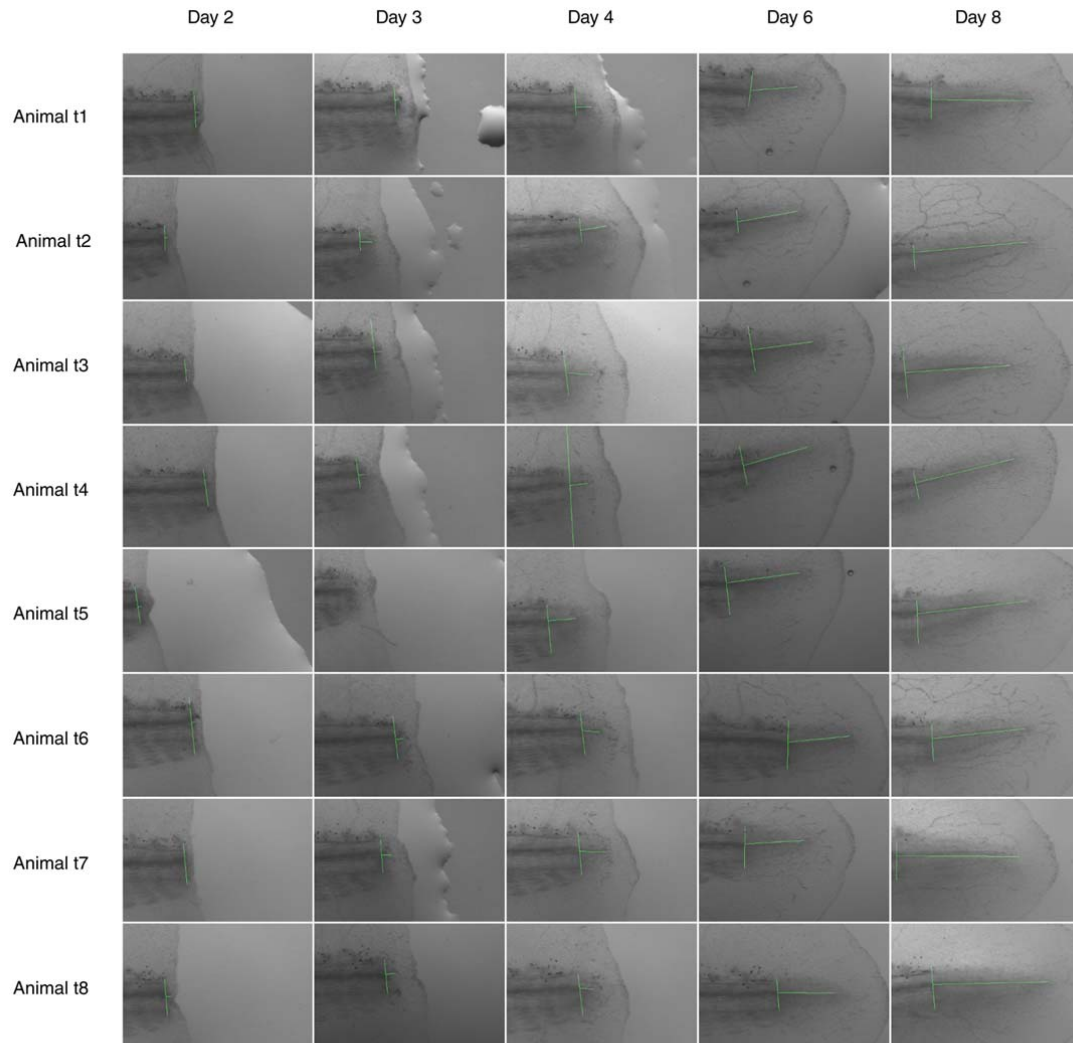
635

636 **Figure 1 – figure supplement 1.** Images used for spinal cord outgrowth measurements in Figure 1B. Each row

637 shows images from an axolotl; each column shows animals from one time point analyzed. Vertical and horizontal

638 lines mark the amputation plane and the spinal cord outgrowth, respectively. High-resolution images are in

639 Supplementary file 1. Animal t3 is shown in the representative images of Figure 1A.

640   **Figure 2 – figure supplement 1**
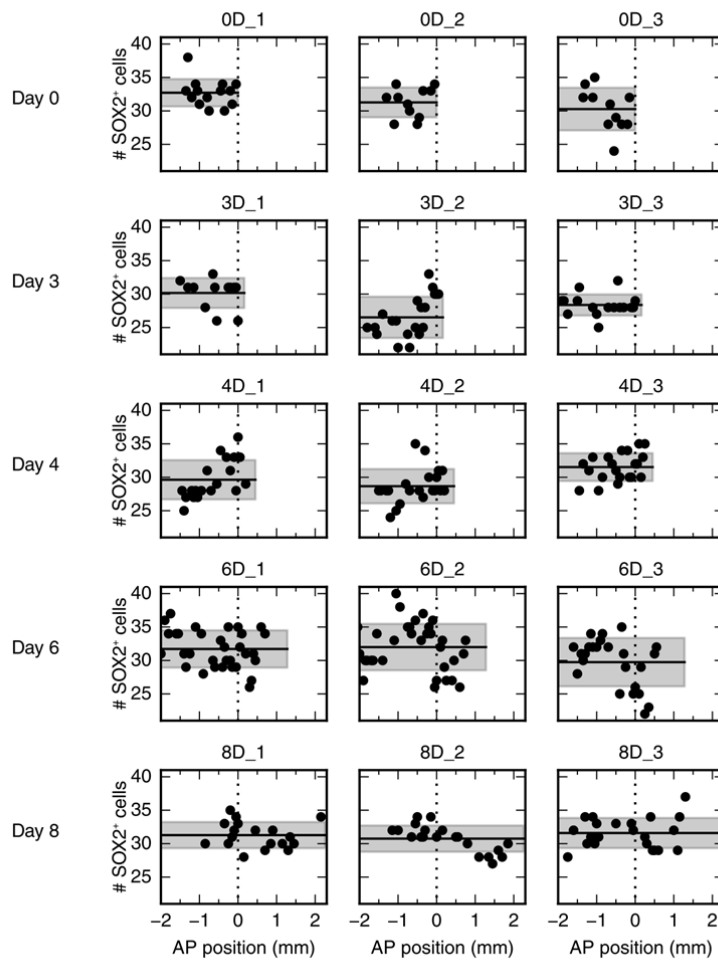
641

642   **Figure 2 – figure supplement 1.** Number of SOX2$^+$ cells per cross section along the AP axis for all 15 animals.

