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Citation	Proceedings of the National Academy of Sciences (2009)
Issue Date	2009-12-11
URL	http://hdl.handle.net/2433/88957
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Type	Journal Article
Textversion	author

Planarian Hedgehog/Patched establishes anterior-posterior polarity by regulating Wnt signaling

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Data deposition:

The sequence data for the reported cDNAs of *D. japonica* have been deposited in the DDBJ/EMBL/GeneBank database under the accession codes AB504738 (*Djptc*), AB504739 (*Djhh*), AB504740 (*Djsufu*), AB504741 (*Djgli*), AB504743 (*DjfzT*), AB504744 (*DjwntP-1*), AB504745 (*Djwnt11-2*), AB505439 (*Djβ-cateninB*), and AB504746 (*DjG3PDH*).

Abstract

Despite long-standing interest, the molecular mechanisms underlying the establishment of anterior-posterior (AP) polarity remain among the unsolved mysteries in metazoans. In the planarians (a family of flatworms), canonical Wnt/β-catenin signaling is required for posterior specification, as it is in many animals. However, the molecular mechanisms regulating the posterior-specific induction of Wnt genes according to the AP polarity have remained unclear. Here, we demonstrate that Hedgehog (Hh) signaling is responsible for the establishment of AP polarity via its regulation of the transcription of Wnt family genes during planarian regeneration. We found that RNAi gene knockdown of *Dugesia japonica patched* (*Djptc*) caused ectopic tail formation in the anterior blastema of body fragments, resulting in bipolar-tails regeneration. In contrast, RNAi of *hedgehog* (*Djhh*) and *gli* (*Djgli*) caused bipolar-heads regeneration. We show that Patched-mediated Hh signaling was crucial for posterior specification, which is established by regulating the transcription of Wnt genes via downstream Gli activity. Moreover, differentiated cells were responsible for the posterior specification of undifferentiated stem cells through Wnt/β-catenin signaling. Surprisingly, *Djhh* was expressed in neural cells all along the ventral nerve cords (along the AP axis), but not in the posterior blastema of body fragments, where the expression of Wnt genes was induced for posteriorization. We therefore propose that Hh signals direct head or tail regeneration according to the AP polarity, which is established by Hh signaling activity along the body's preexisting nervous system.

Introduction

Planarians are one of the most useful animals for investigation of the molecular and cellular mechanisms by which the anterior-posterior (AP) polarity of the body is established during regeneration. It is well known that planarians regenerate a head or a tail from the anterior and posterior ends, respectively, of the amputated stump, with maintenance of the original AP polarity. More than a century ago, T.H. Morgan reported one of the earliest descriptions of 'polarity' in planarian regeneration (1), based on his findings that very thin cross-sectional fragments made by cutting along the AP axis regenerated bipolar two-headed planarians (2), termed "Janus-heads" (an allusion to the Roman god Janus; 3, 4). Since then, much effort has been focused on this fascinating maintenance of the original polarity, and its disruption, such as the induction of Janus-heads formation by treatment with colchicine or colcemid (5, 6), although the molecular mechanisms still remain unknown.

Recently, canonical Wnt/β-catenin signaling was shown to be required for posterior specification in planarians. Wnt/β-catenin signaling is evolutionarily well conserved and plays a role in establishing the AP axis during development in several animal species. In the planarian *Schmidtea mediterranea*, gene knockdown by RNA interference (RNAi) of *Smed-βcatenin-1* causes Janus-heads formation during regeneration (4, 7, 8). RNAi of posterior-specific *Smed-wntP-1* also causes the Janus-heads phenotype, as well as tail-less regeneration in *Smed-wntP-1* or *wnt11-2* RNAi regenerants (9). These findings provided the first insight into the molecular mechanisms directing the AP polarity during planarian regeneration. However, it remained necessary to discover the regulatory system for the posterior-specific

activation of Wnt signals to fully understand the mechanism of the establishment of AP polarity in planarians.

Here, we investigated the upstream regulator(s) of Wnt/β-catenin signaling. We found that Hedgehog (Hh) signaling plays a crucial role for posterior specification in planarians by regulating the transcription of posterior-specific *Wnt* genes. In addition, we discuss the mechanism of the establishment of AP polarity during planarian regeneration.

Results

"Janus-tails" formation in *Djptc* RNAi planarians. To get a better understanding of the molecular mechanisms directing AP polarity, we screened a planarian *Dugesia japonica* EST library (10) for genes robustly responsible for AP polarity. Body fragments (head, trunk and tail fragments) from transversely amputated planarians were examined for regeneration at both the anterior and posterior ends after RNAi by dsRNA injection (11). A homology search and the RNAi screen revealed that *D. japonica patched* (*Djptc*) is required for head regeneration in planarians (Fig. 1). *Djptc*, which is expressed ubiquitously (Fig. S1), codes for a Patched (Ptc) -like receptor, and possesses a conserved sterol-sensing domain characteristic of membrane proteins such as Ptc and NPC-1 (Fig. S2).

To characterize the defect in head regeneration of *Djptc* RNAi [*Djptc(RNAi)*] planarians, we examined the expression patterns of anatomical and molecular markers for AP identity. The expression of *DjPC2*, a central nervous system (CNS) marker (12), showed that a cephalic brain did not develop in the "anterior blastema" (a mass of undifferentiated cells at the anterior cut edge of a stump) of *Djptc(RNAi)* trunk

regenerants (*i.e.*, regenerating fragments from the middle part of amputated planarians), although ventral nerve cords (VNCs) did regenerate (Fig. 1*H*). Expression of *Djndk*, a head marker (13), was absent from the anterior portion of *Djptc(RNAi)* trunk regenerants (Fig. 1*I*). Moreover, two tail marker genes, *DjAbd-Ba* (14) and *Dj frizzled-T* (*DjfzT*), a tail-specific Frizzled receptor gene (4), were ectopically induced in anterior blastemas by *Djptc* RNAi (Fig. 1 *J* and *K*). Regarding gut morphology (the digestive system, consisting of a single anterior and two posterior gut branches; Fig, 1*F*), staining of the gut tract with anti-DjAADCA antibody (15) revealed that *Djptc(RNAi)* regenerants showed posterior fate in the anterior region, as indicated by their bifurcated branching (Fig. 1*L*). These results show that not only is the head-identity lost upon *Djptc(RNAi)*, but the polarity is reestablished to yield two oppositely oriented posterior ends, leading to the generation of the "Janus-tails" formation in *Djptc(RNAi)*. Actually, *Djptc(RNAi)* regenerants occasionally developed an oppositely oriented pharynx, anterior to the original one (arrow in Fig. 1*H*), suggesting the intercalation of an extra pharynx according to the reestablished polarity.

Gradual transformation to posterior polarity after *Djptc* RNAi. To gain more insight into the polarization, we analyzed the expression patterns of relevant marker genes and their expression levels by reverse transcription and quantitative PCR (RT-qPCR) during early regeneration of trunk fragments after *Djptc* RNAi. *DjAbd-Ba* expression was induced ectopically and increased gradually in the anterior end of *Djptc(RNAi)* fragments during regeneration (Fig. 1 *P* and *S*). In contrast, the expression of the anterior-specific gene *DjZicB* (16) was not induced in the anterior blastema at regeneration day-1, but rather decreased to the same level as that in the posterior

blastema at day-3 in *Djptc(RNAi)* (Fig. 1 *Q* and *S*). However, *Djptc(RNAi)* showed no effect on the relative gene expression (RGE) level of brain marker *DjotxA* (17) at day-1, but showed a decrease by day-3 of regeneration (Fig. 1*S*). Also, *Djndk* expression showed moderate induction in the anterior blastema of *Djptc(RNAi)* fragments at day-1, but thereafter decreased gradually (Fig. 1 *R* and *S*). These findings indicate that *Djptc* RNAi does not cause a complete reversal of polarity from the beginning of regeneration. Rather, the initial anterior polarity of body fragments is gradually transformed into posterior polarity during regeneration.

