LETTER

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The molecular logic for planarian regeneration along the anterior–posterior axis

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The planarian *Dugesia japonica* can regenerate a complete individual from a head, trunk or tail fragment via activation of somatic pluripotent stem cells[1,2]. About a century ago, Thomas Hunt Morgan attempted to explain the extraordinary regenerative ability of planarians by positing two opposing morphogenetic gradients of formative "head stuff" and "tail stuff" along the anterior–posterior axis[3,4]. However, Morgan's hypothesis remains open to debate. Here we show that extracellular signal-related kinase (ERK) and Wnt/β-catenin signalling pathways establish a solid framework for planarian regeneration. Our data suggest that ERK signalling forms a spatial gradient in the anterior region during regeneration. The fibroblast growth factor receptor-like gene *nou-darake*[5] (which serves as an output of ERK signalling in the differentiating head) and posteriorly biased β-catenin activity[6–8] negatively regulate ERK signalling along the anterior–posterior axis in distinct manners, and thereby posteriorize regenerating tissues outside the head region to reconstruct a complete head-to-tail axis. On the basis of this knowledge about *D. japonica*, we proposed that β-catenin signalling is responsible for the lack of head-regenerative ability of tail fragments in the planarian *Phagocata kawakatsui*, and our confirmation thereof supports the notion that posterior β-catenin signalling negatively modulates the ERK signalling involved in anteriorization across planarian species. These findings suggest that ERK signalling has a pivotal role in triggering globally dynamic differentiation of stem cells in a head-to-tail sequence through a default program that promotes head tissue specification in the absence of posteriorizing signals. Thus, we have confirmed the broad outline of Morgan's hypothesis, and refined it on the basis of our proposed default property of planarian stem cells.

The planarian *Dugesia japonica* has four distinct body regions arranged in an anterior to posterior sequence: head containing a brain and eyes (H), prepharyngeal (Pr), pharyngeal containing a pharynx (Ph), and tail (T) regions[5,9–12] (Fig. 1a) and possesses high regenerative ability[13,14]. After amputation, anterior-facing wounds form a head blastema, whereas posterior-facing wounds form a tail blastema, a phenomenon known as regeneration polarity. Thereafter, the complete anterior–posterior pattern regenerates within a week by filling in the prepharyngeal and pharyngeal regions between the head and tail[15,16]. This sequence of events requires appropriate activation of somatic pluripotent stem cells (neoblasts), the sole source of regenerative cells[1,2].

Recent RNA interference[17,18] (RNAi) studies revealed that Wnt/β-catenin signalling regulates regeneration polarity in planarians[6–8,19]. RNAi of planarian *β-catenin* caused transformation of the tail region into a head-like region[6,7,19]. β-catenin presumably forms a decreasing tail-to-head activity gradient as a result of the expression of posterior *Wnt* genes[8], as inferred from the expression pattern of *DjAbd-Ba*, a target *Hox* gene of posterior Wnt/β-catenin signalling in neoblast progeny[11,19] (Fig. 1b), and accordingly fits the instructive role of the "tail stuff". By contrast, no molecules have been reported to function as the "head stuff".

