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A LIM-homeobox gene is required for differentiation of Wnt-expressing cells at the posterior end of the planarian body

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SUMMARY

Planarians have high regenerative ability, which is dependent on pluripotent adult somatic stem cells called neoblasts. Recently, canonical Wnt/β-catenin signaling was shown to be required for posterior specification, and Hedgehog signaling was shown to control anterior-posterior polarity via activation of the *Djwnt1/P-1* gene at the posterior end of planarians. Thus, various signaling molecules play an important role in planarian stem cell regulation. However, the molecular mechanisms directly involved in stem cell differentiation have remained unclear. Here, we demonstrate that one of the planarian LIM-homeobox genes, *Djislet*, is required for the differentiation of *Djwnt1/P-1*-expressing cells from stem cells at the posterior end. RNA interference (RNAi)-treated planarians of *Djislet* [*Djislet(RNAi)*] show a tail-less phenotype. Thus, we speculated that *Djislet* might be involved in activation of the Wnt signaling pathway in the posterior blastema. When we carefully examined the expression pattern of *Djwnt1/P-1* by quantitative real-time PCR during posterior regeneration, we found two phases of *Djwnt1/P-1* expression: the first phase was detected in the differentiated cells in the old tissue in the early stage of regeneration and then a second phase was observed in the cells derived from stem cells in the posterior blastema. Interestingly, *Djislet* is expressed in stem cell-derived DjPiwiA- and *Djwnt1/P-1*-expressing cells, and *Djislet(RNAi)* only perturbed the second phase. Thus, we propose that *Djislet* might act to trigger the differentiation of cells expressing *Djwnt1/P-1* from stem cells.

KEY WORDS: Planarian, Regeneration, LIM-homeobox transcription factor, Islet, Wnt signaling

INTRODUCTION

LIM-homeobox gene family members play an important role as transcription factors in tissue-specific differentiation and body patterning during development in both vertebrates and invertebrates (Curtiss and Heilig, 1998; Kadrmas et al., 2004). The Islet family of LIM-homeobox transcription factors has been well conserved throughout evolution (Srivastava et al., 2010). In particular, Islet1 (Isl1) protein is the earliest marker for motor neuron differentiation (Karlsson et al., 1990; Ericson et al., 1995; Yamada et al., 1993; Thor and Thomas, 1997; Pfaff et al., 1996). However, Isl1 function is not restricted to developing neuronal structures, as *Isl1* is also required for pituitary precursor cell proliferation (Takuma et al., 1998) and pancreas organogenesis (Ahlgren et al., 1997). Recent studies have demonstrated that Isl1 plays a key role in cardiac development and serves as a marker for pluripotent cardiovascular progenitors in several species, including mouse, rat, and human (Cai et al., 2003; Buckingham et al., 2005; Moretti et al., 2006; Kattman et al., 2006; Sun et al., 2007). Therefore, Isl1 is required for the proliferation, survival and migration of progenitor cells or multipotent stem cells to form various organs.

Planarians are a superb model for studying the mechanisms of stem cell systems and regeneration because of their robust ability to regenerate themselves using pluripotent stem cells, called neoblasts (Baguñà et al., 1989; Agata and Watanebe, 1999; Reddien and Sánchez Alvarado, 2004; Agata et al., 2006). Planarians can regenerate all organs, including the central nervous system, gut and muscle within one week of amputation (Agata et al., 2003; Umesono and Agata, 2009). Previously, several marker genes of neoblasts were identified in the planarian *Dugesia japonica* (Shibata et al., 1999; Salvetti et al., 2000; Ogawa et al., 2002; Orii et al., 2005; Rossi et al., 2007; Yoshida-Kashikawa et al., 2007; Hayashi et al., 2010; Rouhana et al., 2010). However, the detailed mechanisms of how stem cells are regulated during regeneration remain unclear.

Recently, it was reported that canonical Wnt/β-catenin signaling is required for posterior specification and regeneration in planarians. RNA interference (RNAi) of *Smed-βcatenin1* [*Smed-βcatenin1(RNAi)*], which is an activator of Wnt signaling, in the planarian species *Schmidtea mediterranea* leads to Janus-heads (an allusion to the Roman god Janus) formation in the tail region (Gurley et al., 2008; Petersen and Reddien, 2008; Iglesias et al., 2008). Comparing the knockdown of various posterior Wnt family genes, *Smed-wntP-1(RNAi)*, which has the most posterior-specific expression, shows Janus-heads formation similar to *Smed-βcatenin1(RNAi)* (Adell et al., 2009; Petersen and Reddien, 2009). *Smed-wnt11-2(RNAi)*, however, shows a tail formation defect and inappropriate midline patterning after posterior amputation (Gurley et al., 2010). Furthermore, it has been shown that Hedgehog (Hh) signaling is upstream of Wnt/β-catenin signaling and is involved in the establishment of anterior-posterior (AP) polarity (Rink et al., 2009; Yazawa et al., 2009). These studies clearly indicate that

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signaling molecules plays a crucial role, not only in regeneration, but also in cell differentiation. The direct regulatory mechanism involved in neoblast differentiation, however, has remained unclear.

In this study, we focused on a LIM-homeobox transcription factor, *Dugesia japonica islet* (*Djislet*). Interestingly, *Djislet(RNAi)* shows a tail-less phenotype in addition to defects of the nervous system. Here, we describe a new insight into the relationship between Wnt signaling and a LIM homeobox transcription factor in the process of stem cell differentiation, and discuss how planarians coordinate the differentiated cells and stem cells to regulate regeneration.

Recently, unifying the nomenclature of the planarian Wnt family genes was proposed by a group using *S. mediterranea*: *Smed-wntP-1*, *Smed-wnt2-1* and *Smed-wntP-2* were renamed *Smed-wnt1*, *Smed-wnt2* and *Smed-wnt11-5*, respectively (Gurley et al., 2010). However, the nomenclature has not yet been well fixed. We had independently isolated two Wnt family genes from *Dugesia japonica* and named them *DjwntA* and *DjwntB* (Kobayashi et al., 2007). Recently, we also cloned other Wnt family genes from *Dugesia japonica* using GS FLX System (Roche 454). Here, we named or renamed the *D. japonica* Wnt genes according to a combination of new and old nomenclature as follows: *Djwnt1/P-1* (previously *DjwntP-1*), *Djwnt2/B* (previously *DjwntB*, the homolog of *Smed-wnt2*), *Djwnt11-5/P-2* (the homolog of *Smed-wnt11-5*), *Djwnt11-1* (the homolog of *Smed-wnt11-1*) and *Djwnt11-2* (unchanged).

MATERIALS AND METHODS

Animals

A clonal strain, sexualizing special planarian (SSP) (2*n*=16), of the planarian *Dugesia japonica* was used (Ito et al., 2001). Planarians were maintained in an asexual state in autoclaved tap water at 24°C. They were fed chicken liver every 2 weeks. Animals were starved for at least 1 week before experiments. Planarians with a body length of ~8 mm were used in all experiments. For regeneration studies, planarians were cut into three fragments (head, trunk and tail) by transverse amputation anterior and posterior to the pharynx. These fragments were allowed to regenerate and then fixed at specific times.

