Wnt and TGFβ coordinate growth and patterning to regulate size-dependent behaviour

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Differential coordination of growth and patterning across metazoans gives rise to a diversity of sizes and shapes at tissue, organ and organisal levels. Although tissue size and tissue function can be interdependent1–5, mechanisms that coordinate size and function remain poorly understood. Planarians are regenerative flatworms that bidirectionally scale their adult body size6,7 and reproduce asexually, via transverse fission, in a size-dependent manner8–10. This model offers a robust context to address the gap in knowledge that underlies the link between size and function. Here, by generating an optimized planarian fission protocol in Schmidtea mediterranea, we show that progeny number and the frequency of fission initiation are correlated with parent size. Fission progeny size is fixed by previously unidentified mechanically vulnerable planes spaced at an absolute distance along the anterior–posterior axis. An RNA interference screen of genes for anterior–posterior patterning uncovered components of the TGFβ3 and Wnt signalling pathways as regulators of the frequency of fission initiation rather than the position of fission planes. Finally, inhibition of Wnt and TGFβ3 signalling during growth altered the patterning of mechanosensory neurons—a neural subpopulation that is distributed in accordance with worm size and modulates fission behaviour. Our study identifies a role for TGFβ3 and Wnt in regulating size-dependent behaviour, and uncovers an interdependence between patterning, growth and neurological function.

The infrequency of planarian fission behaviour has largely precluded its mechanistic dissection. However, recently optimized worm husbandry techniques augmented fission activity11,12, and enabled us to study the integration of worm size with fission behaviour. Large planaria (Schmidtea mediterranea) from recirculation culture systems exhibited robust and reproducible increases in fission activity when transitioned to static culture systems and starved (Fig. 1a, Supplementary Video 1). Live imaging provided detailed characterisation of the fission process. Planarians first elongate and adhere their posterior tissue to a substrate. Next, periodic body contractions concentrate body mass towards the head region while thinning out tissues immediately anterior to the adherent tail. After 20–40 minutes, progressive stretching ruptures connecting tissue with rapid recoil, which separates the anterior parent from the posterior fission progeny (Extended Data Fig. 1a, Supplementary Video 1).

Observation of fission behaviour in worms of increasing size showed that the length of first posterior fission fragments did not correlate with parent length (Fig. 1b, d). Instead, larger worms produced additional progeny, each approximately 1 mm in length, such that the number of progeny after 2 weeks linearly correlated with parent size (Fig. 1c, e, Extended Data Fig. 1b–d). Thus, the size of fission fragments is fixed independently of anterior–posterior position or parent length. The frequency of the production of fission fragments—that is, the fission rate—did correlate with worm length (Extended Data Fig. 1e, f), and both the time to the first fission event and the time between sequential fission events was inversely related to parent size (Extended Data Fig. 1g–1). Automated webcam imaging of individual worms allowed us to generate timelines chronicling successful (upward displacement) and unsuccessful (downward displacement) fission attempts (Fig. 1f, Supplementary Video 2). Fission attempts occurred only in worms above 4–5 mm in length, which indicates a minimal size required for fission (Fig. 1g, h, Extended Data Fig. 2a, b). Furthermore, larger worms produced fission progeny more frequently owing to more fission attempts (Fig. 1h, Extended Data Fig. 2c, d), rather than higher rates of success (Fig. 1i). Together, these results confirm that planarian fission is a size-dependent behaviour, with both progeny number and fission rate coupled to parent size.

We tested the hypothesis that patterning cues are required to coordinate worm size and planarian fission. Genes from the Wnt13–16, TGFβ17–19 and Hh20 signalling pathways that regulate anterior–posterior polarity regeneration phenotypes, but no gross morphological defects in parent RNAi worms (Extended Data Fig. 4b–d). Therefore, we conclude that Wnt and TGFβ3 signalling components modulate fission behaviour independently of overt body plan repolarization.