643   Each row shows data from three animals at a given time point. Data from animals 0D_1 and 4D_3 are shown as

644   representative data in Figure 2B and B', respectively.

Figure 2 – figure supplement 2
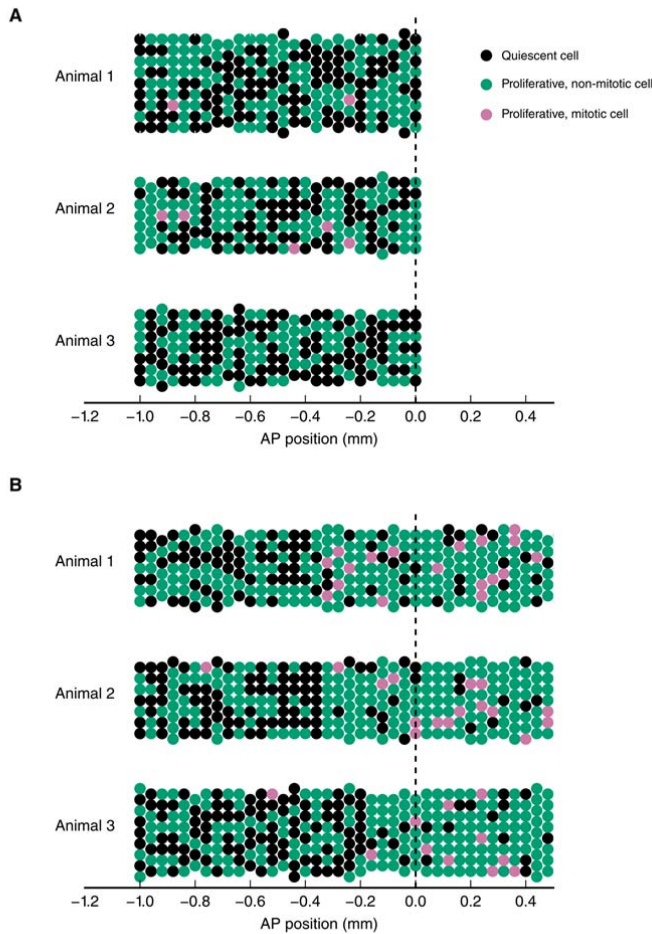
646

647 **Figure 2 – figure supplement 2.** Simulation of the spatial model of cell counts to analyze the spatiotemporal

648 pattern of cell proliferation. (A) Simulations of a spatially homogeneous zone of proliferation for 3 animals.

649 Population mean number of stem cells per cross section, NS_{pop} = 7, inter-animal standard deviation for number of

650 stem cells per cross section, σ_{pop} = 1, intra-animal standard deviation number of stem cells per cross section,

651 σ = 0.5, probability of a cell to be proliferative (expected growth fraction), p_p = 0.5, inter-animal standard deviation

652 of p_p, σ_p = 0.04, probability of a proliferative cell to be mitotic (expected mitotic index), p_m = 0.015, inter-animal

653 standard deviation of p_m, σ_m = 0.003. (B) Simulations of two adjacent spatially homogeneous zones of

654 proliferation for 3 animals. Parameters for the anterior zone are the same as in (A). Probability of a cell to be

655 proliferative and probability of a proliferative cell to be mitotic in the posterior zone are elevated to p_p = 0.8 and p_m

656 = 0.1, respectively. The mean switchpoint location is 300 µm anterior to the amputation plane and the

657 corresponding inter-animal standard deviation is 100 µm. As expected, there are more proliferative and mitotic

658 cells in the posterior zone. Simulation results can statistically be compared with the cell counts we obtained from

659 experimentally observed animals to infer growth fraction, mitotic index and switchpoint (Figure 2F-F'').