Djptc functions upstream of Wnt signaling. Wnt/β-catenin signaling is crucial for posterior specification in planarians (4, 7-9), as it is in many animals. β-catenin is a key mediator of the canonical Wnt/β-catenin cell-to-cell signal transduction cascade in animals (18). To examine the possible relationship between *Djptc* and Wnt/β-catenin signaling in planarians, we conducted double gene knockdown by co-injection of dsRNAs. We confirmed Janus-heads formation in trunk regenerants after RNAi of *Djβ-cateninB* (Fig. 2*C*), an ortholog of *Smed-βcatenin-1* (4, 7, 8). Simultaneous *Djptc* and *Djβ-cateninB* RNAi also caused Janus-heads formation (Fig. 2*D*), indicating that *Djptc* functions upstream of *Djβ-cateninB* signaling.

To examine the *Djptc*/*Wnt* relationship, we analyzed the transcription of posterior-specific *Wnt* genes (7, 9), *DjwntP-1* and *Djwnt11-2* in RNAi trunk regenerants 24 hours after amputation (Fig. 2*E*). Quantification of the RGE level at both ends of *Djptc(RNAi)* trunk regenerants revealed that *Djptc* RNAi increased the expression of *DjwntP-1* and *Djwnt11-2* ectopically in the anterior blastema. We found that this ectopic induction of *Djwnt11-2*, but not *DjwntP-1*, was abrogated by simultaneous

Djβ-cateninB RNAi. The same tendencies were also observed in the posterior end. These results indicate that *Djptc*-mediated signaling regulates the transcription of posterior-specific *Wnt* genes, as is thought to occur in flies (19), and that transcription of *Djwnt11-2* is further regulated by downstream *Djβ-cateninB*. These results suggest that *Djptc* regulates the transcription of *DjwntP-1*, which may act as a primary stimulus to activate Wnt/β-catenin signaling.

Hh signaling is responsible for posteriorization. In general, Ptc is known to be a receptor in the evolutionarily well-conserved Hh signaling pathway (20-24). The basic machinery of the Hh signaling pathway functions via a series of repressive interactions: the ligand Hh, a transmembrane transducer Smoothened (Smo) and an activated form of the transcription factor Gli, act as positive regulators, whereas other components such as the Hh receptor Ptc and a Gli-interacting protein, Suppressor of Fused (SuFu), act as negative regulators. We identified planarian homologs of *hedgehog* (*Djhh*), *suppressor of fused* (*Djsufu*), and *gli* (*Djgli*) from our EST database. A *smoothened* homolog was not identified from our EST database, although one can be found in an *S. mediterranea* genome database (http://smedgd.neuro.utah.edu/). *Djsufu* and *Djgli* were expressed nearly ubiquitously in planarians as similarly to *Djptc* (Fig. S1). In contrast, *Djhh* expression was specifically localized in a subset of cells in intact planarians (Fig. S1).

To determine whether Hh signaling is involved in AP polarity during regeneration, we performed RNAi analyses for Hh signaling component genes. In regenerants with RNAi of the positive regulators *Djhh* and *Djgli*, weak RNAi treatment caused tail malformation, while strong RNAi treatment and a second round of regeneration caused Janus-heads formation (Fig. 3). In the head fragments after the first round of

regeneration, RNAi of *Djhh* and *Djgli* caused Janus-heads formation with the generation of ectopic eyes in the posterior blastemas, as well as loss of the tail identity, as shown by the loss of *DjfzT* expression (Fig. 3 *B* and *C*). Almost half of RNAi trunk regenerants finally showed loss of the tail identity after the second round of regeneration (Fig. 3 *B'* and *C'*), although they showed no clear defect during the first round of regeneration. Simultaneous RNAi for *Djhh* and *Djgli* enhanced this phenotype, including ectopic eye formation around the posterior end of trunk regenerants (Fig. 3 *D* and *D'*). Thus, *Djhh* and *Djgli* are necessary for posterior specification.

In the case of RNAi of the negative regulator *Djsufu*, as assessed by *Djndk* expression in trunk regenerants, Janus-tails formation was also observed similarly to that in *Djptc(RNAi)* (Fig. 3 *F* and *G*; Fig. S3), a finding that accords with the similarity of the phenotypes seen in *ptch1* and *sufu* mouse mutants (25, 26). Furthermore, simultaneous RNAi of *Djgli* was sufficient to suppress this ectopic anterior-tail formation (Fig. 3 *L* and *M*), but not simultaneous *Djhh* RNAi (Fig. 3 *I* and *J*). In addition, quantification of the RGE level after *Djptc* RNAi revealed that the expression of *Djptc* itself tended to increase (Fig. 3*N*), presumably due to the auto-inhibitory regulation of *Djptc*, which has been described in flies and mice (27, 28). In contrast, the RGE levels of *Djsufu* and *Djgli* were not affected in the anterior blastema in *Djptc(RNAi)* (Fig. 3*N*). Together with the conservation of the Hh signaling cascade among animal species (20-24), these findings strongly suggest that conserved Hh signaling is responsible for posterior specification in planarian regeneration, and Janus-tails formation was caused by activation of downstream DjGli ectopically in the anterior blastema.

Hh signaling regulates transcription of Wnt genes. To examine the possibility that the transcription of *Wnt* genes is a primary output of the Hh signaling, we analyzed the expression of *Wnt* genes in RNAi animals for Hh signaling component genes at regeneration day-1. Actually, the transcription of *DjwntP-1* was repressed at the posterior end of fragments as a result of the loss of Hh signaling activity (Fig. 4 *A-H*). *DjwntP-1* expression was absent in the posterior ends of regenerants from both head and trunk fragments after RNAi of *Djhh* (Fig. 4 *B* and *F*) or *Djgli* (Fig. 4 *C* and *G*), as well as simultaneous *Djhh* and *Djgli* RNAi (Fig. 4 *D* and *H*). Conversely, RGE quantification after loss of negative regulators of the Hh signaling pathway indicated that the transcription of *DjwntP-1* and *Djwnt11-2* was aberrantly upregulated in the anterior blastema of trunk regenerants in *Djptc(RNAi)* or *Djsufu(RNAi)* (Fig. 4*I*). Also, simultaneous *Djgli(RNAi)* suppressed the ectopic upregulation of these posterior *Wnt* genes in the anterior blastema in *Djptc* or *Djsufu (RNAi)* (Fig. 4*I*). These results indicated that Hh signaling specifies posterior identity through regulating the transcription of *Wnt* genes in planarian regeneration.

Differentiated cells provide signal transduction for posterior specification. The ability of planarians to regenerate missing parts relies on pluripotent stem cells distributed throughout the body (3, 29, 30), and the surrounding differentiated cells might provide appropriate cues for the differentiation of stem cells (31). To investigate how differentiated cells contribute to the regulation of Wnt signaling for the establishment of AP polarity, we analyzed X-ray-irradiated planarians, in which stem cells are specifically eliminated. *Djhh* was apparently expressed in the X-ray-resistant cells within the VNCs, which were stained with anti-Dj Synaptotagmin (DjSyt)

antibody (Fig. 5 *A-D*; Fig. S4)*,* and *Djptc* expression was detected in X-ray-resistant cells along the VNCs (Fig. S4). Interestingly, the expression of *DjwntP-1* was induced exclusively in X-ray-resistant cells at the posterior end of head regenerants 24 hours after amputation, whereas *Djwnt11-2* induction occurred mainly in X-ray-sensitive cells (Fig. 5*E*). These findings imply that posterior identity is established in differentiated cells by the induction of *DjwntP-1* expression even in the absence of stem cells in the posterior end.