Serendipitously, we discovered that compression of planaria reveals cryptic mechanically vulnerable planes that divide the worm at regularly spaced intervals along the anterior–posterior axis (Fig. 3a, b, Supplementary Video 3). The number of these ‘compression planes’ scaled with worm size (Fig. 3b, c) and their position along the anterior–posterior axis overlapped with the position of fission planes (Fig. 3d). Furthermore, incomplete fission formed tears similar to those observed with compression (Extended Data Fig. 5a). Therefore, we conclude that compression planes are fission planes revealed by mechanical compression. Fission plane number and distribution correlated with worm length during tissue rescaling and regeneration. After starvation, worms reduced body length and lost fission planes to restore number and distribution (Extended Data Fig. 5b–d). To assay regeneration of the fission plane, we amputated worms around the pharynx such that 90% of fragments contained a single plane (Extended Data Fig. 5e–g). One week after amputation, worms remodelled, doubled in length and increased fission plane number (Extended Data Fig. 5f–j). Subsequent feeding increased worm length and fission plane number (Extended Data Fig. 5f–j). After starvation, worms exhibited little to no elongation.

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or plane addition despite rescaling and regenerating their other tissues (Extended Data Fig. 5f–j). In summary, fission planes are pre-established in planarians and correlate dynamically with worm size and form.

Given the role of Wnt and TGFβ signalling in body patterning, we tested whether genes of these signalling pathways regulate fission planes. Worms treated with RNAi were mechanically compressed and the quantity and relative distribution of fission planes was measured (Fig. 3e–g). Notably, whereas RNAi of actR-1 and smad2/3 moderately reduced the number of fission planes, RNAi of Wnt signalling components had no effect on fission plane number or position (Fig. 3e, g, Extended Data Fig. 6a). Even knockdown of wnt11-6 by three rounds of amputation and regeneration did not alter fission-plane patterning (Fig. 3f, g, Extended Data Fig. 6b). Hypomorph RNAi knockdown of β-catenin, actR-1 or smad2/3 revealed little or no effect on the size of fission fragments (Extended Data Fig. 6c–e), which further supports the conclusion that neither Wnt nor TGFβ signalling regulate fission behaviour through the anterior–posterior patterning of fission planes.

We tested whether Wnt and TGFβ signalling instead regulated the frequency of fission attempts. Using the automated webcam image-capture system (Fig. 1f), we recorded fission behaviour in RNAi-treated worms (Fig. 4a). RNAi of β-catenin, actR-1, smad2/3 and wnt11-6 reduced fission attempts, whereas RNAi of apc increased fission attempts (Fig. 4b–d, Extended Data Fig. 7a–l, Supplementary Videos 4–6). RNAi of β-catenin and smad2/3, which resulted in observable morphological abnormalities, also significantly reduced the fission-success ratio (Figs. 2d, 4e, Extended Data Fig. 7k–n). dsh-B RNAi reduced the fission success ratio without altering the number or frequency of fission attempts (Fig. 4d, e, Extended Data Fig. 7k–n). Finally, apc RNAi reduced the time between fission attempts by approximately 50%, and worms initiated fission attempts independently of remaining tissue, markedly reducing their success ratio (Fig. 4e,
Fig. 2 | Wnt signalling and TGFβ3 signalling components modulate fission activity. a, RNAi screen workflow (see also Extended Data Fig. 3). b, c, Heat maps depicting fission activity after RNAi treatment for both the two-phase primary (b) and secondary (c) RNAi screens. Normalized cumulative fissions over time are displayed for individual worms from each RNAi condition (n = 10 worms for phase I, n = 12 worms for phase II and secondary screen). Targets in secondary screening (independently repeated three times) depicted in green (activators) and red (inhibitors). P values determined by two-way analysis of variance (ANOVA) interaction factor. Ctrl, control. d, Representative parent images on days 0 and 14 of the fission assay (n = 10–12, independently repeated 3 times). Scale bars, 1 mm.