γ-ray irradiation

Animals were irradiated with 15 gray of γ-rays using a cesium source (Gammacell 40 Exactor, Best Theratronics). Irradiated animals lost almost all regenerating ability and showed significantly decreased expression levels of stem cell marker genes. At least four days after irradiation, animals were amputated for regenerating studies.

cDNA clones

cDNA clones encoding the respective proteins *Djislet* (accession number AB610877), *DjsFRP-B* (AB610880) and *Djα-tubulin* (AB610878) were identified in a previously constructed library of expressed sequence tags (ESTs) (Mineta et al., 2003). Partial cDNA fragments encoding *Djwnt1/P-1* (AB504744), *Djwnt11-5/P-2* (AB610882), *Djwnt11-1* (AB610881) and *Djwnt11-2* (AB504745) were cloned by PCR. The respective PCR primers were designed based on the sequences obtained using a GS FLX System (Roche 454). These cDNA fragments were cloned using the pCR2.1-TOPO vector (Invitrogen).

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled riboprobes (Roche Diagnostics). They were prepared using PCR products from cDNA pBluescript SK(–) vectors containing the inserts from EST clones of interest, or pCR2.1-TOPO vectors newly cloned as described above. Animals were treated with 2% HCl in 5/8 Holtfreter's solution for 5 minutes at 4°C and fixed in 5/8 Holtfreter's solution containing 4% paraformaldehyde and 10% methanol for 30 minutes at room temperature. Hybridization and detection of DIG-labeled RNA probes were carried out as previously described (Umesono et al., 1997; Agata et al., 1998). In addition, all samples were processed with in situ chip (S) (ALOKA) for treatment of a large number of specimens.

Double staining for detection of mRNA expression was performed essentially as described (Yazawa et al., 2009). 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, TSA kit No. 2 (Molecular Probes) and then an HNPP Fluorescent Detection Set (Roche Diagnostics) were used according to the respective manufacturer's instructions. Cell nuclei were stained with Hoechst 33342. Before treating with an HNPP fluorescent detection set, appropriate samples were subjected to immunohistochemistry using anti-DjPiwiA monoclonal antibody (1:1000), anti-DjSYT (1:2000) and anti-α-tubulin Ab-2 (NeoMarkers, 1:200) as previously described (Yoshida-Kashikawa et al., 2007; Tazaki et al., 1999; Cebrià and Newmark, 2005). In brief, specimens were incubated with the primary antibody overnight at 4°C and then with fluorescence-labeled secondary antibodies (Alexa Fluor 488 or 633; Molecular Probes, 1:500). Fluorescence was detected with a confocal laser scanning microscope FV1000 (Olympus).

RNA interference (RNAi)

Double-stranded RNA (dsRNA) was synthesized from in vitro transcription reactions with a MEGAscript RNAi Kit (Ambion), using PCR products with flanking T7 promoters from appropriate cDNA clones. Feeding RNAi was performed according to bacterial-feeding protocols (Reddien et al., 2005; Gurley et al., 2008) and a dsRNA-feeding protocol (Rouhana et al., 2010). Ten animals were each fed 13 μl of dsRNA-food, which was made up of 4 μg of dsRNA, 50% chicken liver paste and 0.2% agarose (Type IX, SIGMA), every 3 days for three feedings. Control animals were fed distilled water (DW; the solvent for dsRNA) instead of dsRNA. When we performed RNAi of Wnt genes (*Djwnt1/P-1*, *Djwnt11-5/P-2*, *Djwnt11-1* and *Djwnt11-2*), dsRNA was injected into planarians essentially as described (Sánchez Alvarado and Newmark, 1999). Control animals were injected with DW. For regeneration studies, planarians were cut 3 days after the last feeding or injection.

Quantitative real-time PCR (qPCR) analysis

In RNAi-regeneration studies, RNAi head fragments (*n*=10) were stump regions of the posterior end at the indicated regeneration time except in the γ-ray irradiation study in which we used whole head fragments (*n*=10). Total RNA was extracted from these stumps or whole regions using ISOGEN-LS (Nippon Gene), and cDNA was synthesized from 500 ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). qPCR analysis of gene expression levels was performed as previously described (Ogawa et al., 2002). The synthesized cDNAs were appropriately diluted (1/60), and then used for gene expression analyses by qPCR. Ten microliters of qPCR mixture including 1× Quantitect SYBR green PCR master mix (Qiagen), 0.3 μmol/l gene-specific primers and 3 μl of diluted cDNA template were analyzed using an ABI PRISM 7900 HT (Applied Biosystems). The reactions were carried out as follows: 50°C for 2 minutes, 95°C for 15 minutes, 50 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute. PCR primers for each target gene are listed in Table S1 in the supplementary material. Measurements were performed in quadruplicate for technical replicates (coefficient of variation was 0.1-2.8% for all primer sets) and were normalized by the expression level of *Djα-tubulin*. The expression levels of target genes were expressed relative to the level in the control, which was taken as 1.0.

Fluorescence-activated cell sorting (FACS)-based single-cell PCR

Dissociation of planarian cells and staining with dyes were performed as previously described (Hayashi et al., 2006). Pieces of the posterior ends from regenerating-head fragments (1 and 3 days after amputation) were collected from 200 fragments and dissociated into single cells. Flow cytometric analyses and collection of single cells for RT-PCR were carried out using a FACS Vantage SE triple-laser cell sorter (Becton Dickinson) as previously described (Hayashi et al., 2006; Hayashi et al., 2010). The PCR primers are listed in Table S2 in the supplementary material.

Statistical evaluation

The quantitative gene expression data from the qPCR analysis were analyzed by one-way analysis of variance (ANOVA) and the statistical significance of differences between test samples was determined by Student's *t*-test. *P* values greater than 0.01 were taken as not significant (NS) by consideration of the fluctuation in *P* values of a housekeeping gene, *DjG3PDH*, expression level of which was regarded as invariant across samples (data not shown). Measurements from quadruplicates were shown as mean ± s.d.

RESULTS

Djislet RNAi causes tail-less regeneration phenotype in planarians

We isolated a clone, encoding a LIM-homeodomain (LIM-HD) protein from the planarian EST library (see Fig. S1 in the supplementary material). The predicted amino acid sequence has two highly conserved LIM domains and a homeodomain (see Fig. S2 in the supplementary material), showing high similarity to the Islet of vertebrates. Thus, we named this gene *Djislet*. To explore the function of *Djislet* in planarians, we investigated expression patterns and the gene knockdown phenotype of *Djislet*. *Djislet* was expressed in the central nervous system (CNS), including the cephalic brain and ventral nerve cords (VNCs), and the marginal zone of the pharynx in intact animals. These expression patterns showed resistance to γ-ray irradiation, suggesting that *Djislet* was largely expressed in differentiated cells in intact animals (see Fig. S3 in the supplementary material). In spite of such an expression pattern, *Djislet(RNAi)* showed a tail-less regeneration phenotype in both head and trunk fragment regenerants (Fig. 1B). To analyze the tail-less phenotype, we examined the structure of the posterior region using anti-α-tubulin antibody to visualize the axon bundles of the VNCs and the transverse commissures (Cebrià and Newmark, 2005). Interestingly, *Djislet(RNAi)* showed fusion of the VNCs (which normally separate) at the region posterior to the pharynx, similar to the *Smed-wnt11-2(RNAi)* phenotype previously described (Fig. 1C) (Adell et al., 2009; Gurley et al., 2010). These results suggest that *Djislet* functions in posterior regeneration and in acquisition of posterior structures. Hence, regenerants from the head pieces ('head regenerants') showed clearer phenotypes (tail-less but with formation of a pharynx) than those from trunk and tail pieces, and therefore we used head regenerants in the following experiments (except for Fig. 5E). From these observations, we confirmed the tail-less phenotype at the gene expression level using qPCR. We examined the expression of genes that show different expression patterns along the anterior-posterior axis (anterior-specific genes: *DjsFRP-A*, *ndk*, *Djwnt2/B* and *DjsFRP-B*; a pharyngeal region-specific gene: *DjFoxA*; posterior-specific planarian Hox genes: *DjAbd-Ba*, *plox4-Dj* and *plox5-Dj*) in the posterior region of the head regenerants (Orii et al., 1999; Koinuma et al., 2000; Nogi and Watanabe, 2001; Cebrià et al., 2002; Kobayashi et al., 2007). The expression of the posterior genes was remarkably reduced, although neither the anterior genes nor *DjFoxA* was affected (Fig. 1D).