To further identify the cell type in differentiated cells involved in the cross-talk between Hh and Wnt signaling for posteriorization, we conducted dual-color *in situ* detection for the expression pattern of *Djhh* and *DjwntP-1* at an early stage of regeneration. *Djhh* expression was significantly induced in the anterior blastema of regenerants, although this expression was absolutely derived from stem cells (Fig. S4). Thus, this anterior-expression of *Djhh* has no contribution to the induction of *DjwntP-1* expression (Fig. 5*E*). At 18 hours of regeneration, the stage before the induction of *Djhh* expression in the anterior blastema (Fig. S1), *Djhh* expression was not induced in the posterior blastema of regenerants, but was detected in neural cells of the preexisting VNCs, as indicated by the colocalization of *Djsyt* mRNA (32) (Fig. 5 *F* and *G*). In contrast, *DjwntP-1* expression was predominantly induced in cells along and between the VNCs at the posterior end of regenerants (Fig. 5 *G* and *H*), but it was also weakly detected in the anterior region (Fig. 5*I*). Especially, expression of *Djhh* and *DwntP-1* was not colocalized in the same cells at the posterior end of regenerating fragments (Fig. 5*G*). These findings suggest that *de novo* expression of *Djhh* is not required for the induction of *DjwntP-1* expression during regeneration.

Discussion

Hh signaling triggers posterior specification. Based on the findings in this report, we propose a model of the signaling cascade for the posterior specification in planarians (Fig. 6*A*). First, Hh signaling triggers posterior specification by activating the transcription of *DjwntP-1* in X-ray-resistant differentiated cells at the posterior end, and then the posterior specification signal is further transmitted by the induction of *Djwnt11-2* in X-ray-sensitive stem cells through β-catenin signaling. Finally, the resultant committed stem cells direct posterior-fate-specification of undifferentiated stem cells through Wnt/β-catenin signaling. Thus, cues for differentiation of stem cells are provided from not only differentiated cells but also stem cells during planarian regeneration.

Hh signaling is thus the activator of a key molecular switch for posterior specification, and Wnt/β-catenin signaling acts as this switch. It is known that Hh signaling regulates the morphogenesis of various tissues and organs through canonical Wnt signaling in many animal species, including the morphogenesis of intrasegmental patterning and neural tube patterning; however, our findings reveal the role of Hh signaling to establish AP polarity throughout the body in planarians.

Putative role of Hh signaling in intercalary regeneration. It has been shown that ectopic transplantation of a tail fragment into the anterior portion of a planarian caused intercalation of a newly formed ectopic pharynx in the region between the graft and the original pharynx (33, 34). We observed similar intercalation of an extra pharynx in *Djptc(RNAi)* animals (Fig. 1*H*). The anterior blastema of *Djptc(RNAi)* regenerants was transformed to become the posterior end and then it induced ectopic pharynx formation

by intercalation between the induced posterior end and the anterior base of the pharynx. In other words, identification of the "posterior terminus" by the induction of *DjwntP-1* in differentiated cells might be responsible for the intercalary regeneration of a tail in planarians. Differentiated cells provide the signal indicating the terminus ("distalization"), and missing parts are regenerated from stem cell populations between the initial tissue and the terminal cells (tissue) according to the positional information ("intercalation"), in accord with a process called "rearrangement of the body regionality" (refer to 35).

Possible mechanisms for the establishment of AP polarity in planarian. The present study clearly demonstrated that Hh signaling functions as a regulator for posteriorization in planarians. How does Hh signaling establish AP polarity during planarian regeneration? *Djhh* expression itself is not induced in the posterior blastema of regenerating fragments when *DjwntP-1* is expressed there. Rather, *Djhh* is expressed in differentiated neural cells of VNCs. Based on these observations, we propose a possible model in which Hh signaling present in the neural cells of the VNCs is activated exclusively at the posterior end, and induces *DjwntP-1*. That is, DjHh signals (protein or mRNA) might be transported posteriorly via the neural axons of the VNCs (Fig. 6*B*), in a similar way to that reported in photoreceptor neurons in fly (36).

These putative Hh signals transported uni-directionally in the VNCs might also maintain AP polarity in intact planarians, by directing the expression of *DjwntP-1* to be strongest at the posterior tip (Fig. 6*B*; 7, 9). Thus, after amputation, the planarian regeneration system does not require a 'reconstitution' of AP polarity in the remaining tissue. Instead, planarians regenerate their body according to the initial AP polarity of

the fragment along the VNCs. This scenario could account for our observation that anterior polarity was gradually lost and transformed into posterior identity during the course of regeneration in the anterior blastema after *Djptc(RNAi)*. That is, regeneration in the anterior blastema of *Djptc(RNAi)* regenerants was initiated according to the initial AP polarity of the body as defined by Hh signaling activity, and subsequently, the newly formed tissue was gradually transformed to possess posterior identity. We speculate that ectopic activation of downstream DjGli progressively activates Wnt signaling in the anterior blastema, which gradually represses the anterior (head) identity and transforms it into tail fate. The transient expression of *DjwntP-1* observed in the anterior end during regeneration (Fig. 5*I*) might be induced by the "leaky" activation of Hh signaling at the cut surface, but then repressed by a resultant depletion of the Hh signaling activity via the uni-directional transport of *Djhh* gene products.

Some puzzling observations made in classical and recent planarian regeneration experiments can be explained by this model. For example, as originally reported by Morgan (2), the Janus-heads formation in regenerants from very thin cross-sectional fragments could be explained by the lack of a sufficient total amount of Hh signal available for transport and delivery in these thin tissues to transform the polarity of the end to a posterior fate. Disruption of microtubules by treatment with colchicine or colcemid causes Janus-heads formation after regeneration (5, 6), which our model suggests would be the result of the inhibition of Hh signal transport along the microtubules in neural cells. Similarly, Janus-heads formation has been shown in regenerants in which Ca^{2+} signaling was perturbed by chemical inhibition of voltage-operated calcium channels (37), implying loss of DjHh axonal transport and/or signal transduction in neural cells of the VNCs. Further studies to clarify whether

uni-directional transport of Hh signals along the AP axis plays a crucial role in establishing the AP polarity in planarians should yield fruitful insights into this long-standing puzzle.

Materials and Methods

Planarians. A clonal strain of the planarian *Dugesia japonica* was used in all experiments. The colony was maintained in autoclaved tap water at room temperature in dim light. In all experiments, planarians of 8-10-mm length that had been starved for at least 1 week were used. For regeneration studies, planarians were cut into 3 fragments (head, trunk and tail) transversely anterior and posterior to the pharynx and allowed to undergo regeneration.

cDNA clones. cDNA clones encoding the respective proteins were identified in a previously constructed library of expressed sequence tags (ESTs) (10). Partial cDNA fragments encoding DjFzT, DjWntP-1 and DjWnt11-2 were cloned by PCR. To obtain the full-length cDNA, 5' and 3' rapid amplification of cDNA ends (RACE) were carried out using a SMART RACE cDNA amplification kit (Clontech).

X-ray irradiation. Worms placed on wet filter paper on ice were irradiated with 12 R of X-rays using an X-ray generator (SOFTEX B-4). Five days after irradiation, worms were subjected to the examinations indicated in the text.

Whole-mount *in situ* hybridization and immunohistochemistry. Whole-mount *in situ* hybridization was performed with digoxigenin (DIG)-labeled riboprobes (Roche

Diagnostics). Animals were treated with 2% 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 3 h at 4°C. Hybridization and detection of DIG-labeled RNA probes were carried out as previously described (38). After whole-mount *in situ* hybridization, specimens were subjected to immunohistochemistry using anti-DjAADCA or anti-DjSyt antibody essentially as described (15, 32). In brief, specimens were incubated with the primary antibody (1:1000 dilution) overnight at 4°C. They were visualized using a fluorescent microscope Biozero BZ-8000 (Keyence) with fluorescence-labeled secondary antibodies (Alexa Fluor 488, 1:500; Molecular Probe).