660   **Figure 2 – figure supplement 3**
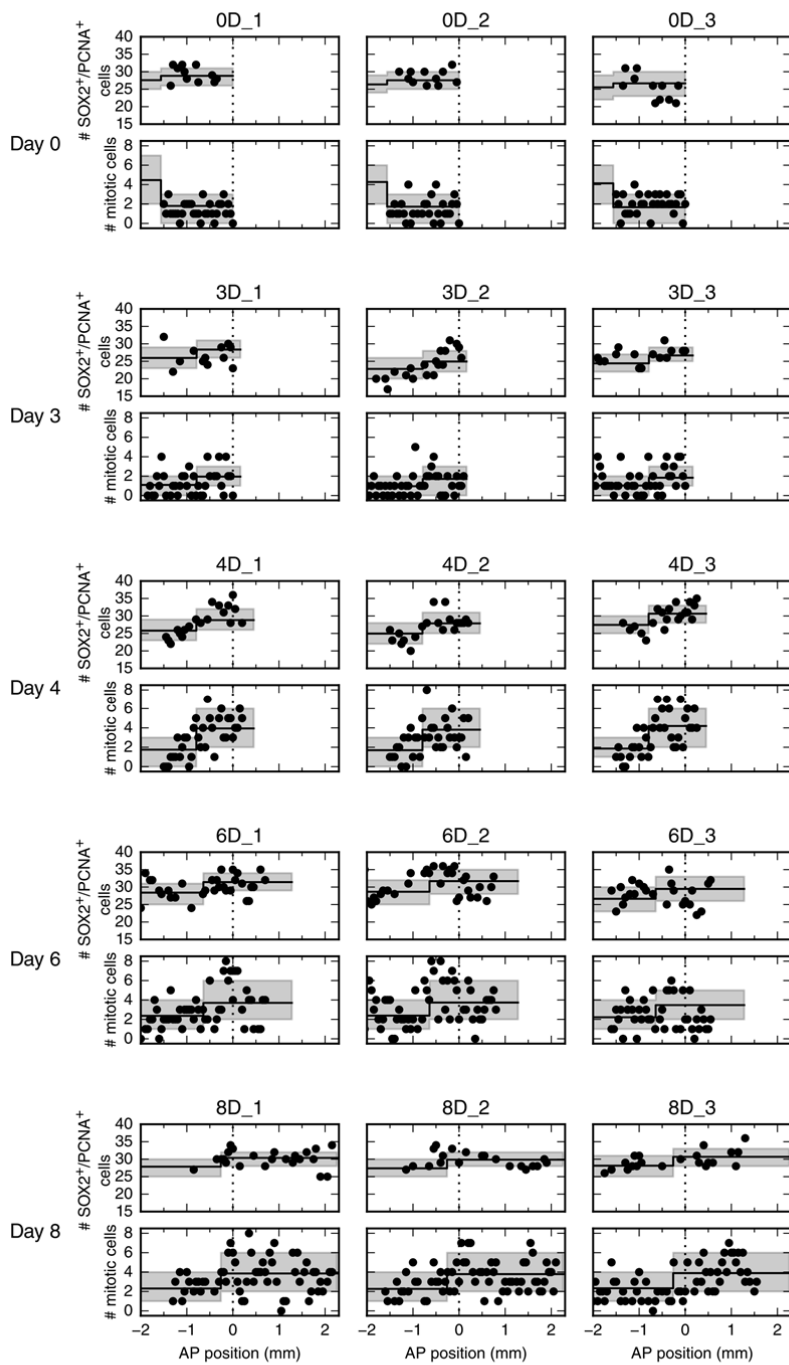
661

662   **Figure 2 – figure supplement 3.** Number of SOX2$^+$/PCNA$^+$ cells per cross section (upper panel) and mitotic cells

663   per section along the AP axis for all 15 animals. Data from animals 0D_1 and 4D_3 are shown in Figure 2D and

664   2D', respectively. Each row shows data from three animals at a given time point.

Figure 2 – figure supplement 4
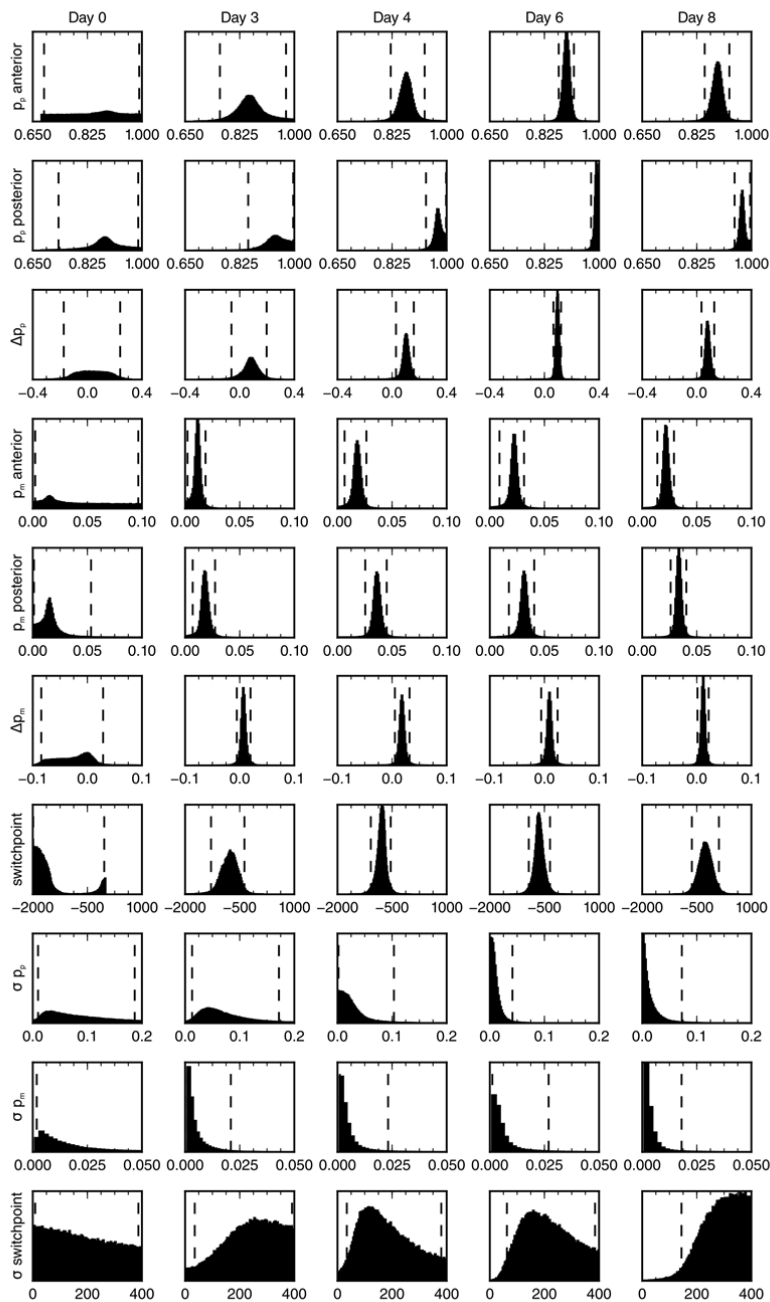
667   **Figure 2 – figure supplement 4.** Posterior marginal distributions for the parameters of the spatial model of cell

668   counts to analyze the spatiotemporal pattern of proliferation. Each row shows a different model parameter. Each

669   column shows a different time point. 3 animals per time point were used in the analysis. Vertical dashed lines

670   show the limits of the 95% credibility interval. The distribution means and the 68% credibility intervals for the

671   growth fraction, mitotic index and the switchpoint are shown in Figure 2F-F'', respectively.