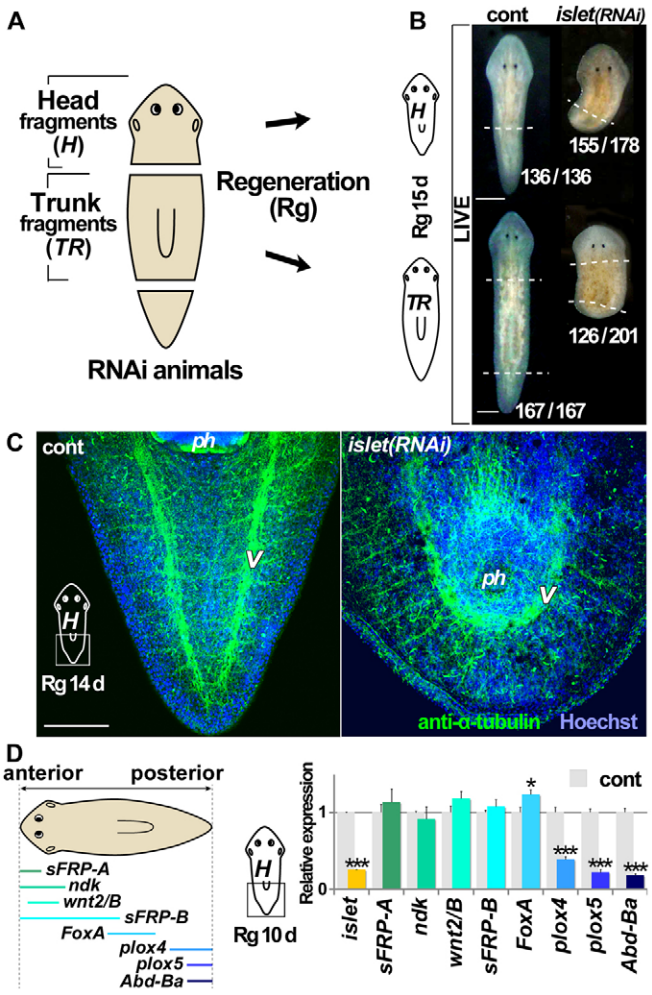

Fig. 1. Gene knockdown of planarian *islet* (*Djislet*) causes tail-less phenotype. (**A**) Schematic of the RNAi-regeneration analysis. Animals treated with RNAi were cut transversely into three fragments: head (H), trunk (TR) and tail. (**B**) Analysis of the *Djislet(RNAi)* phenotype at 15 days after amputation. Animals at the top are head regenerants and those at the bottom are trunk regenerants. Numbers represent the fraction of tested regenerants showing the indicated phenotype. Dashed lines indicate the amputation sites. (**C**) Whole-mount immunostaining with anti-α-tubulin at the tail region in the head regenerants at 14 days. The nuclear staining is shown by Hoechst (blue). Boxed area of the schematic in the left panel indicates the site for confocal imaging. ph, pharynx; v, ventral nerve cord. Scale bars: 200 μm. (**D**) Relative gene expression analysis of *Djislet(RNAi)* animals by qPCR in the posterior region (the boxed area in the diagram) of the head regenerants at 10 days. On the left is a schematic of the expression sites of the genes along the anterior-posterior axis, which are indicated as horizontal bars for each gene. On the right, the expression levels of target genes are shown relative to the level in control head regenerants (taken as 1.0). **P*<0.01, ****P*<0.0001 versus control (gray bars).

The tail-less phenotype caused by *Djislet(RNAi)* is related to *Djwnt1/P-1* function

Because *Djislet(RNAi)* showed a tail-less phenotype, we speculated that this phenotype might be related to Wnt signaling. In *S. mediterranea*, *Smed-wnt1* was expressed most posteriorly among the posterior Wnt genes, and only *Smed-wnt1(RNAi)* animals showed Janus-heads formation in addition to the tail-less phenotype (Adell et al., 2009; Petersen and Reddien, 2009). Thus, *Smed-wnt1* is speculated to be a candidate for the most upstream gene for posteriorization among posterior Wnt family genes. Interestingly, we observed that *Djislet* and *Djwnt1/P-1* were expressed in the dorsal-midline of the posterior blastema at day 3 with the same pattern (Fig. 2A,B, arrowhead). Furthermore, the expression of *Djwnt1/P-1* in the dorsal-midline was eliminated after *Djislet(RNAi)* (Fig. 2B, center). By contrast, *Djwnt1/P-1(RNAi)* animals showed overexpression of *Djislet* in

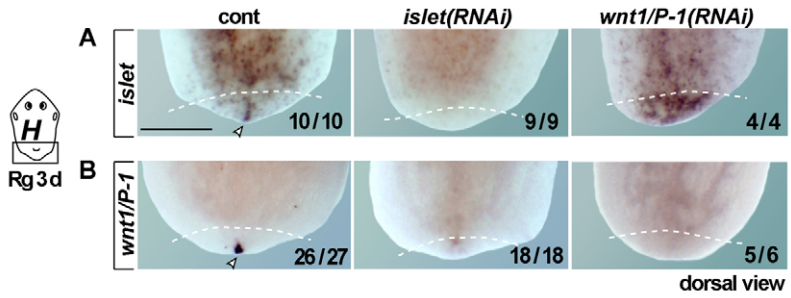

Fig. 2. *Djislet(RNAi)* caused a remarkable reduction of the expression of *Djwnt1/P-1*. (**A**,**B**) The expression patterns of *Djislet* (A) and *Djwnt1/P-1* (B) in the posterior blastema (the boxed area in the diagram) of the head regenerants (H) at day 3 after *Djislet* RNAi and *Djwnt1/P-1* RNAi by whole-mount in situ hybridization (dorsal view). Arrowheads indicate the specific expression in the dorsal midline of the posterior blastema. Note that *Djislet(RNAi)* animals showed the formation of a posterior blastema. Dashed lines indicate the amputation site. Numbers represent the fraction of tested regenerants showing the indicated phenotype. Scale bar: 200 μm.

the posterior blastema (Fig. 2A, right). This overexpression seemed to be a secondary effect, caused by anteriorization after *Djwnt1/P-1(RNAi)*. Thus, the tail-less phenotype caused by *Djislet(RNAi)* might be related to the ablation of *Djwnt1/P-1* expression in the posterior blastema.