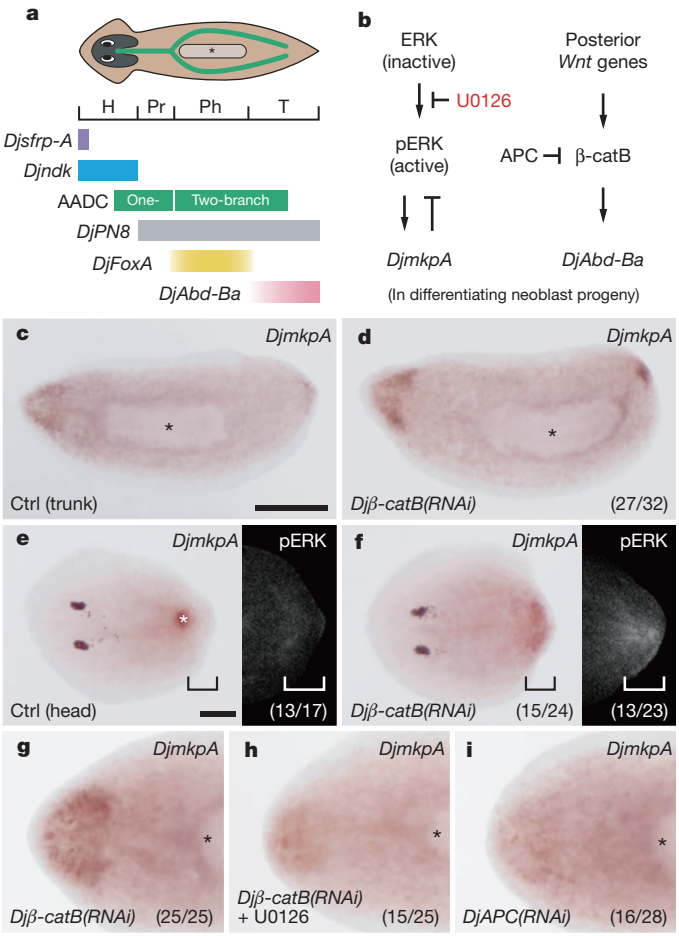

Figure 1 | β-catenin signalling affects ERK signalling during regeneration. **a**, Anterior–posterior regional subdivisions. **b**, Signalling pathways. **c**–**i**, *DjmkpA* expression at 3 days of regeneration (Re-3d). **c**, Control trunk fragment (30/30). **d**, *Djβ-catB(RNAi)* trunk fragment. **e**, Control head fragment. Strong *DjmkpA* expression occurred transiently in the tail blastema[20], and then decreased at this time (26/26). White asterisk indicates *DjmkpA* expression in cells associated with pharynx regeneration (left). Most animals (13/17) showed no obvious pERK signals around posterior-facing wounds (bracket, right). **f**, *Djβ-catB* RNAi increased *DjmkpA* expression (left) and pERK level in the presumptive tail region (bracket, right). **g**–**i**, Regenerating head in trunk fragments. Black asterisks (**a**, **c**, **d** and **g**–**i**) indicate a pharynx. Anterior is to the left. Scale bars: **c**, 500 μm; **e**, 200 μm.

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We previously reported that mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) signalling is highly activated at wounds and induces *DjmkpA*, a *D. japonica* MAPK phosphatase gene, in head and tail blastema cells (Fig. 1c); activated ERK (phosphorylated ERK; pERK) and *DjmkpA* form a negative feedback circuit that controls blastema cell differentiation (Fig. 1b), resulting in regeneration of the head or tail at a wound[20]. Thus, hitherto unknown links between ERK and posterior β-catenin signals might direct the appropriate differentiation of neoblasts to reconstruct a complete head-to-tail axis, and here we tested this possibility during regeneration of *D. japonica*.

To sensitively visualize ERK activation *in vivo*, we monitored *DjmkpA* expression. RNAi of *D. japonica β-cateninB*[19] (*Djß-catB*) clearly increased *DjmkpA* expression in the presumptive regenerating tail via ERK activation (Fig. 1c–f). *Djß-catB* RNAi also increased *DjmkpA* expression in the regenerating head region via ERK activation (Fig. 1c, d) because this increase was strongly suppressed by treatment with MEK inhibitor U0126 (Fig. 1g, h), which inhibits ERK signalling[20] (Fig. 1b and Supplementary Fig. 1). Conversely, increased β-catB activity induced by RNAi of *D. japonica adenomatous polyposis coli* (*DjAPC*) strongly decreased *DjmkpA* expression in regenerating head (Fig. 1i) and interfered with head regeneration (Supplementary Fig. 2). These observations suggest that the posterior β-catenin signalling level may directly affect the ERK signalling level, and the absence of β-catenin signalling allows neoblasts to achieve the ERK activation necessary for head regeneration at anterior-facing wounds.