28

672 **Figure 2 – figure supplement 5**
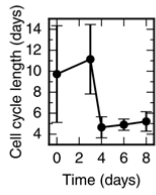
673

674 **Figure 2 – figure supplement 5.** Cell cycle length time-course calculated from the proliferation rate time-course

675 shown in Figure 2G.

676   **Figure 3 – figure supplement 1**
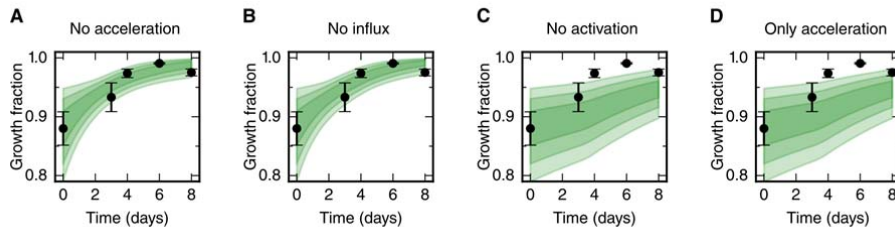
677

678   **Figure 3 – figure supplement 1.** Prediction of growth fraction in the high-proliferation zone for four model
679   scenarios with selected mechanisms switched off (green shaded areas). Black dots show the same experimental
680   data as in Figure 3B. Scenarios in panels A-D correspond to the scenarios in Figure 3D-G, respectively.
681   Switching off the acceleration of the cell cycle length and switching off the cell influx hardly have an effect on
682   the growth fraction time course (A,B). As expected, switching off the activation of quiescent stem cells has a
683   strong impact on growth fraction time-course (C,D). This is consistent with the fit of a non-zero rate activation rate
684   k to this data.

685   **Figure 3 – figure supplement 2**
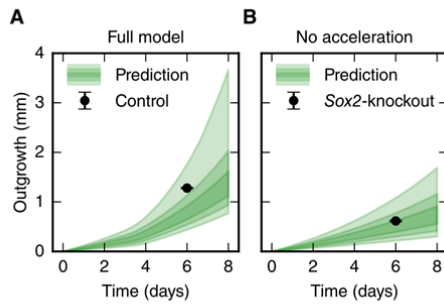
686

687   **Figure 3 – figure supplement 2.** Comparison of the spinal cord outgrowth prediction by our model with the

688   measured outgrowth reported by Fei et al., 2014. **(A)** Outgrowth prediction of the full model (green, same as in

689   Figure 3C) and rescaled outgrowth in control condition (black dot, n = 12 axolotls). **(B)** Outgrowth prediction of the

690   model for the case that cell cycle acceleration is switched off (green, same as in Figure 3D) and rescaled, density

691   corrected outgrowth in a *Sox2*-knockout condition (black dot, n = 24 axolotls).

Additional files

Supplementary file 1

Tiff stack of individual high-resolution images that are shown in Figure 1 – figure supplement 1

(http://dx.doi.org/10.5281/zenodo.59817). It can be opened with Fiji or ImageJ.

Supplementary file 2

Zip archives containing all raw images used for the clone tracking

(http://dx.doi.org/10.5281/zenodo.59824). Images for each individual animal are in separate zip

archives. Zip archive file names correspond to the arbitrarily chosen animal IDs used in the clone

trajectory dataset (see Supplementary notebook "clone_velocities"). The image filename indicates the

time point of the measurement together with the animal ID. A representative example is shown in

Figure 2I. The image files can be opened with AxioVision Microscopy software (Zeiss).

A

Day 2

Day 4

Day 6

Day 8

B

Outgrowth (mm) vs Time (days)