The tail-less phenotype caused by *Djislet(RNAi)* is different from the *Djwnt1/P-1(RNAi)* phenotype at a later stage

We did, however, find a difference between the *Djislet(RNAi)* and *Djwnt1/P-1(RNAi)* phenotypes. Although both phenotypes showed an inhibition of posterior regeneration, at a later stage of posterior regeneration, *Djwnt1/P-1(RNAi)* showed Janus-heads formation in addition to some tail-less regenerants (Fig. 3A). By contrast, *Djislet(RNAi)* did not show Janus-heads formation at all (Fig. 1B, Fig. 3A). Staining of the regenerants for expression of *Dj frizzled-T* (*DjfzT*), a posterior-specific Wnt receptor gene (Yazawa et al., 2009), showed that there was a dramatic reduction of the tissue expressing *DjfzT* in both *Djislet(RNAi)* and *Djwnt1/P-1(RNAi)* (Fig. 3B). This implies that both RNAi phenotypes showed a defect in the tail region. The *Djislet(RNAi)* animals, however, always formed a pharynx, like the control, whereas the *Djwnt1/P-1(RNAi)* animals did not (Fig. 3B′). Therefore, we decided to classify the two tail-less phenotypes caused by *Djwnt1/P-1(RNAi)* and *Djislet(RNAi)*, as pharynx/tail-less (P/T-less) and tail-less (T-less) phenotype, respectively. To address what caused this difference, we examined the expression pattern of *Djwnt1/P-1* in the early stages of regeneration. The expression pattern of *Djwnt1/P-1* at day 1 showed a different modality compared with that at day 3. In contrast to day 3, the head fragments displayed a dot-like pattern on the ventral side of the posterior end at day 1, as previously reported in *S. mediterranea* (Fig. 3C) (Petersen and Reddien, 2009; Gurley et al., 2010). Interestingly, this dot-like expression pattern was not affected by *Djislet(RNAi)* (Fig. 3C). These results suggest that the difference between *Djislet(RNAi)* and *Djwnt1/P-1(RNAi)* phenotype was determined by whether or not *Djwnt1/P-1* was expressed in the early stages of regeneration. Thus, we propose that the phenotypes of Janus-heads and P/T-less were caused by ablation of the *Djwnt1/P-1* dot-like expression in the early stage of regeneration, and that the *Djislet(RNAi)* T-less phenotype was caused by ablation of the subsequent concentrated expression pattern of *Djwnt1/P-1*.

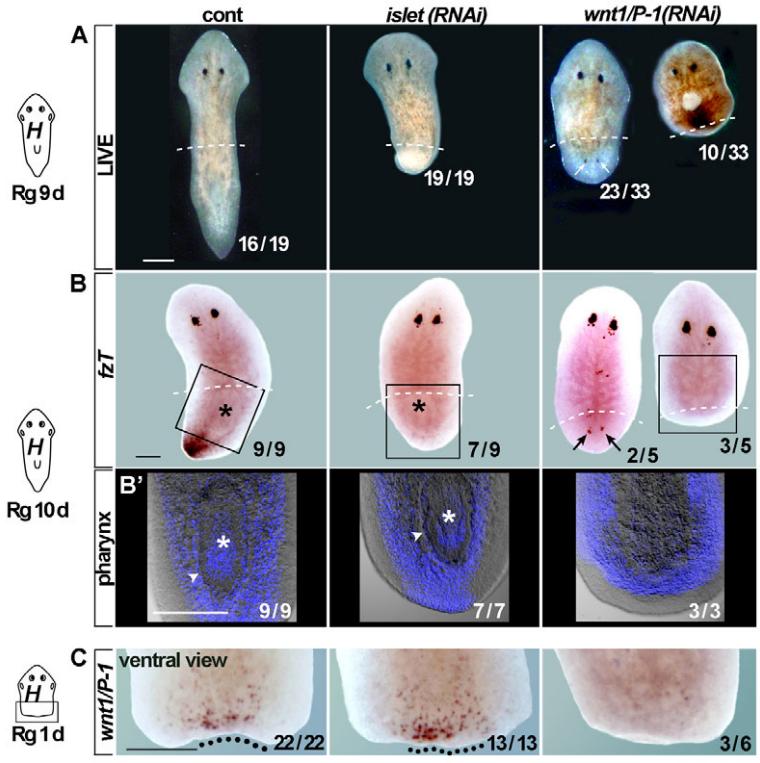

Fig. 3. *Djislet(RNAi)* phenotype is different from *Djwnt1/P-1(RNAi)*. (**A**) Live images of *Djislet(RNAi)* and *Djwnt1/P-1(RNAi)* animals obtained from the head regenerants (H) at day 9. (**B**) Expression pattern of *DjfzT* (*fzT*), a posterior-specific marker gene, in the head regenerants at day 10 by whole-mount in situ hybridization (dorsal view). (**B′**) Confocal images of boxed areas in B. Nuclei are stained with Hoechst (blue). Asterisks indicate the newly developed pharynx. Note that the pharynxes of the control and *Djislet(RNAi)* animals are shown by the elliptical borderline (arrowhead) and the Hoechst signal of these central regions (left/center). *Djwnt1/P-1(RNAi)* animals did not show this region (right). (**C**) The expression pattern of *Djwnt1/P-1* in the posterior end of head regenerants (indicated in the diagram) at day 1 (ventral view). Dotted line indicates the dot-like pattern of *Djwnt1/P-1* expression in the stump region of the ventral side. Dashed lines indicate the amputation site. Arrows indicate ectopic eyes. Numbers represent the fraction of tested regenerants showing the indicated phenotype. Scale bars: 200 μm.

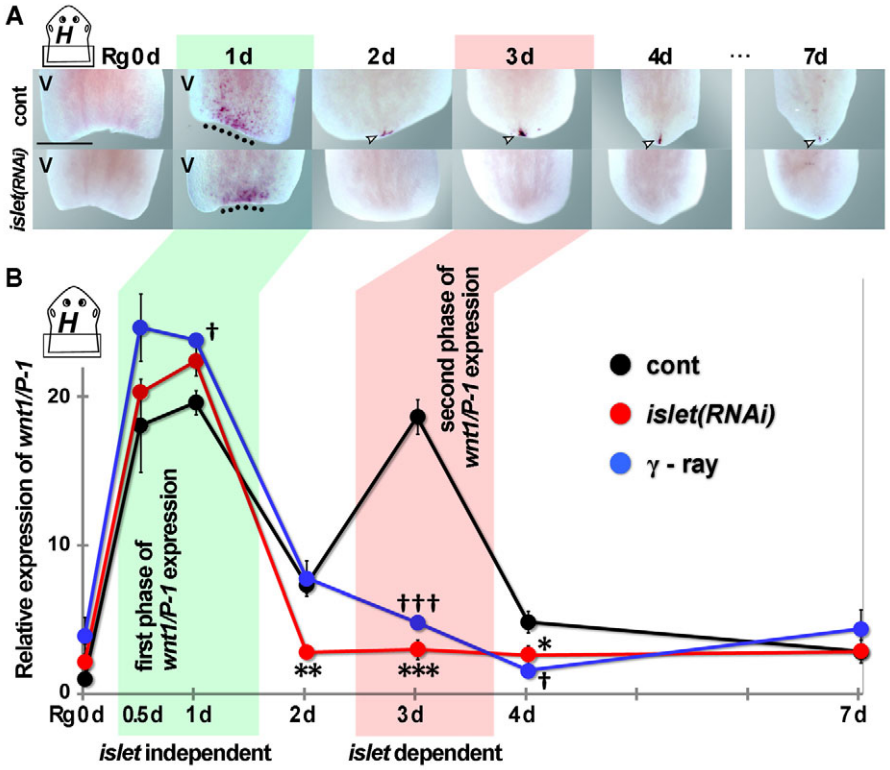

Fig. 4. *Djislet* is required for the second phase of *wnt1/P-1* expression.
(**A**) Expression patterns of *Djwnt1/P-1* during posterior regeneration in the head fragment (indicated in the diagram) in *Djislet(RNAi)*. Dotted lines indicate the dot-like pattern of *Djwnt1/P-1* expression on the ventral side of the posterior end. Arrowheads indicate the concentrated pattern in the dorsal midline of the posterior blastema. V, ventral view; other panels show dorsal view. Scale bar: 200 μm. (**B**) Relative expression levels of *Djwnt1/P-1* in the posterior end (indicated in the diagram) during posterior regeneration after treatment with *Djislet(RNAi)* or γ-ray irradiation. Expression levels are shown relative to the level in the posterior end of the control at day 0 (taken as 1.0). **P*<0.01, ***P*<0.001, ****P*<0.0001, *Djislet(RNAi)* vs control; †*P*<0.01, †††*P*<0.0001: γ-ray vs control.