We next examined whether ERK signalling is also required for regeneration of the prepharyngeal and pharyngeal regions. U0126 (25 μM) caused failure of head and tail regeneration (Fig. 2a, b and Supplementary Fig. 3) without affecting neoblast survival, as assayed by the expression of *DjpiwiA*, a neoblast-specific marker gene[1] (Supplementary Figs 1b and 4c). Such inhibitor treatment also prevented pharynx regeneration from both head and tail fragments (Fig. 2a, b and Supplementary Fig. 3), and blocked reconstruction of the single anterior gut branch from pre-existing gut tissues in regenerating tail fragments (Fig. 2a, b). Further analysis of regenerating tail fragments revealed that lower concentrations of U0126 (5–6.25 μM) severely inhibited formation of both an anterior gut within the prepharyngeal region and a pharynx, but not head regeneration (Fig. 2c), suggesting that quantitative differences of ERK activity influence reconstruction of the anterior–posterior pattern. Quantitative reverse transcription-polymerase chain reaction (qRT–PCR) analysis revealed a decreasing head-to-tail gradient of *DjmkpA* expression in response to ERK activation, a pattern reversed compared with that of *DjAbd-Ba* expression (Fig. 2d). Indeed, a lower concentration of U0126 inhibited *DjmkpA* expression most strongly outside the head region (Fig. 2e, f), in the same region where severe regeneration defects occurred (Fig. 2c). Thus, graded ERK activation along the anterior–posterior axis may instruct the reconstruction of the prepharyngeal and pharyngeal regions between the head and tail.

D. japonica nou-darake (*Djndk*) encodes a fibroblast growth factor (FGF) receptor-like transmembrane protein, but lacks the cytoplasmic kinase domain characteristic of the FGF receptor family[5]. Our previous finding that *Djndk* RNAi induced ectopic differentiation of head tissues, such as eyes and brain cells, outside the head region[5], a phenotype opposite to that of ERK inhibition, led us to test the relationship between ERK and *Djndk* for specifying the intermediate subdivisions along the anterior–posterior axis during regeneration.

We demonstrated that *de novo* expression of *Djndk* serves as an output of the ERK/*DjmkpA* feedback circuit in the differentiating head blastema (Supplementary Fig. 4). In control tail fragments at 3 days of regeneration, *DjmkpA* was highly expressed with a well-demarcated posterior end in the head region, and expressed at progressively lower levels towards the pharyngeal region (Fig. 3a). *Djndk* RNAi abolished the clearly defined boundary of *DjmkpA* expression between the head and prepharyngeal region (Fig. 3b), presumably due to increased