Legend: t1, t2, t3, t4, t5, t6, t7, t8

Day 0

Day 4

Day 8

High-proliferation zone

-800μm -500μm   0μm

AP position
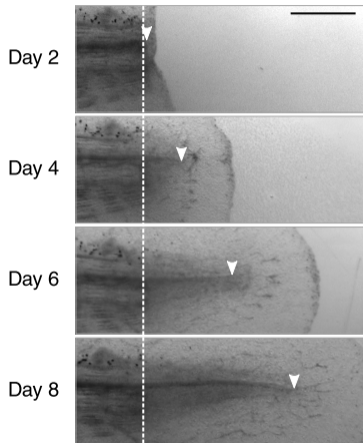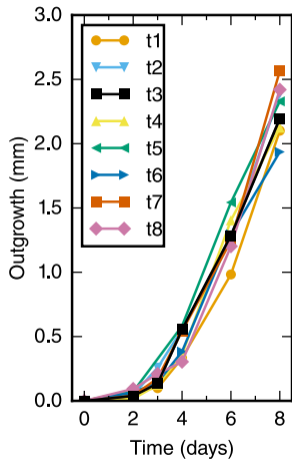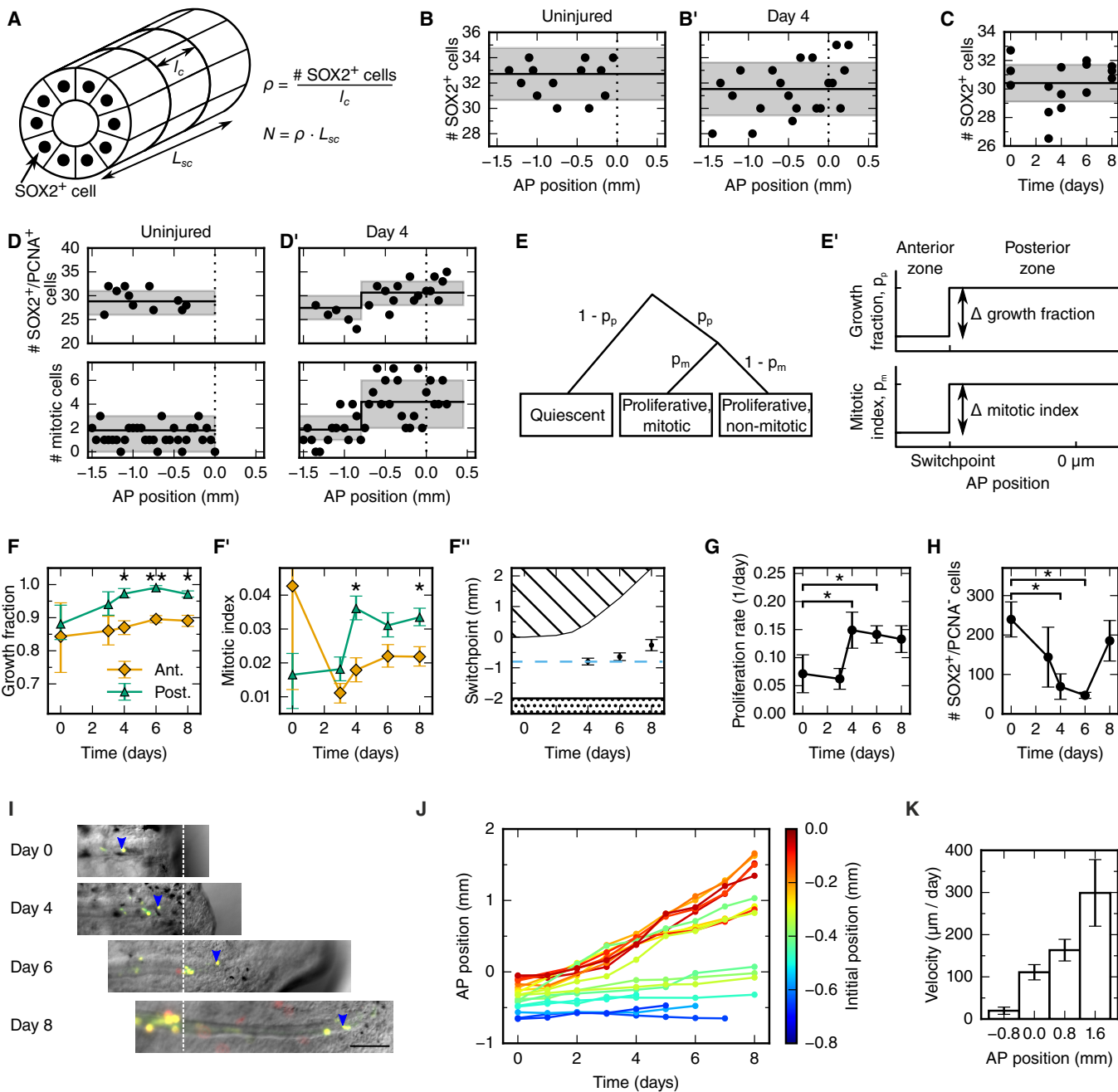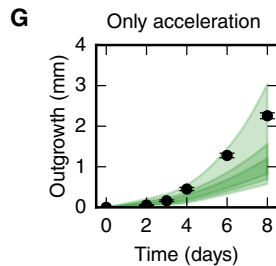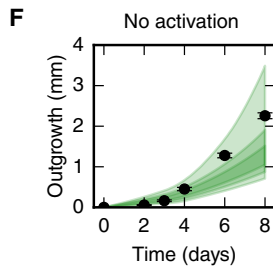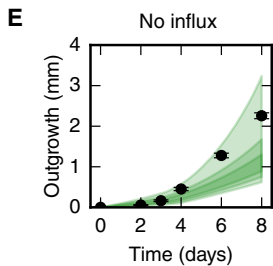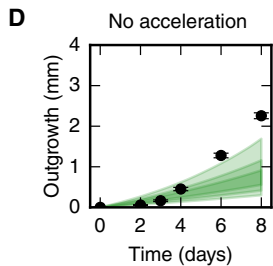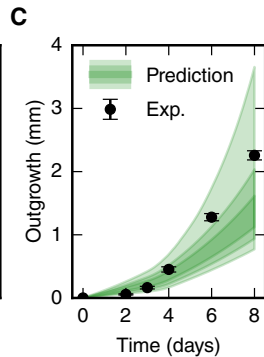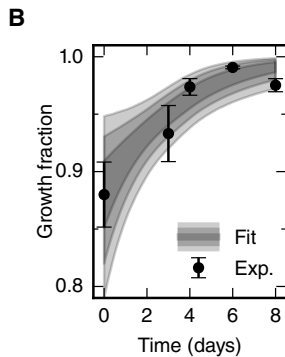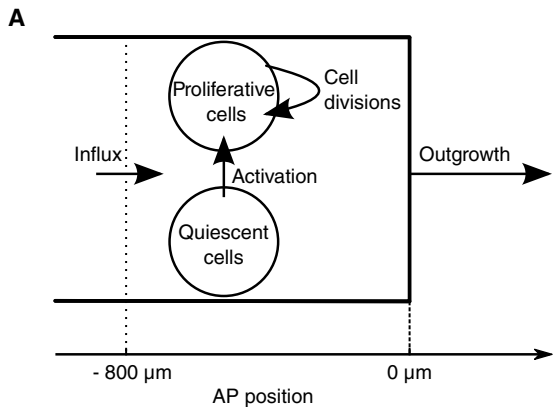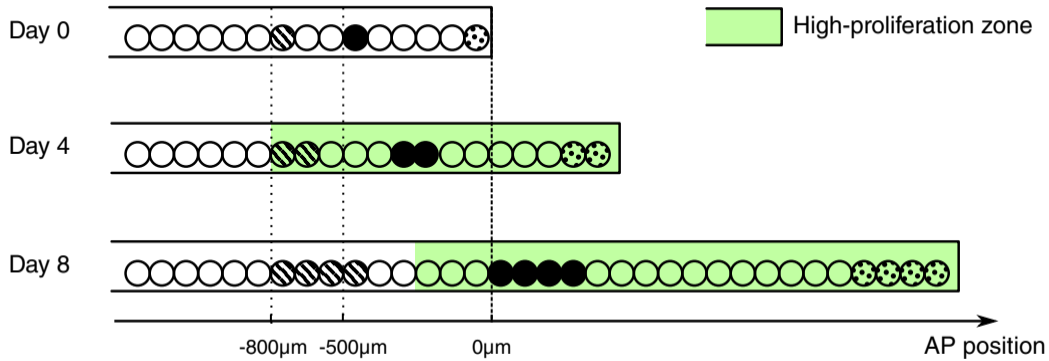
	Day 2	Day 3	Day 4	Day 6	Day 8
Animal t1					
Animal t2					
Animal t3					
Animal t4					
Animal t5					
Animal t6					
Animal t7					
Animal t8					