Dual-color detection of mRNA expression was performed essentially as described. For the detection of DIG- or fluorescein-labeled RNA probes, samples were incubated with specific antibodies conjugated with alkaline phosphatase or horseradish peroxidase (1:2000; Roche Diagnostics). To develop fluorescent color, a TSA kit No. 2 (Molecular Probes) and an HNPP Fluorescent Detection Set (Roche Diagnostics) were utilized according to the manufacturer's instructions. Cell nuclei were labeled with Hoechst No. 33342. Fluorescence was detected with a confocal laser scanning microscope FV1000 (Olympus).

RNA interference (RNAi). Double-stranded RNA (dsRNA) was synthesized from appropriate cDNA clones, and was injected into planarians essentially as previously described (usual-dose RNAi; 11). Control animals were injected with distilled water (DW; the solvent for dsRNA). For regeneration studies, planarians were cut 3 days after the third injection. For the high-dose RNAi in single *Djhh* and *Djgli* RNAi experiments, the synthesized dsRNAs were dissolved in half the volume of DW compared to the

volume used for usual-dose RNAi. Injected animals were allowed to undergo a second round of regeneration: the animals were cut transversely, and then both the anterior and posterior blastemas of the fragments were cut off at regeneration day-4.

Reverse transcription and quantitative PCR (RT-qPCR) analysis. Planarians were cut into 3 fragments and allowed to undergo regeneration for various numbers of days (*e.g.*, day-0, just after the cutting; day-1, around 24 h after amputation) as indicated in the text. Pieces of the anterior or posterior ends from RNAi trunk fragments ($n \geq 20$), or the posterior end from head fragments of X-ray-irradiated planarians ($n=10$ each), which included regenerating blastemas, were then collected. Total RNA was extracted from each pool of blastemas using ISOGEN-LS (Nippon Gene), and cDNA was synthesized from 1 µg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative analysis of the amount of each gene product was performed essentially as described previously (39) using the 7900HT Real-Time PCR System (Applied Biosystems). Each reaction (10 µl) contained 1x QuantiTect SYBR Green PCR Master Mix (Qiagen), gene-specific primer at 0.3 µ*M*, and 1 µl of diluted (1:20) cDNA template, and was subjected to PCR as follows: 95°C for 15 min, 50 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 1 min. PCR primers for each target gene are listed in Supplementary Table S1. The primer set for the *Djptc* gene was not complementary to its dsRNA, rather, primers were designed to correspond to the 3' end of the *Djptc* gene and the dsRNA was designed to correspond to the 5' end. Measurements were performed in quadruplicate for technical replicates (coefficient of variation was 0.1~2.8% for all primer sets), and were normalized to the expression level of *DjG3PDH* (32). In the analyses of trunk regenerants, the expression level in the

anterior end of the control fragment was taken as the standard for the relative gene expression (RGE) value, except in the case of *Djwnt11-2*, for which the level of the posterior end of the control fragment was taken as the standard because its expression was not detectable in the anterior end. In head regenerants, the expression level in the control fragment was taken as the standard. The RGE values were calculated according to Ogawa *et al*. (39). In brief, the level of the target gene transcript detected was compared with the baseline level detected in the standard, and the RGE index of the standard was set to 1.0 (dashed line in each figure). The RGE index reported here is the mean of quadruplicate measurements.

Acknowledgements

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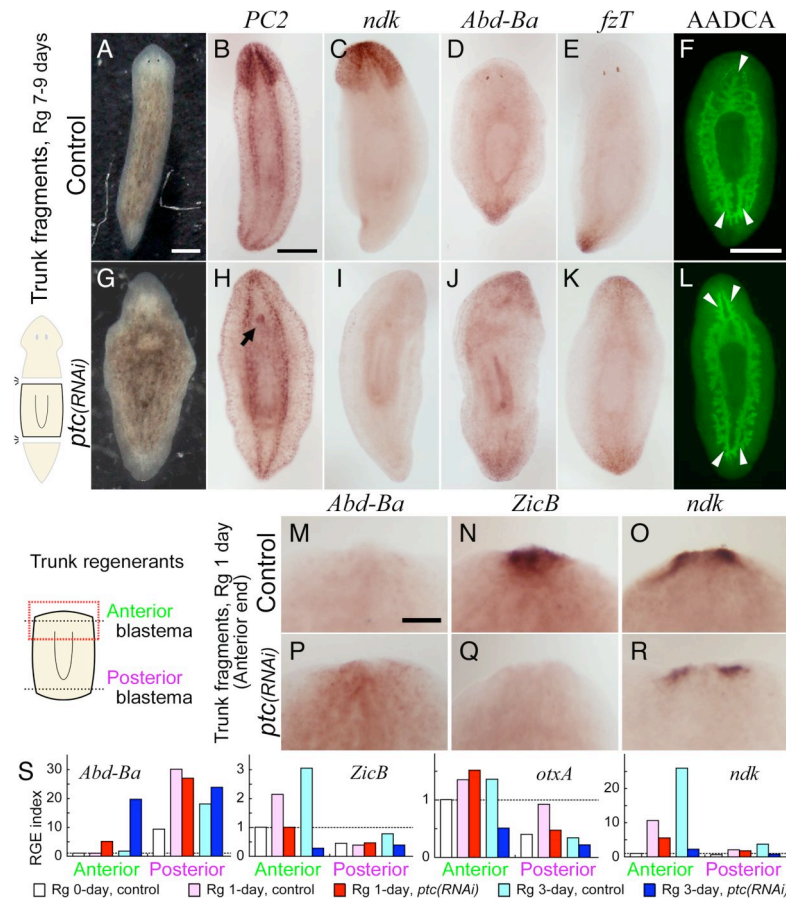
Figures

Fig. 1. "Janus-tails" phenotype is caused by *Djptc* RNAi. Anterior end is placed at the top in all panels. Rg, regeneration. (*A-L*) *Djptc(RNAi)* caused abnormal anterior blastema formation in trunk fragment regenerants at 7-9 days post-amputation. (*A* and *G*) Live animals. (*B-F*, and *H-L*) Characterization by i*n situ* detection of *DjPC2* (CNS marker; *B* and *H*), *Djndk* (a head marker; *C* and *I*), *DjAbd-Ba* (a tail marker; *D* and *J*) and *DjfzT* (a tail marker; *E* and *K*), and by immunostaining for DjAADCA (digestive gut branch staining; *F* and *L*). Arrowheads indicate the single anterior and two posterior gut branches (*F*), and the bifurcated branching of the main gut tract in the anterior region as posterior fate (*L*). Arrow indicates an extra pharynx (*H*). Bars, 500 µm. (*M-R*)