Djislet is required for the second phase of *Djwnt1/P-1* expression

Previous studies demonstrated that the expression of *Smed-wnt1* has two phases: an γ-ray-insensitive wound-induced expression (the dot-like pattern) phase and an γ-ray-sensitive expression (the concentrated pattern) phase in *S. mediterranea* (Petersen and Reddien, 2009; Gurley et al., 2010). Therefore, we examined in detail the gene expression patterns and expression levels of *Djwnt1/P-1* in *Djislet(RNAi)* animals during posterior regeneration by in situ hybridization and qPCR analysis (Fig. 4). We observed two phases of *Djwnt1/P-1* expression conforming closely to the expression patterns of *Smed-wnt1* seen by in situ hybridization (Fig. 4A). Moreover, the concentrated pattern was remarkably reduced by *Djislet(RNAi)*, whereas the dot-like expression pattern was not affected (Fig. 4A).

Next, we validated these results quantitatively by qPCR analysis, because previous studies did not quantitatively analyze the *wnt1/P-1* expression levels during posterior regeneration. We found that the expression level of *Djwnt1/P-1* showed two clear waves: the first at day 1 and the second at day 3 (Fig. 4B, black line). These waves were designated the first phase of *wnt1/P-1* expression and the second phase of *wnt1/P-1* expression, respectively (Fig. 4, green and pink band). To investigate what cell types contribute to these expression patterns, we identified cell types by γ-ray sensitivity. In planarians, stem cells can be eliminated within at least 4 days of γ-ray irradiation (Shibata et al., 1999; Hayashi et al., 2006). We also investigated the expression levels of *Djwnt1/P-1* in *Djislet(RNAi)* animals and γ-ray-irradiated animals. Both γ-ray-irradiated and *Djislet(RNAi)* animals clearly showed ablation only of the second phase of *wnt1/P-1* expression, but not the first phase, in agreement with in situ hybridization analysis (Fig. 4B, blue and red lines). Consequently, we speculated that these two phases of *wnt1/P-1* expression can be classified into *islet*-independent and *islet*-dependent phases, respectively.

Djislet functions in the stem cell-derived cells expressing *Djwnt1/P-1*

Because *Djislet* regulates the second (γ-ray sensitive) phase of *wnt1/P-1* expression, we speculate that *Djislet* functions in the stem cell-derived cells. To address this matter, we examined the expression pattern of *Djislet* in both the first phase (day 1) and second phase (day 3), with and without γ-ray irradiation (Fig. 5A-D). *Djislet* expression showed a similar pattern to *Djwnt1/P-1* expression (dot-like pattern) in control animals at day 1 (Fig. 5A,B, dotted lines) as well as the dorsal-midline pattern at day 3 (Fig. 2, Fig. 5A,B, arrowheads). However, in γ-ray-irradiated animals, the expression of *Djislet* in the posterior end of regenerants was completely eliminated, in contrast to the first phase of *wnt1/P-1* expression (Fig. 5C,D). We thus confirmed that *Djislet*-expressing cells in the posterior region are γ-ray-sensitive cells.

Next, we investigated the co-expression of *Djislet* and *Djwnt1/P-1* using dual fluorescence in situ hybridization and confocal imaging (Fig. 5E). In the head regenerants, as expected, we could see a few cells that co-expressed both genes at the tip of the posterior blastema on the dorsal side at day 3, although we did not find cells that co-expressed both *Djwnt1/P-1* and *Djislet* on the ventral side at day 1 (see Fig. S6 in the supplementary material). Then we performed dual in situ hybridization combined with DjPiwiA immunostaining, because DjPiwiA is known to be a stem cell and stem cell-derived cell marker (Fig. 5E) (Yoshida-Kashikawa et al., 2007). We found a few triple-positive cells (*Djwnt1/P-1*, *Djislet* and DjPiwiA protein) in the proximal region of the posterior blastema of the trunk regenerants at day 3 (Fig. 5E,E′, arrowhead). Because the expression levels of DjPiwiA protein in the triple-positive cells were relatively weaker than those of undifferentiated stem cells (Fig. 5E, anti-DjPiwiA, upper side of panel), these triple-positive cells were likely to be stem cell-derived cells (see Discussion). Moreover, the expression level of DjPiwiA protein gradually decreased from the triple-positive cells towards

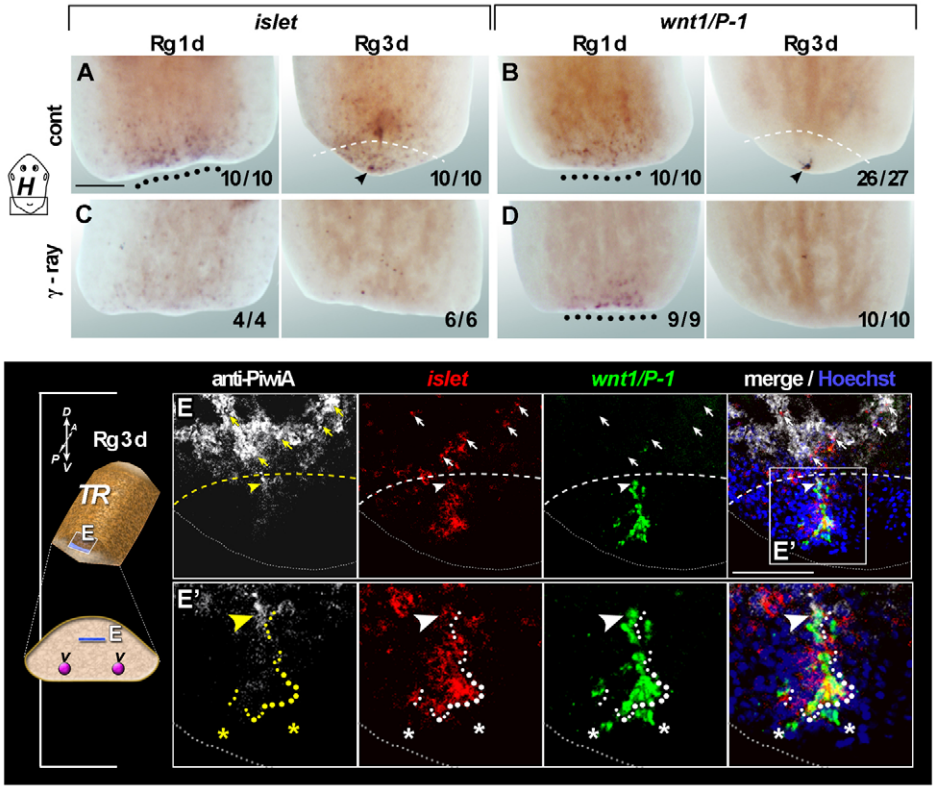

Fig. 5. *Djislet* is co-expressed with *Djwnt1/P-1* in stem cell-derived cells. (**A-D**) The expression pattern of *Djislet* and *Djwnt1/P-1* in the posterior end (indicated in the diagram) in γ-ray irradiated animals (C,D) and non-irradiated control animals (A,B). Dotted lines indicate the dot-like expression pattern in the stump region. Black arrowheads indicate the concentrated pattern in the dorsal midline of the posterior blastema. Numbers represent the fraction of tested regenerants showing the indicated phenotype. (**E**,**E′**) Confocal imaging shows the expression pattern of *Djislet* (red) and *Djwnt1/P-1* (green) by dual in situ hybridization and combined with immunostaining for DjPiwiA protein (white) in the posterior end of the trunk regenerants. The nuclear staining is shown by Hoechst (blue). Images show a dorsal layer at the tip of the posterior blastema at day 3 after amputation (shown in the illustration on the left). E′ shows magnified views of boxed area in E. White or yellow arrowheads indicate the triple-positive cells (*Djislet*, *Djwnt1/P-1* and DjPiwiA). Arrows indicate *Djislet* and DjPiwiA double-positive cells. Dotted lines indicate *Djislet* and *Djwnt1/P-1* double-positive cells. Asterisks indicate *Djwnt1/P-1* single-positive cells. The right-hand panels are merged images. v, ventral nerve cord. Dashed lines indicate the amputation site. Scale bars: 100 μm.