A

Animal 1

Animal 2

Animal 3

● Quiescent cell
● Proliferative, non-mitotic cell
● Proliferative, mitotic cell

−1.2   −1.0   −0.8   −0.6   −0.4   −0.2   0.0   0.2   0.4
AP position (mm)

B

Animal 1

Animal 2

Animal 3

−1.2   −1.0   −0.8   −0.6   −0.4   −0.2   0.0   0.2   0.4
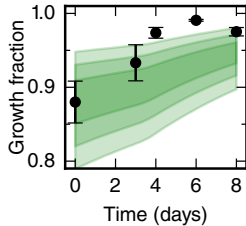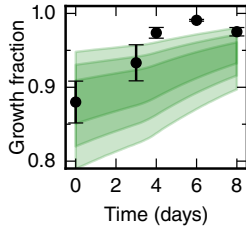AP position (mm)
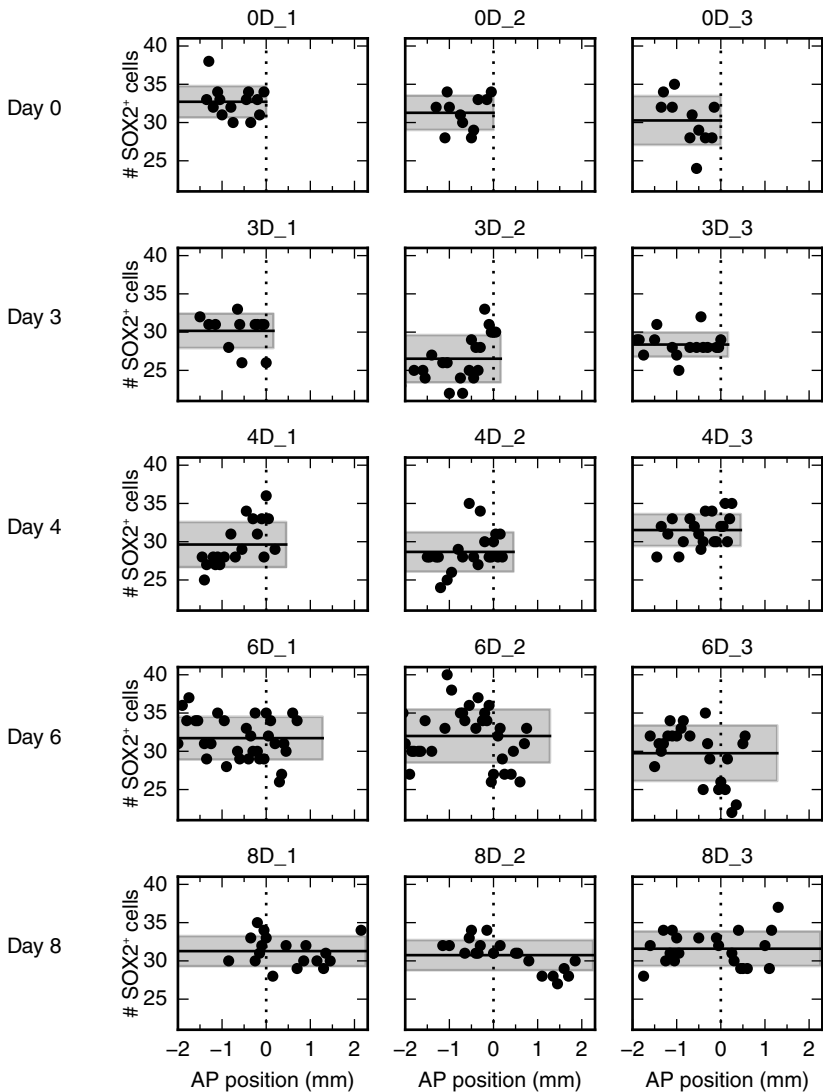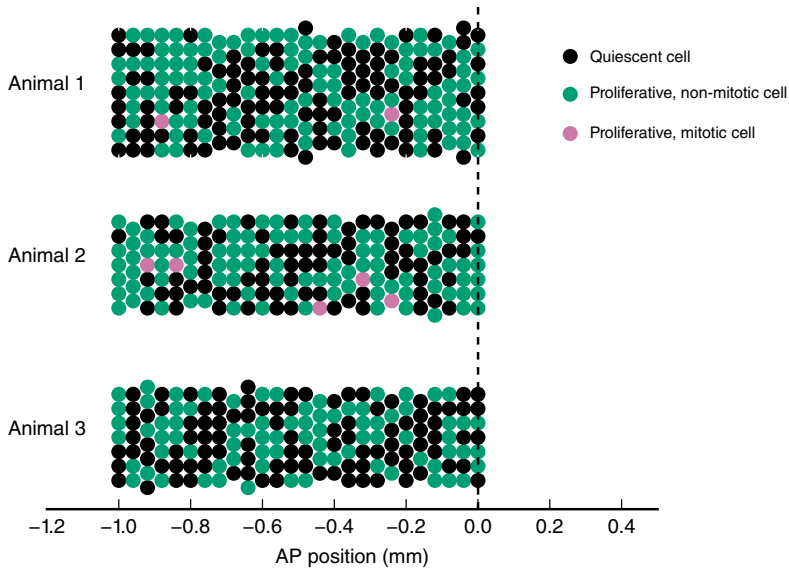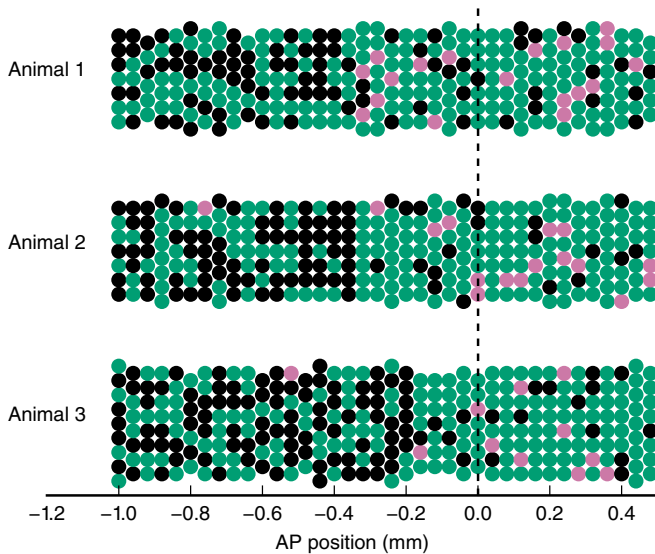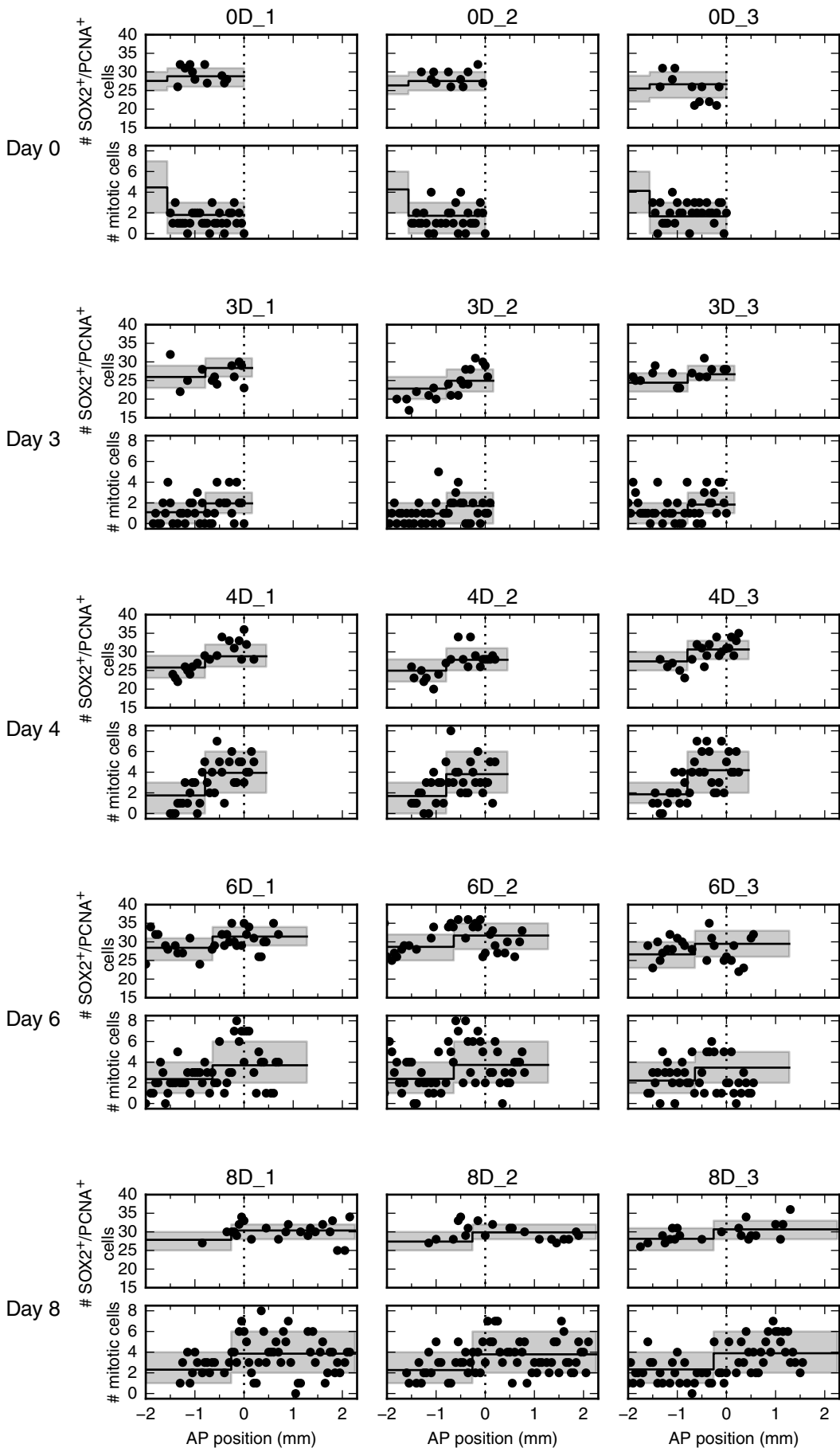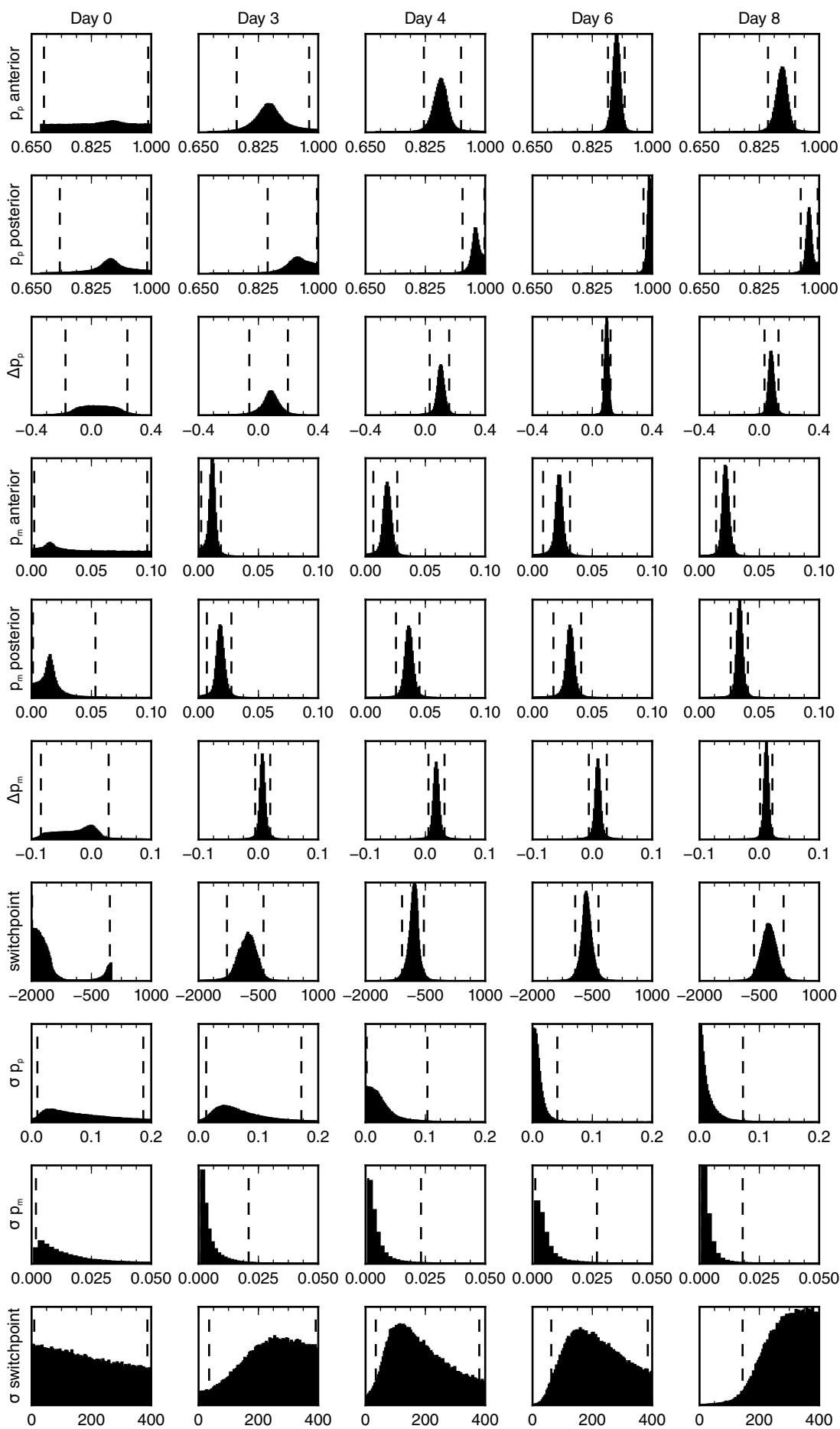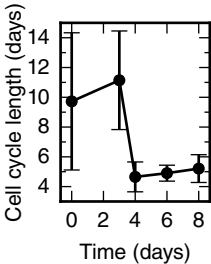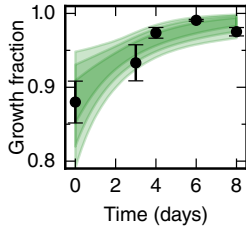
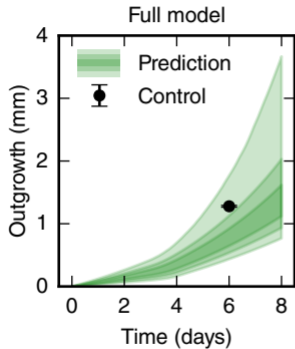**A** No acceleration   **B** No influx   **C** No activation   **D** Only acceleration
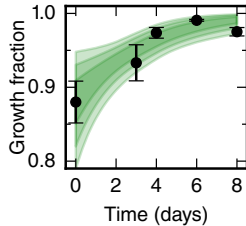
A Full model

B No acceleration