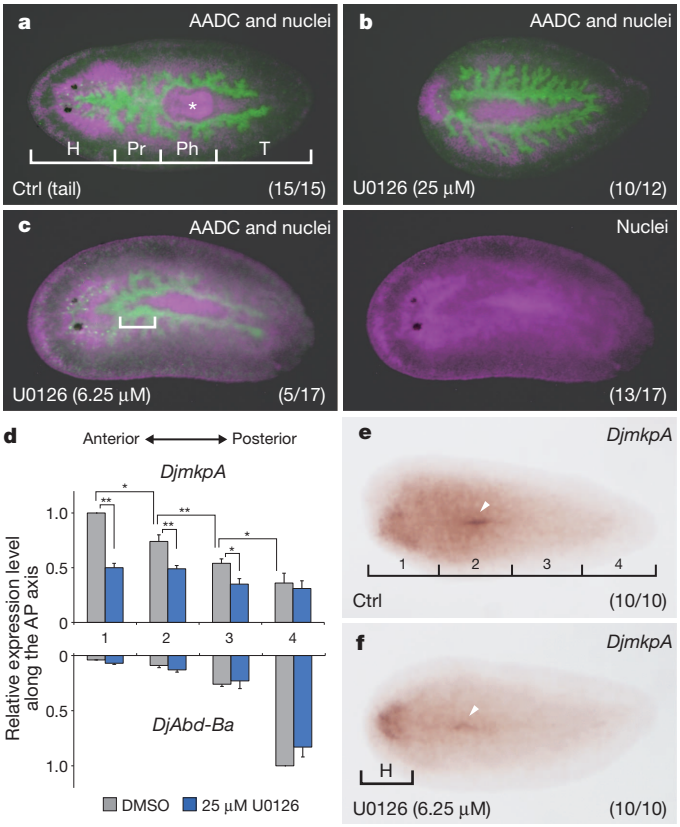

Figure 2 | Effects of pharmacological ERK inhibition on anterior–posterior patterning during regeneration. **a**, Control tail fragment at Re-7d (15/15). **b**, 25 μM U0126 treatment blocked regeneration of the H, Pr and Ph regions at Re-7d, but allowed the regenerant to retain the two original posterior branches of the gut (10/12), as assayed by anti-AADC antibody staining (green), and caused failure to regenerate a pharynx (Hoechst 33342 staining (magenta) (12/12)). **c**, 6.25 μM U0126 treatment caused the regenerant to have two gut branches in the presumptive Pr region (5/17; bracket, left), but left head regeneration normal at Re-7d (100% of animals, $n = 17$). This treatment also caused failure to regenerate a pharynx (13/17, right). The brain in the H region and the pharynx (asterisk in **a**) are visualized by Hoechst 33342 staining (magenta). **d**, Relative gene expression levels at Re-3d, as determined by qRT–PCR. Regenerants were divided into quarters along the anterior–posterior (AP) axis and total RNA was isolated from each quarter. Bars show mean ± s.d. of replicate qRT–PCR assays ($n = 3$) of pooled RNA from each quarter of 12 animals (Student's *t*-test, $*P < 0.05$, $**P < 0.01$). **e**, **f**, *DjmkpA* expression in control versus 6.25 μM U0126-treated regenerants at Re-3d. Arrowheads indicate *DjmkpA* expression in cells associated with pharynx regeneration. Anterior is to the left.

DjmkpA expression in the prepharyngeal region. qRT–PCR confirmed that *Djndk* RNAi increased the ERK-dependent *DjmkpA* expression (Fig. 3c). Later, *Djndk* RNAi caused formation of ectopic head tissues predominantly in the prepharyngeal region[5,14] (Supplementary Fig. 5b) and marked loss of prepharyngeal identity[9,16] (Fig. 3d–f). These observations suggest that *Djndk* RNAi caused transformation of the prepharyngeal region into a head-like region through ERK activation in a non-cell autonomous manner. Moreover, *Djndk* RNAi rescued the loss-of-pharynx phenotype in a moderately ERK-deficient background (Fig. 3c, g–i). Thus, we speculate that *Djndk* might encode a protein that concentrates in the head region and binds to molecules secreted in that area (as yet unidentified) that promote ERK activation in neoblasts involved in anteriorization.

We also showed that posterior β-catenin signalling acts to specify the pharyngeal, but not prepharyngeal, region during head regeneration from tail fragments. *Djß-catB* RNAi decreased the posteriorly biased *DjAbd-Ba* expression (Fig. 2d), and conversely increased the expression of *DjmkpA* and *DjsfrpA* (a readout of ERK signalling in