the tip of the posterior blastema, along with *Djislet* and *Djwnt1/P-1* double-positive cells (Fig. 5E′, dotted lines). The posterior-most *Djwnt1/P-1*-expressing cells did not express *Djislet* (Fig. 5E′, asterisks). Given the gradually decreasing expression of DjPiwiA protein, the posterior-most cells might be terminally differentiated cells. The overlapping expression of *Djislet* and *Djwnt1/P-1* raises the possibility that *Djislet* functions to regulate only the second phase of *Djwnt1/P-1* expression, which is activated in stem cell-derived cells in the posterior end of the regenerants.

Djislet regulates the posterior genes in the γ-ray-sensitive posterior blastema cells

To understand the relationship between *Djislet* and other posterior-specific Wnt and Hox genes, we examined the effect of *Djislet(RNAi)* on the expression of posterior genes. In contrast to the significant reduction of *Djwnt1/P-1* expression in *Djislet(RNAi)* animals from day 2 (Fig. 4), no major difference was observed in the expression of *Djwnt11-1*, *Djwnt11-2* or *DjAbd-Ba* between control and *Djislet(RNAi)* animals in the posterior blastema at day 2 (Fig. 6A-C). However, at day 3, the *Djislet(RNAi)* animals had lost the expression of these genes (Fig. 6A-C). By contrast, the expression of *Djwnt11-5/P-2* was not affected by *Djislet(RNAi)* in the posterior blastema (Fig. 6D).

We examined the gene expression level of posterior genes during regeneration in the posterior blastema treated with *Djislet(RNAi)* and γ-ray irradiation (Fig. 6E). The seven posterior genes were categorized into three classes: the class I gene is not directly under the regulation of *Djislet* and is expressed mainly in γ-ray-insensitive cells (*Djwnt11-5/P-2*), class II genes are partly downregulated in *Djislet(RNAi)* and expressed in both γ-ray-sensitive and -insensitive cells (*Djwnt11-1*, *DjfzT*, *plox4-Dj* and *plox5-Dj*), class III genes are strongly downregulated in *Djislet(RNAi)* and expressed mainly in γ-ray-sensitive cells (*Djwnt11-2* and *DjAbd-Ba*). The genes of classes II and III showed no difference in expression level between control and *Djislet(RNAi)* animals until day 2 [Fig. 6E, no significant change in *Djislet(RNAi)* vs control], but then showed clearly decreased expression by *Djislet(RNAi)* at day 3 [Fig. 6E, #: *Djislet(RNAi)* at day 3 vs *Djislet(RNAi)* at day 2]. Moreover, upon irradiation, the expression of genes in class III was barely detected (Fig. 6E, no significant change in γ-ray vs γ-ray at day 0) and genes in class II showed increased but still lower expression levels compared with control (Fig. 6E, †: γ-ray vs γ-ray at day 0, *: γ-ray vs control). A previous study reported that expression of both *Smed-wnt11-1* and *Smed-wnt11-2* is absent in irradiated head regenerants (Gurley et al., 2010). The difference in results of