Posteriorized anterior end in *Djptc(RNAi)* trunk regenerants 1-day after amputation. Expression patterns of *DjAbd-Ba* (*M* and *P*), *DjZicB* (anterior marker; *N* and *Q*) and *Djndk* (*O* and *R*) in the anterior ends. Bar, 200 μm. (*S*) Time course of relative gene expression (RGE) level reveals gradual posteriorization of anterior blastema after *Djptc(RNAi)* during early regeneration (1- and 3-day). The expression levels of target genes (*DjAbd-Ba*, *DjZicB*, *DjotxA* and *Djndk*) in anterior or posterior blastemas were normalized and expressed relative to the level in the anterior end of control trunk regenerants at day-0 taken as 1.0 (white column of "Anterior" bars; indicated by dashed line). Mean of quadruplicates.
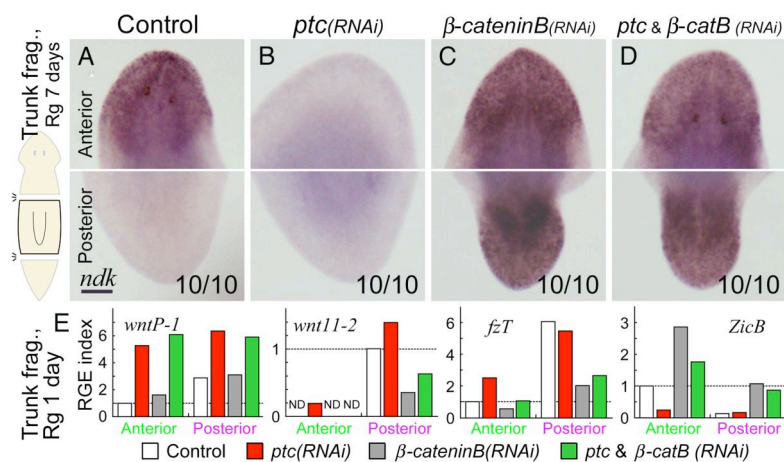
Fig. 2. *Djptc* functions upstream of Wnt/β-catenin signaling. (*A-D*) *Djndk* expression in anterior (upper) and posterior (lower) regions of RNAi trunk regenerants at day-7. *Djptc(RNAi)* caused Janus-tails formation (*B*), whereas *Djβ-cateninB(RNAi)* caused Janus-heads formation (*C*) with or without simultaneous *Djptc(RNAi)* (*D*). Numbers represent the fraction of tested regenerants showing the indicated phenotype. Bar, 200

µm. (*E*) RGE levels of *Wnt* genes in anterior and posterior blastemas of RNAi trunk regenerants at day-1, with the anterior (*DjZicB*) and posterior (*DjfzT*) marker genes shown as positive controls for RNAi phenotype. ND, not detected.
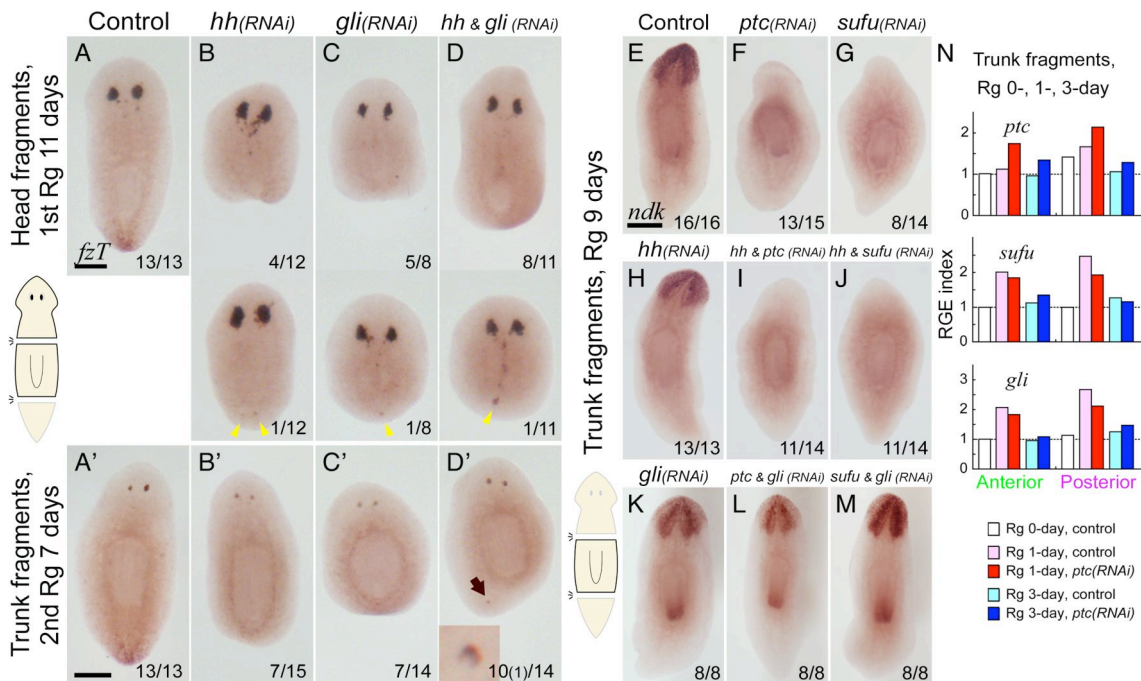

Fig. 3. Hh signaling is involved in the establishment of AP polarity. (*A-D*) Loss of Hh signaling activity caused Janus-heads or loss of the tail identity in regeneration. *DjfzT* expression in head fragments at day-11 of the first round of regeneration (*A-D*), and trunk fragments at day-7 of the second round of regeneration (*A'-D'*) after strong inhibition of the positive regulators of the Hh signaling cascade (RNAi of *Djhh* or *Djgli*). Note that the generation of ectopic eyes in the posterior blastemas (arrowheads) and extra eye formation (arrow; insert) near the posterior end (7%; 1/14, *D'*). Bars, 300 µm. (*E-M*) Janus-tails formation after RNAi of negative regulators of Hh signaling

cascade (*Djptc* or *Djsufu*) and its suppression by simultaneous RNAi of positive regulators. *Djndk* expression in trunk fragments at regeneration day-9. Bar, 300 μm. (*N*) RGE index of Hh signaling component genes in anterior and posterior blastemas after *Djptc(RNAi)* as in Fig. 1*S*.
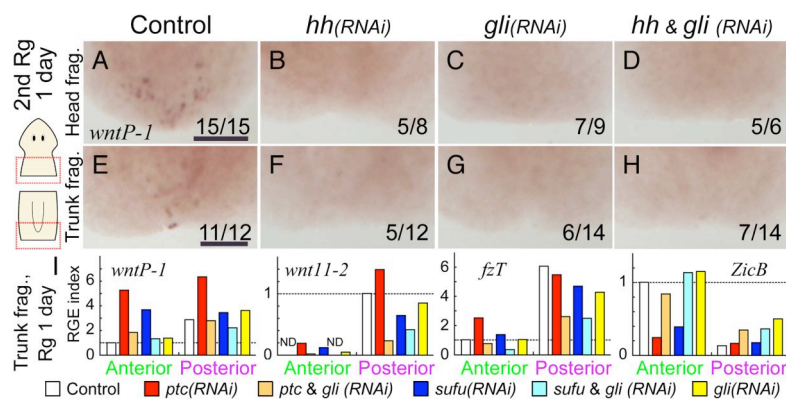