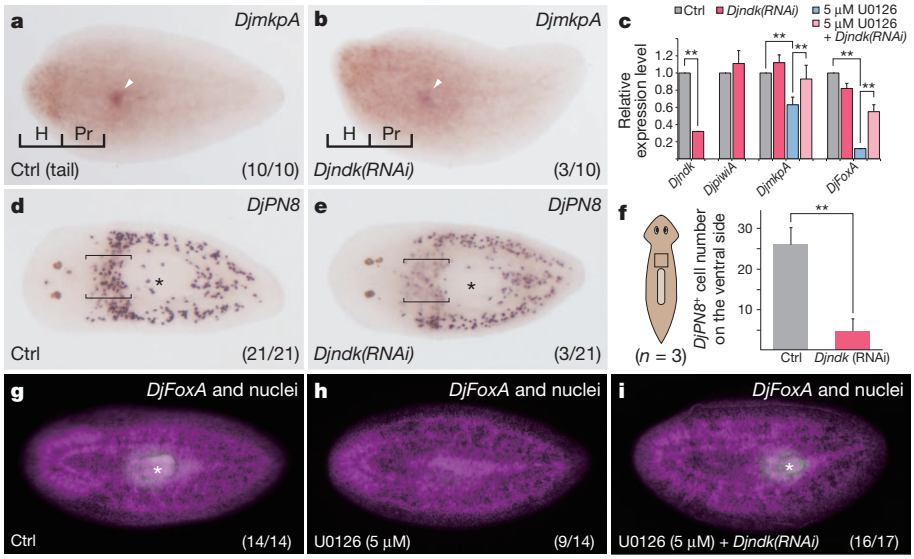

Figure 3 | Effects of *Djndk* RNAi on anterior–posterior patterning during regeneration. **a**, **b**, *DjmkpA* expression at Re-3d. Arrowheads indicate *DjmkpA* expression in cells associated with pharynx regeneration. **c**, Relative gene expression levels at Re-3d. Bars show mean ± s.d. of replicate qRT–PCR assays ($n = 3$) of pooled RNA from 15 animals (Student's *t*-test, $*P < 0.05$, $**P < 0.01$). **d**, **e**, Ventral view of *DjPN8* expression at Re-7d. Brackets indicate *DjPN8* expression in the prepharyngeal region that was severely affected by *Djndk* RNAi. *Djndk* RNAi caused formation of an ectopic eye posterior to an original eye. **f**, The number of *DjPN8*$^+$ cells in the boxed area ($n = 3$ animals in each assay; Student's *t*-test, $**P < 0.01$). Error bars represent s.d. **g**–**i**, Staining with *DjFoxA* probe (green) and Hoechst 33342 nuclear staining (magenta) at Re-7d. Asterisks (**d**, **e**, **g** and **i**) indicate a pharynx. Anterior is to the left.

the head blastema[20]), suggesting that anterior ERK signalling was increased in *Djß-catB(RNAi)* animals (Fig. 4a–c; Supplementary Fig. 6). However, these knockdown animals failed to form a pharynx rudiment at 3 days of regeneration (Fig. 4d, e), as assayed by the expression of *DjFoxA* (a marker for pharyngeal cells[10]). Combined 5 μM U0126 treatment and *Djß-catB* RNAi additively decreased *DjFoxA* expression (Fig. 4c), suggesting that interplay between ERK and posterior β-catenin signals is required to regenerate pharyngeal cells. At 7 days of regeneration, *Djß-catB(RNAi)* animals showed roughly normal regeneration of the head and prepharyngeal regions (100% of animals, $n = 30$) and ectopic fusion of the two posterior branches of the gut in the presumptive pharyngeal region (Fig. 4f), a phenotype opposite to