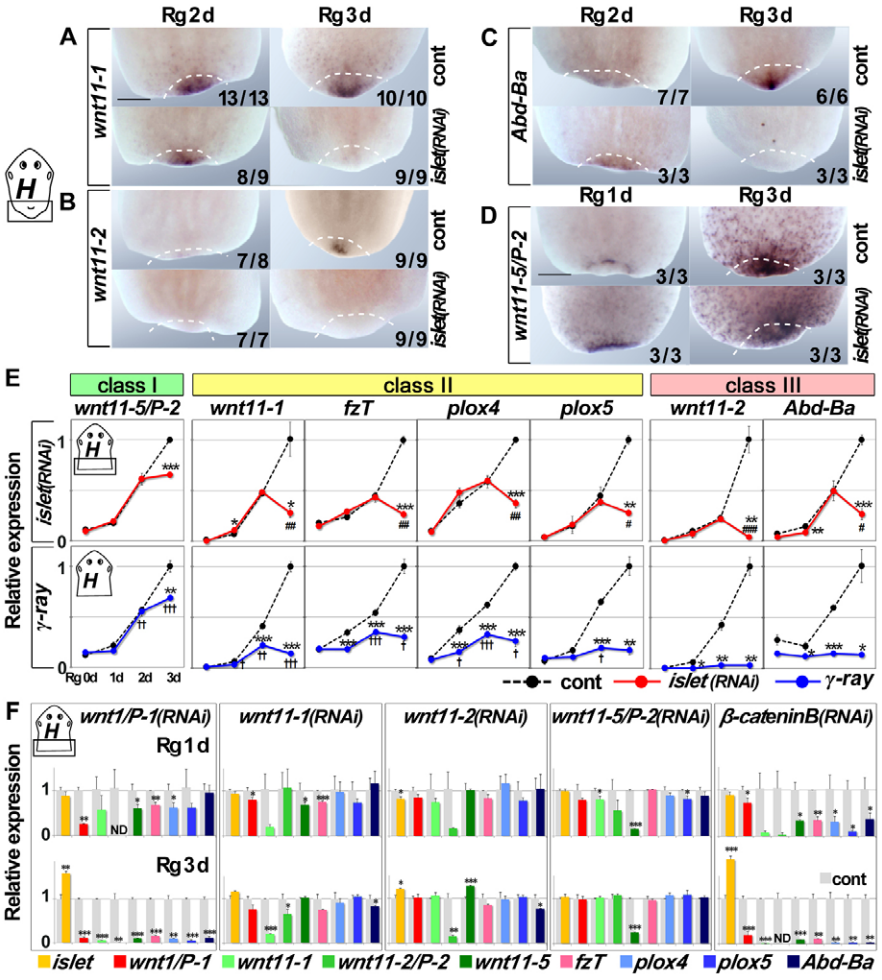

Fig. 6. *Djislet* maintains posteriorization in the posterior blastema. (**A-D**) Expression patterns of *Djwnt11-1* (A), *Djwnt11-2* (B), *DjAbd-Ba* (C) and *Djwnt11-5/P-2* (D) in the posterior blastema (indicated in the diagram) after *Djislet* RNAi. All images are dorsal views. Dashed lines indicate the amputation site. Numbers represent the fraction of tested regenerants showing the indicated phenotype. Scale bars: 200 μm. (**E**) Relative gene expression levels during regeneration measured by qPCR in the posterior blastema after *Djislet* RNAi (upper panels), and in whole regenerants after γ-ray irradiation (lower panels). Gene expression levels are expressed relative to levels in control at day 3 after amputation (taken as 1.0). Three types of gene groups were observed: class I (*Djwnt11-5/P-2*), class II (*Djwnt11-1*, *DjfzT*, *plox4-Dj* and *plox5-Dj*) and class III (*Djwnt11-2* and *DjAbd-Ba*). **P*<0.01, ***P*<0.001, ****P*<0.0001, vs control; $^{\#}$*P*<0.01, $^{\#\#}$*P*<0.001, $^{\#\#\#}$*P*<0.0001, *Djislet(RNAi)* at day 3 vs *Djislet(RNAi)* at day 20; †*P*<0.01, ††*P*<0.001, †††*P*<0.0001, γ-ray vs γ-ray at day 0. (**F**) Relative gene expression analysis by qPCR in the posterior blastema treated with RNAi of four posterior-specific Wnt genes (*Djwnt1/P-1*, *Djwnt11-1*, *Djwnt11-2* and *Djwnt11-5/P-2*) and *Djβ-cateninB* at day 1 (upper) and at day 3 (lower) after amputation. Note that the expression level of *Djislet* was not reduced in the knockdown animals of any of the genes, and was rather increased by anteriorization of their posterior end in *Djwnt1/P-1(RNAi)* or *Djβ-cateninB(RNAi)* at day 3. The expression levels were normalized and are shown relative to the level in the posterior end of the control (taken as 1.0). ND, not detected. **P*<0.01, ***P*<0.001, ****P*<0.0001, vs control (gray bars).

wnt11-1 expression between that study and our own might be caused by the difference of the sensitivity of gene expression between qPCR and in situ hybridization analysis. By contrast, the expression levels of *Djwnt11-5/P-2* (class I) remained unchanged from the control until day 2 [Fig. 6E, no significant change in *Djislet(RNAi)* or γ-ray vs control], and showed no significant change or a slight increase from day 2 to day 3 in *Djislet(RNAi)* and irradiated animals (Fig. 6E). This finding in irradiated animals was in close accord with the reported features of *Smed-wnt11-5* (Petersen and Reddien, 2009; Gurley et al., 2010). These results suggest that *Djislet* is required for the maintenance and upregulation of the posterior genes expressed in the γ-ray-sensitive posterior blastema cells.

Djwnt1/P-1 signal is upstream of the posterior Wnt genes

To investigate epistasis in these four posterior Wnt genes (see Fig. S4 in the supplementary material), we examined their gene expression levels by qPCR in the posterior blastema in animals with RNAi for each Wnt gene and *Djβ-cateninB* (Fig. 6F). *Djwnt1/P-1(RNAi)* animals exhibited a decrease in the expression levels of the other Wnt and posterior genes at day 1 and day 3, as did *Djβ-cateninB(RNAi)* animals (Fig. 6F). Moreover, *Djwnt1/P-1(RNAi)* did not show an increase in the expression of the anterior genes at day 1, although it showed a slight decrease in the expression of the posterior genes (Fig. 6F; see Fig. S9 in the supplementary material) and this decrease was not influenced by anteriorization. In addition, *Djβ-cateninB(RNAi)* had little effect on the expression of *Djwnt1/P-1* at day 1, as previously described (Petersen and Reddien, 2009; Rink et al., 2009). This result showed that, at least in the first phase, *Djwnt1/P-1* is expressed independently of the β-catenin pathway, in contrast to the other Wnt and posterior genes. By contrast, RNAi knockdown of the Wnt genes *Djwnt11-1*, *Djwnt11-2* and *Djwnt11-5/P-2* did not affect the expression levels of the other Wnt and posterior genes (Fig. 6F). Collectively, these results suggest the possibility that *Djwnt1/P-1* signal is upstream of the β-catenin pathway and activates the expression of the other Wnt and posterior genes via the β-catenin pathway.

DISCUSSION

Islet functions in the Wnt-expressing cells of planarian

The relationship between LIM-homeobox transcription factors and Wnt signaling has been reported in the developmental processes of several organisms (Riddle et al., 1995; Adams et al., 2000; Matsunaga et al., 2002; O'Hara et al., 2005). Furthermore, β-catenin is involved in *Isl1* expression in cardiac progenitors in the mouse embryo (Lin et al., 2007; Kwon et al., 2009). However, the Islet family homologs have not been reported to regulate Wnt

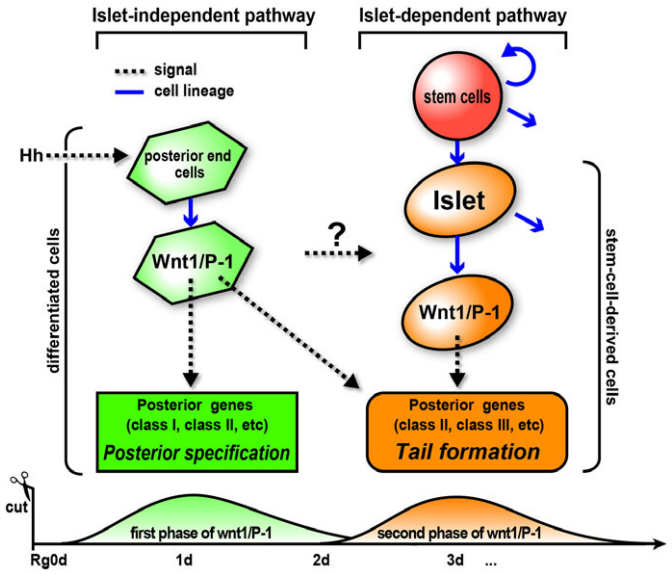

Fig. 7. Putative model of the sequential specification of posteriorization in planarians. The signal cascade model for posterior regeneration in planarians inferred from the present study. The cell lineage pathway of posterior regeneration can be divided into two phases: the *islet*-independent first phase of *wnt1/P-1* expression and the *islet*-dependent second phase of *wnt1/P-1* expression.

signaling. In this study, we demonstrated for the first time that planarian *islet* regulates Wnt signaling, which is required for posterior regeneration.

During posterior regeneration in planarians, *Djislet*-expressing cells appeared specifically in the posterior end (Figs 2, 5; see Fig. S3 in the supplementary material). These cells were γ-ray sensitive (Fig. 5A,C) and located close to cell populations that strongly expressed PiwiA mRNA and/or protein (Fig. 5E, arrows; see Fig. S5 in the supplementary material). These data suggest that *Djislet* might be involved in differentiation of stem cells. Moreover, our results also showed that triple-positive cells (for *Djislet*, *Djwnt1/P-1* and DjPiwiA protein) were observed in the posterior blastema (Fig. 5E′). In previous studies, PiwiA protein has been found to be expressed, not only in stem cells which are expressing *piwiA* mRNA, but also in post-mitotic progeny: the stem cell-derived cells (Guo et al., 2006; Yoshida-Kashikawa et al., 2007; Scimone et al., 2010). The blastema cells are almost all post-mitotic cells, as indicated by the absence of mitotic cell markers (Salvetti et al., 2000; Eisenhoffer et al., 2008; Tasaki et al., 2011a; Tasaki et al., 2011b). Therefore, the DjPiwiA protein-expressing cells in the blastema are most likely to be stem cell-derived cells. For this reason, it is thought that the triple-positive cells (*Djislet*, *Djwnt1/P-1* and DjPiwiA) are true stem cell-derived cells (Fig. 5E′). In the mouse embryo, previous studies have demonstrated that Islet1 is required for proliferation, survival, migration and differentiation of multipotent cardiac progenitor cells involved in forming the heart (Cai et al., 2003; Buckingham et al., 2005; Kwon et al., 2009). Therefore, we speculate that *Djislet* plays an important role in regulating the stem cell differentiation of the *Djwnt1/P-1*-expressing cell lineage. The fact that *Djislet*-expressing cells are found in the posterior blastema in addition to the *Djwnt1/P-1*-expressing cells suggests that *Djislet* also has other function(s), such as a role in neural cell differentiation (see Fig. S5G in the supplementary material).