Fig. 3 | Pre-established fission planes determine progeny size independently of Wnt and TGFβ signalling. a, Schematic of compression assay (Supplementary Video 3). b, Pre- and post-compression worm (inset) and compression planes revealed in 3–6-mm worms (independently repeated 5 times). c, Compression plane number relative to worm length (n = 117 worms). PCC, linear regression (red lines), R² values and 95% confidence interval (black lines) are shown. d, Fission (n = 196 fission progeny from 50 worms) and compression plane (n = 173 planes from 30 worms) overlap along the anterior–posterior axis of the worm. e, f, Representative images of post-compression worms after knockdown of Wnt and TGFβ3 signalling components using the specified number of RNAi feedings and rounds of regeneration (n depicted by dot plot quantification; experiment performed three times (e) or once (f)). Scale bars, 1 mm. g, Plot of the number of fission planes per worms length after RNAi treatment of 2 experiments (n = 20 and 10 worms to the left and right of the dotted line, respectively). P values determined by two-sided t-test. NS, not significant. Data are mean ± s.d. (d) or mean ± s.e.m. (g).
CNS expressing Wnt and TGFβ (Fig. 8h, i). Together, these data support a model in which an anterior marker indicates that half of the CNS is sufficient to initiate fission. Finally, removal of anterior tissue that contains the cephalic ganglia delayed the onset of fission behaviour (Extended Data Fig. 8a, b). Restoration of fission activity coincided with regeneration and re-establishment of anterior, pc2 co-localized, tsh expression (Extended Data Fig. 8g). Notably, removal of anterior tissue that contained just one cephalic ganglion did not alter the total number of fission progeny produced (Extended Data Fig. 8c–f), which indicates that half of the CNS is sufficient to initiate fission. Finally, RNAi against coe, a transcription factor essential for the patterning of the CNS22,23, markedly reduced planarian fission (Extended Data Fig. 8h, i). Together, these data support a model in which an anterior CNS expressing Wnt and TGFβ signalling components regulates fission initiation.

Extended Data Fig. 7i–n, Supplementary Video 6). These findings demonstrate that Wnt and TGFβ signalling regulate the frequency of fission behaviour. We proposed that components of the Wnt and TGFβ signalling pathways might regulate fission behaviour through the planarian CNS. Double fluorescent in situ hybridization (FISH) with the CNS marker pc2 confirmed that Wnt and TGFβ fission regulators were detected in pc2-positive cells in the anterior CNS (Extended Data Fig. 8a, b). Removal of anterior tissue that contains the cephalic ganglia delayed the onset of fission behaviour (Extended Data Fig. 8c–f). Restoration of fission activity coincided with regeneration and re-establishment of anterior, pc2 co-localized, tsh expression (Extended Data Fig. 8g). Notably, removal of anterior tissue that contained just one cephalic ganglion did not alter the total number of fission progeny produced (Extended Data Fig. 8c–f), which indicates that half of the CNS is sufficient to initiate fission. Finally, RNAi against coe, a transcription factor essential for the patterning of the CNS22,23, markedly reduced planarian fission (Extended Data Fig. 8h, i). Together, these data support a model in which an anterior CNS expressing Wnt and TGFβ signalling components regulates fission initiation.