Fig. 4. Hh signaling regulates transcription of *Wnt* genes. (*A-H*) *DjwntP-1* expression was lost by RNAi of *Djhh* or *Djgli* in the posterior end of both head (*A-D*) and trunk (*E-H*) regenerants on day-1 of the second round of regeneration. Bars, 200 μm. (*I*) RGE quantification of *Wnt* genes in anterior and posterior blastemas of trunk fragments at regeneration day-1 after RNAi of Hh signaling components genes, with the anterior (*DjZicB*) and posterior (*DjfzT*) marker genes as positive controls for RNAi phenotype. ND, not detected.
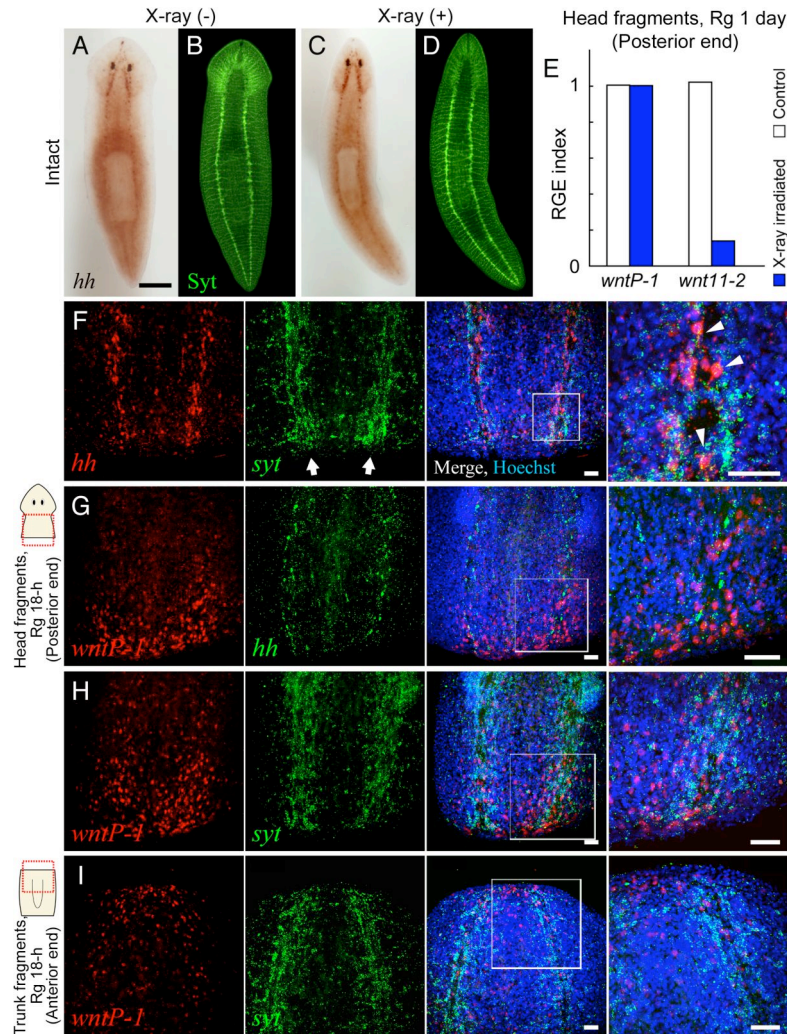
Fig. 5. Posteriorizing signal originates in differentiated cells (neuron). (*A-D*) Expression of *Djhh* was observed in X-ray-resistant differentiated cells of the VNCs. *In situ* detection of *Djhh* in non-irradiated (*A*) and irradiated (*C*) planarians, followed by labeling of the CNS with anti-DjSynaptotagimin antibody (neural marker; Syt; *B* and *D*). Bar, 1 mm. (*E*) RGE index of *Wnt* genes in the posterior blastema of head fragments from X-ray-irradiated animals 1-day after amputation. (*F-I*) Confocal images of expression patterns of *Djhh*, *DjwntP-1* or *Djsyt* by dual *in situ* detection in the posterior (*F-H*) and anterior (*I*) end in regenerants from head and trunk fragments, respectively, at 18 h after amputation. Panels show individual expression patterns (red or green color)

and their merged image with the image of nuclear staining (Hoechst; blue). The rightmost panel is a magnified view of each boxed area. Arrow indicates VNC (*F*). Note that *Djhh* was expressed in neural cells of the VNCs, which were labeled with *Djsyt* probe (arrowheads; *F*), but not in the posterior blastema (*F* and *G*). Also, *DjwntP-1* was predominantly expressed in the posterior end surrounding the VNCs (*G* and *H*), but was also weakly expressed in the anterior end (*I*). Bars, 50 μm.
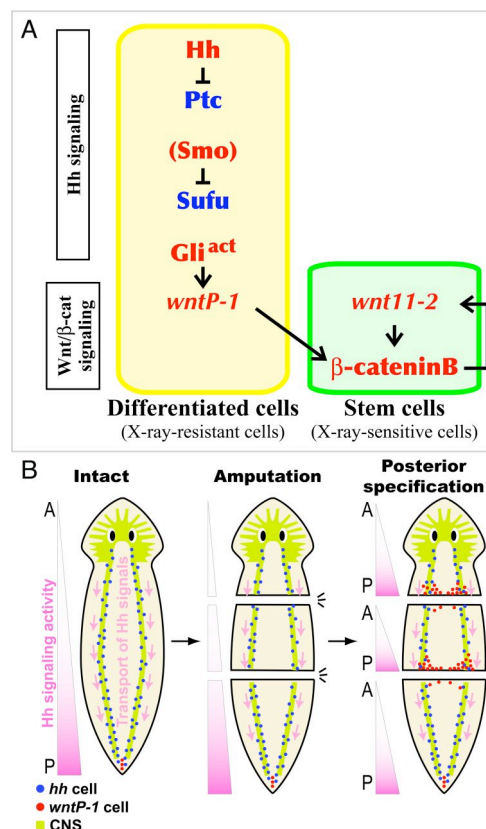

Fig. 6. Models for two aspect of the establishment of AP polarity by Hh signaling in planarians. (*A*) The signaling cascade model for posterior specification in planarian regeneration inferred from the present study. Hh signaling triggers posteriorization by

inducing transcription of *Wnt* genes in differentiated cells (yellow-shaded box), from which the signal is transmitted to stem cells (green-shaded box) through Wnt/β-cateninB signaling for tail regeneration. Positive and negative regulators of posterior specification are written with red and blue letters, respectively. (*B*) A possible model for the establishment of AP polarity by Hh signaling in planarians. *DjwntP-1*-expressing cells (red dots) at the posterior end suggests that Hh signaling is exclusively activated there (pink gradients on the left), although *Djhh*-expressing cells (neural cells; blue dots) are localized all along the VNCs (CNS is indicated by light green). In this model, *Djhh* gene products would be transported posteriorly via VNCs (pink arrows), leading to the induction of posterior specification at the posterior end.
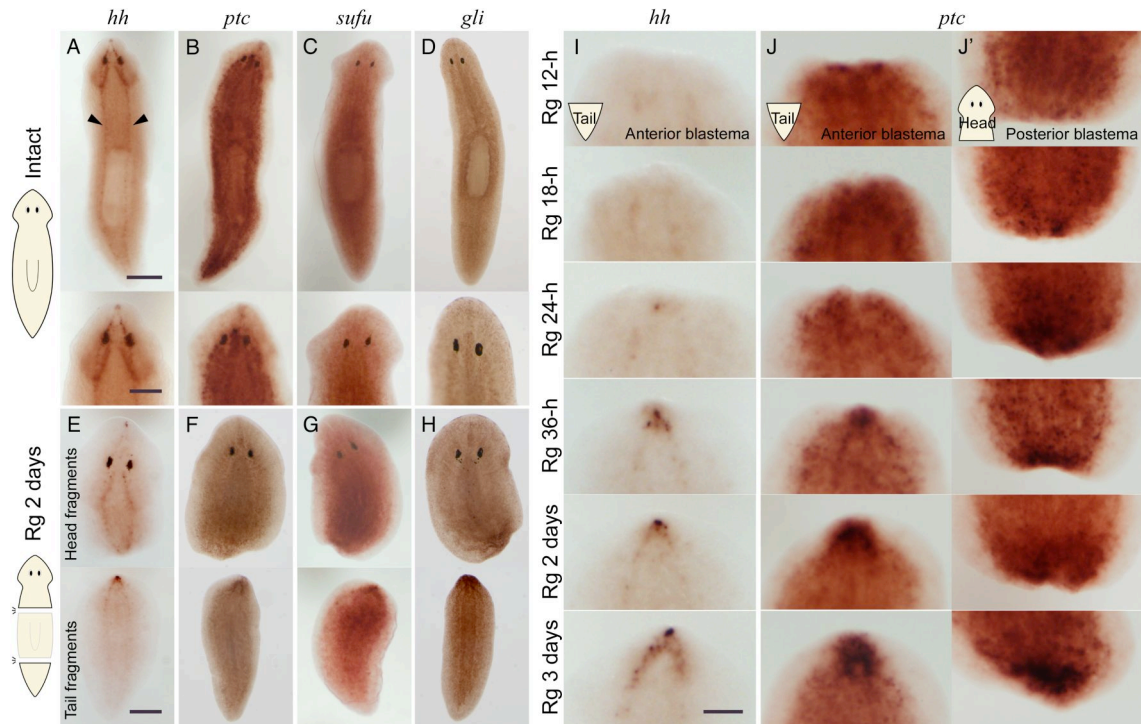
Supplemental Figures