Only the second phase of *wnt1/P-1* expression is affected by *Djislet*

Djwnt1/P-1-expression has two phases, the first phase and the second phase, during posterior regeneration. These two phases of *wnt1/P-1*-expression have been described previously (Petersen and Reddien, 2009; Gurley et al., 2010). However, the detailed mechanism of the regulation of posterior regeneration via two phases of *wnt1/P-1* expression has not been elucidated because the separation of these two phases has been difficult. However, we have succeeded in segregating these two phases using *Djislet(RNAi)*. We found that *Djislet* is expressed only in the second phase of *wnt1/P-1*-expressing cells (Fig. 5; see Fig. S6 in the supplementary material) and regulates only the second-phase of *wnt1/P-1* expression (Fig. 4). We speculate that the first phase expression in differentiated cells is required for recruiting stem cells into *Djwnt1/P-1*-expressing cells, and then a kind of positive circuit of Wnt-signaling might be established. *Djslet* is required for differentiation of the *Djwnt1/P-1*-expressing cells from the Piwi-positive stem cells. Here, we propose one possible model of the sequential posterior regeneration process divided into two phases based on *Djislet* function (Fig. 7).

The activity of the *islet*-independent first phase of *wnt1/P-1* expression

In the early stage of posterior regeneration (day 1), the first phase of *wnt1/P-1* expression is upregulated in the differentiated cells at the posterior end independently of *Djislet* function (Fig. 4). Recently, it was demonstrated that this early expression of *wnt1/P-1* is under the control of Hh signal activity, and that elimination of *wnt1/P-1* function caused by inhibition of Hh signaling induces anteriorization of the posterior end (Rink et al., 2009; Yazawa et al., 2009).

Interestingly, absence of the first phase of *wnt1/P-1* expression caused anteriorization (Janus-heads and P/T-less phenotype). Moreover, expression of *Djwnt11-5/P-2* occurred in γ-ray-irradiated head regenerants, indicating that posterior fate is determined independently of stem cells (Fig. 6) (Petersen and Reddien, 2009; Gurley et al., 2010). Thus, we propose that the first phase of *wnt1/P-1* is involved in the decision of posterior specification through the activation of the expression of posterior genes (class I, class II and an unidentified factor) via the β-catenin pathway in the differentiated cells (Figs 6, 7). In support of this model, the synergy between *Smed-wnt1* and *Smed-wnt11-5* for the decision of posterior specification has been reported (Petersen and Reddien, 2009). In addition, *Smed-wnt1(RNAi)* animals that were P/T-less had smaller than normal posterior blastemas (Adell et al., 2009). By contrast, *Djislet(RNAi)* animals showed posterior blastema formation with a transient increase in the expression of posterior genes (Fig. 6). We speculate that the first phase of *wnt1/P-1* signaling might be involved in blastema formation accompanying the activation of the posterior genes in stem cell-derived cells (Fig. 7).

The activity of the *islet*-dependent second phase of *wnt1/P-1* expression

In the middle stage of posterior regeneration (day 3), the second phase of *wnt1/P-1* expression emerges in place of the first phase (Fig. 4), i.e. the posteriorization signal from the differentiated cells is inherited by stem cell-derived cells in the tip of the posterior blastema. At present, the mechanism of this inheriting is unclear. Perhaps β-catenin and some additional signal molecules are needed to generate the second phase of *wnt1/P-1*-expressing cells (Fig. 6F) (Petersen and Reddien, 2009). However, our results clearly demonstrate that when the *Djislet* function is absent, the posteriorization signal cannot be maintained in the blastema (Fig.

6). As a result, these animals show a T-less phenotype (Fig. 1). Moreover, *Djwnt11-5/P-2* (class I) seems not to be expressed under control of *Djislet* expression (Fig. 6). *Djwnt11-5/P-2* is expressed mainly in the differentiated cells (Fig. 6E) (Petersen and Reddien, 2009; Gurley et al., 2010), and is also expressed in the pharyngeal region. By contrast, the other posterior genes (class II and III) show tail-specific expression (see Fig. S4 in the supplementary material) (Orii et al., 1999; Nogi and Watanabe, 2001; Yazawa et al., 2009). Hence, we propose that *Djislet* has distinct local effects on the stem cell-derived posterior blastema cells that form the tail structure via tail-specific-posterior genes (class II, III and unidentified factor(s); Figs 6, 7). Furthermore, it is known that *Smed-wnt1*-expressing cells are rapidly turned over in intact animals (Petersen and Reddien, 2009; Gurley et al., 2010). We also found that *Djislet* was co-expressed with *Djwnt1/P-1* in the γ-ray-sensitive cells in intact animals and appeared to regulate *Djwnt1/P-1* expression in these cells (see Fig. S10 in the supplementary material). However, the morphological phenotype does not appear in intact animals after *Djislet* RNAi (see Fig. S11 in the supplementary material). These results raise the possibility that *islet*-dependent *wnt1/P-1* expression is not related to posterior specification in either homeostasis or regeneration. A recent study showed that *Smed-wnt11-2(RNAi)* results in the posterior VNCs converging and fusing at the midline (Adell et al., 2009; Gurley et al., 2010). Our results also indicated that *Djislet(RNAi)* and partial RNAi of *Djwnt1/P-1* animals showed the fusion of VNCs (Fig. 1C; see Fig. S7 in the supplementary material), suggesting that this might be a secondary action of *Djwnt11-2* defects caused by ablation of the second phase of *wnt1/P-1* signaling. Actually, *Djwnt11-2* expression was remarkably downregulated by *Djislet(RNAi)* or *Djwnt1/P-1(RNAi)* (Fig. 6E,F). Thus, it seems that *islet*-dependent *wnt1/P-1* signaling mediates proper tail formation through the function of posterior genes.

Partial RNAi of *Djwnt1/P-1* induced a T-less phenotype similarly to *Djislet(RNAi)* (see Figs S7, S8 in the supplementary material). This result is also consistent with the possibility that the T-less phenotype is a weak anteriorization phenotype, although the animals did not ectopically express anterior-specific genes (Fig. 1D; see Fig. S8 in the supplementary material). Therefore, we have to leave open the alternative possibility that the tail defects caused by *Djislet(RNAi)* might be a secondary consequence of a weak anteriorization of the axis. However, when we carefully quantified the expression level of *Djwnt1/P-1* after partial RNAi of *Djwnt1/P-1* by qPCR, we found that the first phase of *wnt1/P-1* expression in differentiated cells was only slightly reduced (11%, not statistically significant) in the partial RNAi animals, although ~36% reduction ($P<0.001$) of the second-phase expression was observed (see Fig. S7D in the supplementary material). Thus, we suppose that partial RNAi of *Djwnt1/P-1* showed a similar phenotype to T-less of *Djislet(RNAi)* owing to retention of the first phase of *wnt1/P-1* expression, and that the RNAi effect was different between differentiated cells and stem cell-derived cells. To address this issue precisely, it will be necessary to examine the function of *Djislet* in transcriptional control more directly using various genomic and proteomic approaches.

Collectively, our findings demonstrate that posterior regeneration occurs by coordination of the above-mentioned two modes of action of *Djwnt1/P-1* activity. *Djislet* seems to be one of the crucial factors regulating this coordinating mechanism through its control of the differentiation of Djwnt1/P-1-secreting cells from stem cells. We believe that the *Djislet* knockdown model proposed here provides new insights into the mechanism of regeneration and tissue repair by pluripotent stem cells.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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