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We tested whether polarity genes could modulate size-dependent behaviour via size-dependent patterning of the CNS. To identify neuronal subpopulations that regulate fission downstream of Wnt and TGFβ, we analysed 17 neuronal markers24–29 in small, medium and large planaria and 10 markers in worms treated with smad2/3 RNAi (Fig. 4f, Extended Data Fig. 9a, b). Patterning of pkd1L-2+, gabrg3L-2 and sargasso-1+ mechano-sensory neurons exhibited the clearest changes in worms of increasing size and after smad2/3 RNAi treatment (Extended Data Fig. 9a, b). In large worms, mechano-sensory neurons are tightly restricted to the anterior and knockdown of either smad2/3 or wnt11-6 broadened their distribution akin to that of smaller worms (Fig. 4g–l). RNAi against pkd1L-2 and gabrg3L-2 (homologous to cation and chloride channel genes, respectively) increased planarian fission activity (Fig. 4m, n), and live imaging of gabrg3L-2 RNAi worms confirmed an increase in fission attempts without a reduction in fission success (Extended Data Fig. 10, Supplementary Video 7). These results indicate that mechano-sensory neurons are differentially patterned during growth, inhibit fission behaviour and require Wnt and TGFβ for their appropriate patterning in accordance with worm size. Therefore, we conclude that Wnt and TGFβ signalling coordinates worm size and behaviour via size-dependent patterning in the adult CNS.
In conclusion, we used planaria as a model for the integration of size, patterning and function and established fission as a robust, reproducible and quantifiable size-dependent behaviour (Fig. 1, Supplementary Video 1). Although previous studies have generated physical models for the process of transverse fission\(^9\), mechanisms that couple worm size and fission frequency have remained unknown. We discovered two independent mechanisms by which fission is coordinated with worm size in \textit{S. mediterranea}. First, previously undescribed iterative structures patterned in accordance with anterior–posterior axis length couple worm size with the number of fission progeny produced (Fig. 3, Supplementary Video 3). Second, the Wnt and TGF\(\beta\) signalling pathways mediate size-dependent patterning of mechanosensory neurons, which regulate fission frequency (Fig. 4, Extended Data Figs. 9, 10). Thus, we demonstrate that differential patterning of key cell populations in accordance with tissue size provides a mechanistic link between worm growth and the acquisition or modulation of tissue function. Together, our results identify a role for Wnt and TGF\(\beta\) patterning genes in the regulation of size-dependent behaviour and show that developmental patterning cues coordinate tissue growth with size-dependent functions.

**Online content**

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METHODS

Worm husbandry. Clonal CIW4 S. mediterranea were maintained in 1 × Montjuïc salts as previously described. CIW4 worms were sourced from a large recirculation culture as previously reported. In brief, worms are housed in three culture trays (244 cm length × 61 cm width × 30.5 cm height) stacked vertically. Water is recirculated through the system by a sump pump, which moves water through a chiller, a canister filter, a UV sterilizer and the three housing trays. Water is then passed through two vertically stacked sieves and a set of filter/loss pads before being returned to the sump pump. Worms were pulled from this system and placed directly into fixation assays, starved for at least seven days before tissue fixation for imaging, or transferred to a unidirectional flow system culture for controlled feeding or RNAi feeding experiments.

Gene cloning and RNAi feeding protocol. Candidate genes analysed in this study were cloned from a CIW4 cdNA library into a pPR-T4P vector as previously described (Supplementary Table 1). These served as template for in vitro synthesis of dsRNA for RNAi feedings. Unc22 dsRNA was used for control RNAi treatment. RNAi food was prepared by mixing 1 volume of dsRNA at 1,600 ng ml⁻¹ with 1.5 volumes of beef liver paste. For RNAi experiments that target neuronal genes, 1 volume of dsRNA at 1,400 ng ml⁻¹ was mixed with 1 volume beef liver paste. The amount of food administered was 10 μl of food per 1 mm of worm length present in the worm flow container. Worms were allowed to feed for 6–10 h with 2 rounds of light stimulation to facilitate additional consumption. Worms were fed every three days for a total of three RNAi feedings, unless otherwise specified. After RNAi feedings, worms were transferred to the relevant biological assay.

Fission assay. A detailed protocol for fission induction has been made available through Protocol Exchange. To induce fission, worms were removed from recirculation culture or unidirectional flow system culture and washed 5–10 times with fresh 1 × Montjuïc salts. Individual worms were placed in 15-cm tissue culture dishes with 50 μl 1 × Montjuïc salts and their body length was measured. Representative images of day-0 parents were captured using a Leica M205 microscope. Plates were stacked 6–12 dishes high and placed in a dark incubator at 20 °C. Daily, plates were removed from the incubator and fission fragments for each worm were counted and removed from the 15-cm dish. For some experiments, images of fission fragments were taken on the day they were collected to allow for quantification of fission fragment length. The 1 × Montjuïc salts in each individual dish was replaced weekly.