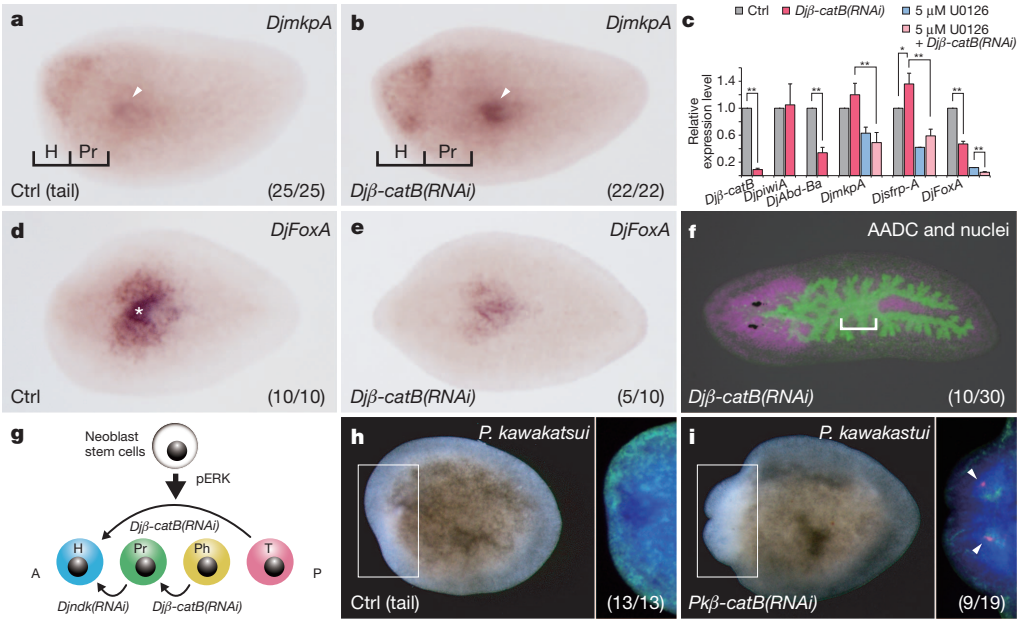

Figure 4 | Phenotypes induced by RNAi of *ß-catenin* in different planarian species. **a**, **b**, *DjmkpA* expression at Re-3d. Arrowheads indicate *DjmkpA* expression in cells associated with pharynx regeneration. **c**, Relative gene expression levels at Re-3d. Bars show mean ± s.d. of replicate qRT–PCR assays ($n = 3$) of pooled RNA from 15 animals (Student's *t*-test, $*P < 0.05$, $**P < 0.01$). **d**, **e**, *DjFoxA* expression at Re-3d. Asterisk (**d**) indicates a pharynx rudiment. **f**, Staining with anti-AADC antibody (green) and Hoechst 33342 nuclear staining (magenta) at Re-7d. The *Djß-catB(RNAi)* animal completely lacks a pharynx, and instead shows ectopic fusion of the two posterior gut branches (bracket). **g**, Molecular logic for planarian regeneration along the anterior–posterior (A–P) axis. **h**, **i**, Left panels, control tail fragment of *Phagocata kawakatsui* at 19 days after amputation and a *Pkß-catB(RNAi)* tail fragment at 16 days after amputation. Right panels, enlarged view of corresponding boxed areas in left panels. The *Pkß-catB(RNAi)* animal regenerated a head-like structure, including a brain visualized by strong staining with Hoechst 33342 (blue) and with anti-synaptotagmin (SYT) antibody (green), and a paired cluster of photoreceptor cells visualized by anti-arrestin staining (magenta; arrowheads). The control animal failed to regenerate a head and showed high background signals of anti-SYT staining. Anterior is to the left.

that induced by ERK inhibition (Fig. 2c), at the expense of the pharynx structure. This suggests that *Djß-catB* RNAi caused transformation of the pharyngeal region into a prepharyngeal-like region through ERK activation.

Altogether, our findings encourage us to refine Morgan's hypothesis at the cellular and molecular levels as follows. We showed that *Djndk* RNAi anteriorized the prepharyngeal region, and *Djß-catB* RNAi anteriorized the pharyngeal and tail regions, with increases of ERK activity level in both cases, suggesting that regenerative cells are prone to undergo differentiation into head tissues in the absence of these two posteriorizing signals (Fig. 4g and Supplementary Fig. 5). Accordingly, we propose that neoblasts might follow a default program of differentiation into head tissues that is triggered by the activation of ERK and calls to mind the old description by Morgan in 1904 that "in the absence of polarity (i.e. posteriorizing signals) the new material (i.e. *de novo* pERK) produces a head" in *Planaria maculata*[21]. Thus, activated-ERK triggering of the default program might correspond to Morgan's "head stuff". To validate this, we will have to demonstrate the existence of a pERK gradient itself in planarians.