Fig. S1. Expression pattern of Hh signaling component genes in *D. japonica*. (*A-D*) Expression pattern in intact animals (upper), and a magnified view of the same animals' head region (lower). *Djhh* expression was specifically localized at the anterior tip of the head, VNCs (arrowheads), medial border of the brain and eye in intact planarians (*A*), whereas the other Hh signaling components, *Djptc* (*B*), *Djsufu* (*C*) and *Djgli* (*D*), were expressed ubiquitously. Bars, 500 μm (whole) and 300 μm (magnified). (*E-H*) Expression pattern in head and tail regenerants at day-2. *Djhh* expression was induced in the anterior blastema only (*E*), whereas the other genes were induced in both anterior and posterior blastemas (*F-H*). Bar, 300 μm. (*I-J'*) Induction of the expression of *Djhh* (*I*) and *Djptc* (*J* and *J'*) during the course of regeneration. *Djhh* expression was first

detected in the anterior blastema of regenerating tail fragments at least 24 hours after amputation and was strongest in the anterior tip of regenerating. The expression of *Djptc* was induced in both anterior blastema (of tail fragments) and posterior blastema (of head fragments) from the earliest stage examined during regeneration. Bar, 200 μm.
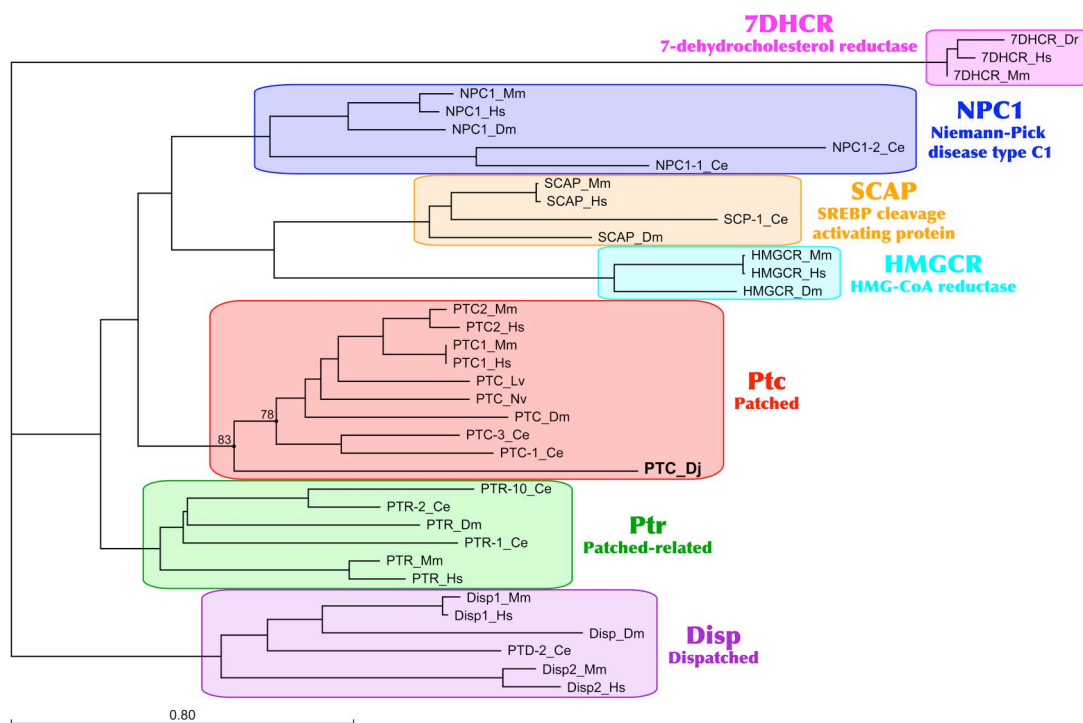

Fig. S2. Phylogenetic tree of SSD-containing proteins. Phylogenetic tree (Neighbor Joining, bootstrap replicas: 100) of conserved sterol-sensing domain (SSD) shows that DjPtc is more closely related to the Patched (Ptc) family (red) than any other proteins. Protein sequences were derived from the GenBank database. 7DHCR, 7-dehydrocholesterol reductase (pink); DISP, Dispatched (purple); HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A-reductase (light blue); NPC1, Niemann-Pick

disease type C1 (blue); PTR, Patched-related (green); SCAP, sterol regulatory element-binding protein (SREBP)-cleavage activating protein (orange). Ce, *Caenorhabditis elegans*; Dj, *Dugesia japonica*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Hs, *Homo sapiens*; Lv, *Lytechinus variegatus*; Mm, *Mus musculus*; Nv, *Nematostella vectensis*.
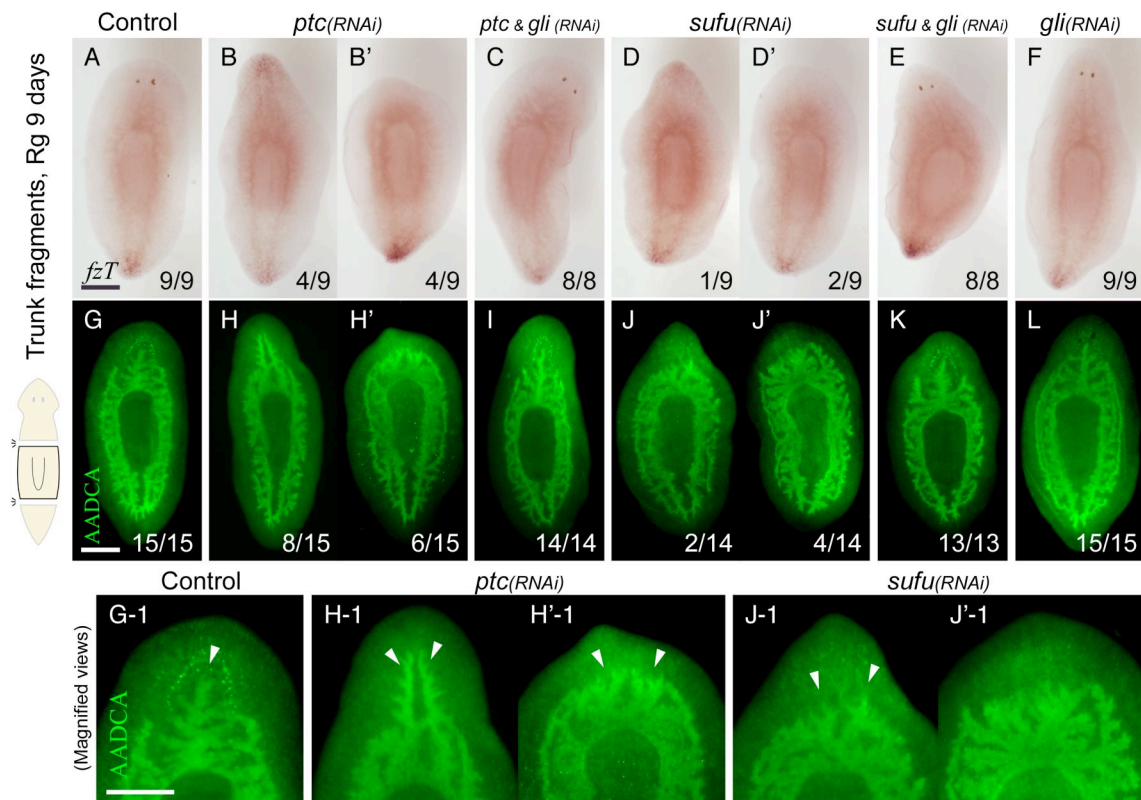