For data analysis, the number of daily cumulative fissions was divided by initial body length and then normalized to the average of the control RNAi fissions. This normalized fission score for each day was converted to a heat colour code. Daily scores for each individual worm were aligned in descending order along the y axis and the average score of each column was calculated and used to sort individual worms in ascending order along the x axis. The average fission score of each RNAi condition was then sorted in ascending order from left to right. This resulted in a heat map visualization ranking the effects of RNAi treatments on fission activity.

Fission plane compression assay. Fission planes were revealed by compression between a plastic tissue culture dish and a glass coverslip (Supplementary Video 3). Worms were inverted with their ventral side up, compressed using four fingertips, between a plastic tissue culture dish and a glass coverslip (Supplementary Video 3). Fission plane compression assay. Position of fission planes was revealed by mechanical compression. Position of fission planes were not blinded to allocation during experiments and outcome assessment.

Quantification of live imaging. Videos of individual worms were manually annotated. For each fission attempt, the start time and completion time were recorded and the success or failure of the attempt was recorded. To depict fission behaviour, a timeline was constructed and a numerical value was given to each frame of a video. A value of 0 was assigned to any frame in which no fission behaviour was observed; a positive value was given to any frame during a successful fission attempt; and a negative value was assigned to any frame during a failed fission attempt (see Fig. 1F). A prolonged diagonal line in a timeline indicates a period in which frames were not acquired owing to failed communication between the image acquisition software and the webcam.

Statistical tests. For all pairwise comparisons, significance was tested using an unpaired Student's t-test. GraphPad Prism was used to calculate PCC values with a two-tailed 95% confidence interval and to perform linear regression analyses. Two-way ANOVA analysis was performed in GraphPad Prism to determine the significance of RNAi treatment over time. No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Source data and construct sequences can be accessed from the Stowers Original Data Repository at http://www.stowers.org/research/publications/libpb-1356. All other data are available from the corresponding author upon reasonable request.

Code availability

Code for the Python 3.6 (https://www.python.org/) script used for a wrapper for FFmpeg (https://www.ffmpeg.org/) for the high-throughput recording of fission behaviour is available at the Stowers Original Data Repository at http://www.stowers.org/research/publications/libpb-1356.

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Authors contributions

Conceptualization, data analysis and interpretation: C.P.A., B.W.B.-P. and A.S.A.; acquisition of data: C.P.A., B.W.B.-P. and J.J.L.; design and fabrication of planarian live-imaging systems: J.J.L.; software: C.J.W.; data curation: J.J.L. and C.J.W.; writing of the original manuscript: C.P.A., B.W.B.-P. and A.S.A.; supervision and funding acquisition: A.S.A.; and revision and editing of the manuscript: all authors.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Characterization of planarian fission biology.