The ability to regenerate a head varies among planarian species[22]. In the planarian *Phagocata kawakatsui*, the tail region shows markedly reduced head-regenerative ability, whereas tail regeneration is normal (Fig. 4h and Supplementary Fig. 7). We reasoned that if head tissue differentiation is a default property of neoblasts in response to ERK activation not only in *D. japonica* but across planarian species (Supplementary Fig. 8), excessive posterior β-catenin signalling might be interfering with the ERK activation necessary for head regeneration from tail fragments of *P. kawakatsui*. Indeed, RNAi of *P. kawakatsui β-cateninB (Pkβ-catB)* enabled *de novo* regeneration of a head-like structure, including brain tissues and a pair of eyes, at anterior-facing wounds of tail fragments (9/19; Fig. 4i). Moreover, seven of these nine regenerants showed negative phototaxis[23] (a typical outcome of the function of the brain and eyes[24]), in contrast to regenerants from control tail fragments (0/13; Supplementary Fig. 9). Thus, we identified a genetic cause of the lack of head-regenerative capacity in this planarian species. We speculate that the balance between anterior ERK signalling and posterior β-catenin signalling may vary among planarian species, resulting in the drastic differences of their head-regenerative capacity.

In summary, we have not only integrated the concept of default differentiation of stem cells into head tissues into Morgan's hypothesis, but have also demonstrated that posterior Wnt/β-catenin signalling interferes with head tissue differentiation in planarians by overriding this default.

METHODS SUMMARY

Planarian culture, drug treatments and quantitative RT–PCR[20], behavioural assay[23], gene silencing by RNA interference[17,18] and whole-mount *in situ* hybridization[25] were performed as reported. The *Pkβ-catB* gene sequence was obtained from transcriptome analysis of *P. kawakatsui* by next generation sequencing with a Roche 454 GS FLX Titanium. Based on the sequence data, a 1,300-base-pair complementary DNA fragment of *Pkβ-catB* was amplified from a cDNA library of non-regenerating intact planarians constructed in lambda ZAP II (Stratagene) using oligo(dT) primers, and used as a template to make double-stranded RNA. Whole-mount immunostaining is described in the Methods section.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Y.U. and K.A. designed the study. Y.U., J.T. and K.H. performed the study in *D. japonica*. S.Y., E.K. and O.N. performed *P. kawakatsui* transcriptome analysis. Y.U., J.T. Y.N. and M.H. performed the study in *P. kawakatsui*. T.I. performed behavioural assays. Y.U. wrote the paper.

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METHODS

Animals. A clonal strain of the planarian *Dugesia japonica* derived from the Iruma River in Gifu prefecture, Japan, which is maintained in autoclaved tap water at 22–24 °C, was used in this study. An isolated colony of the planarian *Phagocata kawakatsui* derived from the Yogo Lake area in Shiga prefecture, Japan, which is maintained in autoclaved tap water at 16–18 °C, was used in this study. These planarians (about 8 mm in length) were starved for at least 1 week before experiments. They were transversely dissected into three pieces: head, trunk (including pharynx) and tail, and allowed to undergo regeneration.

Treatment with a chemical inhibitor. The MAPK/ERK kinase (MEK) inhibitor U0126 (Cell Signaling Technology) was dissolved in dimethyl sulphoxide (DMSO) and used at the indicated concentration(s) for each experiment. Amputated planarians were allowed to regenerate in tap water supplemented with the inhibitor or DMSO alone immediately after amputation for the indicated period of regeneration for each experiment.

Identification of *P. kawakatsui β-cateninB* (*Pkβ-catB*) gene. Transcriptome analysis of *P. kawakatsui* was performed by next generation sequencing with Roche 454 GS FLX Titanium. For this purpose, messenger RNA was isolated from 190 trunk fragments containing a pharynx and from 178 tail fragments. The *Pkβ-catB* gene sequence was obtained from the Roche 454 sequencing reads by using the full-length amino acid sequence encoded by the *D. japonica β-catB* gene as a query through tblastn search. A 1,300-bp cDNA fragment of *Pkβ-catB* was amplified from a cDNA library of non-regenerating intact planarians constructed in lambda ZAP II (Stratagene) using oligo(dT) primers and used in this study.