Fig. S3. Phenotypes induced by RNAi of Hh signaling component genes were characterized with respect to head-less morphologies in RNAi trunk regenerants. RNAi of *Djptc* and *Djsufu* caused various phenotypes in the regeneration of the anterior region, ranging from Janus-tails formation, to posteriorization of the anterior region, and loss of the head identity. RNAi trunk fragment regenerants at day-9 were examined for *DjfzT*

expression (*A-F*), followed by immunostaining using anti-DjAADCA antibody to analyze gut-branching morphology (*G-L*). A portion of the anterior region of specimens after immunostaining is magnified in the lower panel (*G-1, H-1, H'-1, J-1* and *J'-1*). *DjfzT* expression was ectopically induced in the anterior blastema of trunk regenerants after *Djptc* (*B*) or *Djsufu* (*D*) RNAi, indicating Janus-tails formation. Actually, these regenerants showed bifurcated branching of the main tract of the gut in the anterior region (*H* and *H-1*). However, *DjfzT* expression was sometimes detected only in the posterior end (without ectopic expression in the anterior blastema) of RNAi regenerants (*B'* and *D'*). They showed a rounded anterior end, and did not generate eyes or induce *Djndk* expression in the anterior region (Fig. 3*N*). Regarding gut morphology, regenerants did show bifurcated branching of the main tract of the gut in the anterior region after *Djptc* (*H'* and *H'-1*) or *Djsufu* (*J* and *J-1*) RNAi, indicating the posteriorization of the anterior region (but not transformation to a tail fate). Furthermore, *Djsufu(RNAi)* regenerants sometimes showed abnormal anterior gut-branching (*J'* and *J'-1*), suggesting an abnormal anterior end, which possessed neither a head (anterior fate) nor a tail (posterior fate), that is, loss of the head identity. These phenotypes would correspond to those of Janus-heads formation and tail-less regeneration in the reported RNAi for *Djhh* and *Djgli* (Fig. 3 *A-D*). Moreover, all of these phenotypes were suppressed by simultaneous *Djgli* RNAi (*C, E, I* and *K*), as indicated by the single main tract of the gut and also the detection of DjAADCA-positive neurons in the cephalic brain. Bars, 300 μm.
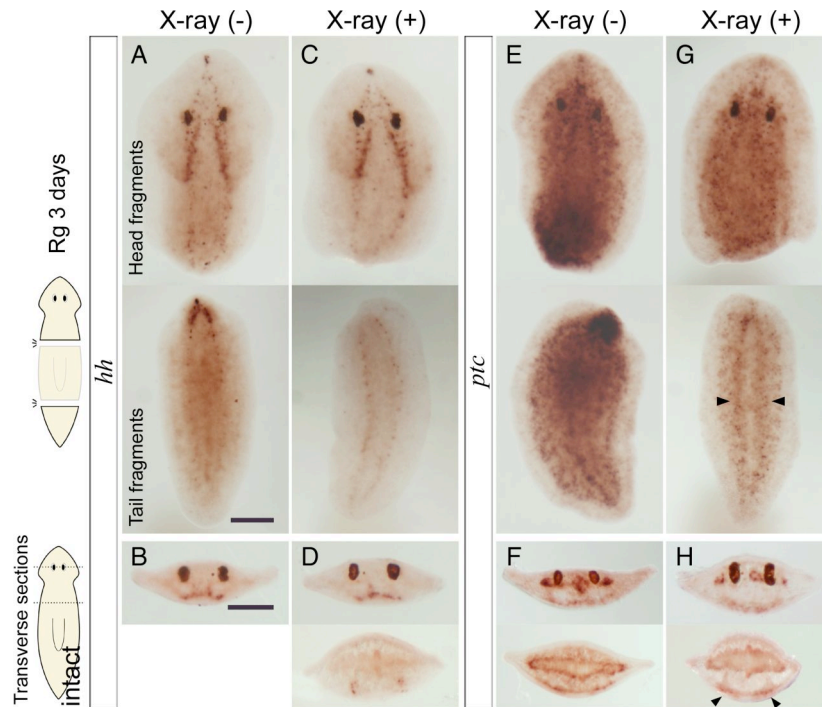
Fig. S4. Gene expression pattern of *Djhh* and *Djptc* in X-ray-irradiated planarians and regenerants. Regenerants from head (upper) and tail (lower) fragments of X-ray-irradiated animals were analyzed for the expression of *Djhh* (*A* and *C*) and *Djptc* (*E* and *G*) 3 days after amputation. Also, intact animals (no amputation) after X-ray irradiation were examined by *in situ* hybridization, followed by transverse sectioning at the head (upper) and pre-pharyngeal (lower) regions as indicated by dotted lines in the cartoon (*B*, *D*, *F* and *H*). *Djhh* expression in the head region and VNCs was not affected by X-ray irradiation (*C* and *D*), indicating its localization in differentiated cells. In contrast, the expression in the anterior blastema of regenerants from tail fragments (tip and brain precursor) was eliminated by X-ray irradiation (*C*), suggesting that these expressing cells were derived from the stem cell population. In contrast, X-ray irradiation not only abolished the upregulation of *Djptc* in both the anterior and

posterior blastemas of regenerants from body fragments after amputation, but also markedly reduced the ubiquitous expression of *Djptc* (*E* and *G*). In X-ray-resistant cells, *Djptc* was predominantly expressed in cells neighboring the VNCs (arrowheads) and gut epithelial cells (*F* and *H*). Taken together, these results indicate that Hh signaling mediated by *Djptc* occurs in both differentiated and undifferentiated cell populations, and suggest that AP polarity is defined by Hh signaling in differentiated cells of the VNCs. Bars, 300 μm.

Table S1. PCR primers used in RT-qPCR analysis

| Gene | Sequence | | Accession No.* |
	Forward	Reverse	
DjG3PDH	accaccaactgtttagctcccttag	gatggtccatcaacagtcttttgc	AB504746
Djndk	tcacaaactccaccgcagtacttt	ggtatggattagcattattgaattgtg	AB071948
DjZicB	gtcgtttctaacgtctaatcaacattctg	cgttctttcattttacattgagattcg	AB231881
DjAbd-Ba	gattatcacgcattgtcagcttatacttc	agctgggaatagaattgtgaggataata	AB049972
DjotxA	ggaacaatttgtccgttctacattagac	tcgtttcaatcttcttatagtcgccata	AB008834
DjwntP-1	agtgacatagtttatttagaagacgttcca	aaatgcgtcctgtagttcccaa	AB504744
Djwnt11-2	gcggaaagattaccatacattggtaaag	caaataacaatgatgcactactaaaggca	AB504745
DjfzT	cgcacaatctaaacagaatgaaactc	tcattgactggaaatgaagaattcg	AB504743
Djptc	gagtaatggctctttggtcaggc	tttaccggtcgctggaatatcc	AB504738
Djsufu	gatgctggcgaaattatgtattcagt	tacagccatatcagaccgacaatga	AB504740
Djgli	tgtcaaaacaaagtcactttcagcaaa	gtaatcccatgcagtatcatatcaacact	AB504741

*Accession number refers to the GenBank database.