a, Live imaging of large planarian worm during fission (representative of 12 experiments; see also Supplementary Video 1). b, Imaging of single individual large planarian and regenerating progeny 0, 4, 8 and 12 days after fission induction (experiment repeated 50 times). c, d, Anterior–posterior length of progeny (c) and time to fission event (d) since induction or the previous fission (n = 50 worms). Fission fragments binned by position along the anterior–posterior axis (the first fission is the most posterior). e, Schematic of fission induction and quantitative scoring system used to compare fission activity between different conditions. f, Cumulative fission fragments produced over 14 days by individual worms binned by parent size (n = 10 per bin). g, h, Time to first fission event (g) or time between sequential fission events (h) for worms 6–8 mm, 9–12 mm or 13–17 mm in length. i, Raw parent length measurement of planarian individuals 6–8 mm, 9–12 mm or 13–17 mm in length. j, Time between first and second fission events for worms 6–8 mm, 9–12 mm or 13–17 mm in length (n = 139 independent measurements from 30 worms). k, l, Time between induction and first fission (k) or between first and second fission (l) plotted relative to parent length (n = 26 and 21 independent measurements from 30 worms). PCC, linear regression and $R^2$ values are provided. $P$ values determined by determined by two-sided t-test. Data are mean ± s.e.m. (c, d, j).
Extended Data Fig. 2 | Quantification of fission behaviour across a range of worm sizes. a, All individual timelines depicting fission activity over 9 days for worms ranging from 2 mm (bottom) to 12 mm (top) in length (n = 39 worms). b, Number of successful fission attempts per worm relative to parent length. c, d, Number of fission attempts (c) and time to first fission attempt (d) for worms binned into small (2–5 mm), medium (6–7 mm) and large (8–12 mm) groups (n = 16 small, 11 medium, 12 large worms). Data are mean ± s.e.m.
Extended Data Fig. 3 | Strategy for a targeted RNAi screen to identify regulators of fission. a, Detailed schematic of RNAi workflow. Worms are grown to an optimal size in the recirculation culture system and transferred to a flow system for RNAi feedings. After 3 RNAi feedings, worms were transferred to a 15-cm dish and worm length was recorded. The number of fissions were recorded daily for 14 days for each worm from each RNAi condition. For data analysis, the number of daily cumulative fissions were divided by initial body length and then normalized to the average of the control RNAi fissions. For data visualization, this normalized fission score for each day was converted to a heat colour code. Daily scores for each individual worm were aligned in ascending order along the y axis. The average score of each column is calculated and used to sort individual worms in ascending order along the x axis. The average fission score of each RNAi condition was then sorted in ascending order from left to right. The result is a heat-map visualization that ranks the effects of RNAi treatments on fission activity. Green arrows indicate positive interactions; red arrows indicate inhibitory interactions.