RNA interference. Double-stranded RNA (dsRNA) was synthesized essentially as previously described[17,18]. Sequences used to generate dsRNAs are shown in Supplementary Fig. 10. dsRNA was suspended in diethylpyrocarbonate (DEPC)-treated H_2O. Intact planarians were injected with dsRNA every other day for a total of 2 times using a Drummond Scientific Nanoject II injector (Drummond Scientific Company). Control animals were injected with DEPC-treated H_2O alone. In the case of *P. kawakatsui*, no injection was performed in control animals. One day after the last injection, planarians were amputated and allowed to undergo regeneration.

Whole-mount *in situ* hybridization. Animals were treated with 2% hydrochloric acid (HCl) in 5/8 Holtfreter's solution for 5 min at 4 °C and fixed in 5/8 Holtfreter's solution containing 4% paraformaldehyde and 5% methanol for less than 2 h at 4 °C. Hybridization and colour detection of digoxigenin (DIG)-labelled RNA probes were carried out as previously described[25]. A mixture of BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Roche), HNPP/Fast Red (Roche) or TSA Labelling (Molecular Probes) was used for colour development of the alkaline phosphatase-conjugated anti-DIG antibody (Roche) or for detection of fluorescence.

Whole-mount immunostaining. Planarians were treated with 2% HCl in 5/8 Holtfreter's solution for 5 min at 4 °C and washed twice with 5/8 Holtfreter's solution at room temperature. They were then fixed with 5/8 Holtfreter's solution containing 4% paraformaldehyde and 5% methanol for less than 2 h at 4 °C. The fix solution supplemented with PhosSTOP phosphatase inhibitor (Roche) was used for the detection of phosphorylated protein. To lower the background, fixed planarians were treated with hybridization buffer for whole-mount *in situ* hybridization overnight at 55 °C. Fixed animals were blocked with 10% Blocking reagent (Roche) in PBST (phosphate buffered saline containing 0.1% Triton X-100) for 1 h at 4 °C, and then incubated with 1/1,000 diluted mouse anti-AADC[12], 1/1,000 diluted mouse anti-DjPiwiA[20], 1/200 diluted rabbit anti-phosphorylated histone H3 (Upstate Biotechnology), 1/1,000 diluted rabbit anti-phosphorylated ERK (pERK)[20], 1/1,000 diluted mouse anti-synaptotagmin[14] or 1/1,000 diluted rabbit anti-arrestin[14] antibody overnight at 4 °C. The samples were washed with PBST for 30 min four times and signals were detected with 1/500 diluted Alexa Fluor 488- or 594-conjugated goat anti-mouse or anti-rabbit IgG (Invitrogen) in 10% goat serum in PBST overnight at 4 °C in the dark. pERK signals were detected with 1/500 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG and HNPP/Fast Red (Roche). The samples were also incubated with 1/1,000 diluted Hoechst 33342 at the same time.

Reverse transcription and quantitative RT–PCR analysis. Total RNA was isolated from 15 whole tail fragments of *D. japonica* using Isogen LS (Nippon Gene). Twelve regenerating tail fragments of *D. japonica* were divided into quarters along the anterior–posterior axis and total RNA was also isolated from each quarter. In the case of *P. kawakatsui*, the stock of mRNA isolated for transcriptome analysis was used. First-strand cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative analysis of the amount of each gene product was carried out as previously described[20] using an iCycler real-time PCR machine (Bio-Rad Laboratories). Measurements were normalized by the expression level of a constitutively transcribed housekeeping gene, *GAPDH*. The mean of three replicate qRT–PCR assays was reported. Oligonucleotide primer sequences used for the assays are listed in Supplementary Fig. 11.

Behavioural assay. Ten millilitres of planarian culture water (23 °C) was put in a 3-cm wide × 6-cm long × 1-cm deep quartz rectangular container painted black except for one clear end. A planarian was put into the container 1.5 cm from the clear end, and the container was exposed to 300 lx of white light from a horizontal position at the clear end of the container. Planarian behaviour was recorded using an overhead digital video camera (Sony) for 90 s.