b, Wnt, TGFβ and Hh signalling pathway diagrams focusing on components targeted for the RNAi screen.
**Extended Data Fig. 4** | Analysis of morphology and/or internal tissues in regenerating fragments and fissioning parents. **a**, Representative images of regenerating tissue fragments from different positions along the anterior–posterior axis at 15 days post-amputation (dpa). Fraction of worms with pictured phenotype along with 1-mm scale bar depicted below each image. **b, c**, In situ staining of CNS (pc2), intestine (porc) and muscle (t-mus) tissues at day 15 of regeneration (b) or the fission assay (c). **d**, High-resolution image of body wall musculature (t-mus) in control RNAi and smad2/3 or β-catenin RNAi treated worms. Representative images (n = 7–13 worms) from a single experiment. All images are oriented ventral side up with anterior on the left side. Scale bars, 0.5 mm.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Effects of growth, starvation and regeneration on fission planes. **a**, Image of planaria after incomplete fission, revealing ventral tear identical to compression planes (observed more than five independent times). **b**, Post-compression worms at 5, 18 and 30 days post-fertilization (dpf) (5 dpf image from same experiment as Fig. 3b). Data are from a single experiment. **c, d**, Bidirectional plot of compression planes versus worm length (n = 25 worms) (c), and relative distribution of planes (d) at 5, 18 or 30 dpf (n = 28, 18, 31, 15 and 19 worms (left to right in d)). **e**, Schematic of experiment tracking establishment of fission planes during tissue regeneration. **f, g**, Representative images (f) and bidirectional plot of compression planes versus worm length (g) after amputation (1 dpa, n = 15 worms), regeneration (8 dpa, n = 19 worms) and growth (fed 14 dpa and 25 dpa, n = 12 and 32 worms) or de-growth (starved 25 dpa, n = 15 worms). Data from a single experiment. **h–j**, Worm length (h), number of compression planes (i) and relative distribution of planes (j) after amputation (1 dpa, n = 15 worms), regeneration (8 dpa, n = 19 worms) and growth (fed 14 dpa and 25 dpa, n = 12 and 32 worms) or de-growth (starved 25 dpa, n = 15 worms). Data are mean ± s.d. (c, g) or mean ± s.e.m (h–j).
Extended Data Fig. 6 | Effects of RNAi of Wnt and TGFβ signalling components on fission planes. a, b, Relative plane distribution after RNAi treatment (n = 20 (a) and 10 (b) worms). c, Representative images of progeny within 24 h of fission and of remaining parent tissue at day 28 after fission induction for worms treated with control, β-catenin, actR-1 or smad2/3 RNAi (experiment independently performed twice). Scale bar, 1 mm. d, e, Length of the first fission progeny (d) or all subsequent progeny (e) in worms treated with control, β-catenin, actR-1 or smad2/3 RNAi (n = 85 fission fragments from 36 worms). P values determined by two-way ANOVA interaction factor (a, b) or two-sided t-test (d, e). Data are mean ± s.e.m.
Extended Data Fig. 7 | Wnt and TGFβ signalling components regulate the frequency of fission initiation. a–h, All individual timelines depicting fission activity over 9–10 days for worms treated with control (a, g), actR-1 (b), smad2/3 (c), β-catenin (d), dsh-B (e), APC (f) or wnt11-6 (h) RNAi. i–n, Graphs depicting the time between sequential fission attempts (i, j), the number of successful fission attempts (k, l) and the number of unsuccessful fission attempts (m, n) in worms fed double-stranded RNA (dsRNA) that targets regulators of fission (n = 421 fission events from 116 worms). Worms were given either 3 (a–f, i, k, m) or 18 (g, h, j, l, n) dsRNA feedings. Batched experiments are plotted separately. P values determined by two-sided t-test. Data are mean ± s.e.m.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | The planarian anterior CNS regulates fission.
a, Whole-brain imaging of pc2 and fission regulator gene expression detected by double FISH (n = 2–4 worms; experiment independently repeated). Scale bars, 100 μm.  
b, Single-cell co-expression of pc2 and fission regulators in the posterior branches of the anterior CNS (n = 3–5 worms). Scale bar, 50 μm.  
c, Fission induction in intact, 100% head-amputated or 50% head-amputated worms over a 9-day observation period (n = 12 worms).  
d–f, Total number of fission progeny over 9 days (d), the time between fission induction and first fission (e), and the time between first and second fission (f) for intact, 100% head-amputated or 50% head-amputated worms (n = 94 fission events from 36 worms).  
g, Regeneration time course in 100% head-amputated worms showing recovery of anterior gene expression of pc2 co-localized with teashirt (n = 4–5 worms; experiment performed once). Scale bar, 500 μm.  
h, Heat maps depicting fission activity after treatment with coe RNAi. Normalized cumulative fissions over time are displayed for individual worms from each RNAi condition (n = 12 worms).  
i, Representative parent images on days 0 and 14 of the fission assay (n = 12, experiment independently performed twice). Scale bars, 1 mm. P value determined by two-sided t-test (d–f) or two-way ANOVA (h). Data are mean ± s.e.m. (d–f).
Extended Data Fig. 9 | Comparison of neuronal subpopulations in worms of increasing size and after smad2/3 RNAi treatment.

**a.** Representative images of neuronal marker staining in small, medium and large worms (n = 3–5 worms; 1 experiment).

**b.** Representative images of a subset of neuronal markers analysed in worms treated with smad2/3 RNAi (n = 3–5 worms; 1 experiment). Scale bars, 0.5 mm.
Extended Data Fig. 10 | *gabrg3L-2* negatively regulates the frequency of fission initiation. a, b, All individual timelines depicting fission activity over 9 days for worms treated with control (a) or *gabrg3L-2* (b) RNAi (*n* = recordings of 10 worms combined from 2 independent experiments).