

# A Pitx transcription factor controls the establishment and maintenance of the serotonergic lineage in planarians

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## ABSTRACT

In contrast to adult vertebrates, which have limited capacities for neurogenesis, adult planarians undergo constitutive cellular turnover during homeostasis and are even able to regenerate a whole brain after decapitation. This enormous plasticity derives from pluripotent stem cells residing in the planarian body in large numbers. It is still obscure how these stem cells are programmed for differentiation into specific cell lineages and how lineage identity is maintained. Here we identify a Pitx transcription factor of crucial importance for planarian regeneration. In addition to patterning defects that are co-dependent on the LIM homeobox transcription factor gene *islet1*, which is expressed with *pitx* at anterior and posterior regeneration poles, RNAi against *pitx* results in *islet1*-independent specific loss of serotonergic (SN) neurons during regeneration. Besides its expression in terminally differentiated SN neurons we found *pitx* in stem cell progeny committed to the SN fate. Also, intact *pitx* RNAi animals gradually lose SN markers, a phenotype that depends neither on increased apoptosis nor on stem cell-based turnover or transdifferentiation into other neurons. We propose that *pitx* is a terminal selector gene for SN neurons in planarians that controls not only their maturation but also their identity by regulating the expression of the Serotonin production and transport machinery. Finally, we made use of this function of *pitx* and compared the transcriptomes of regenerating planarians with and without functional SN neurons, identifying at least three new neuronal targets of Pitx.

**KEY WORDS:** Planaria, *Schmidtea mediterranea*, Regeneration, Serotonergic neuron, Pitx, *Islet1*, Stem cell differentiation

## INTRODUCTION

Transcription factors are key regulators of differentiation processes and the misexpression of a small number of transcription factors can change the fate of a cell completely (reviewed by Sancho-Martinez et al., 2012). The family of paired class homeobox/pituitary homeobox (Pitx) transcription factors has been implicated in a number of differentiation decisions in mammals. For instance, Pitx proteins can act as terminal selectors, which are transcription factors that regulate both the terminal differentiation of a late progenitor cell and the establishment and maintenance of its cell type-specific functions (reviewed by Flames and Hobert, 2011; Hobert, 2011;

Hobert et al., 2010). In the mouse, Pitx2 and Pitx3 are terminal selectors for GABAergic and dopaminergic neurons, respectively (Smidt et al., 2004a; Smidt et al., 2004b; Westmoreland et al., 2001), and the ETS domain factor Pet1 (Fev – Mouse Genome Informatics) controls terminal selection of mouse serotonergic neurons (Alenina et al., 2006; Deneris, 2011; Hendricks et al., 1999; Liu et al., 2010; Smidt et al., 2004a; Smidt et al., 2004b; Westmoreland et al., 2001). Serotonergic and dopaminergic neurons have been implicated in a number of human diseases and conditions such as schizophrenia, depression (Lucki, 1998) and Parkinson's disease (Braak and Del Tredici, 2009).

Regeneration hardly occurs in mammals (Arvidsson et al., 2002; Sofroniew, 2009) and neurodegenerative diseases have been a major focus of medical research. By contrast, planarian flatworms can regenerate any missing body part, including a brain. The basis for this amazing ability are adult stem cells called neoblasts, which make up 20–30% of the total cell population and at least some of which are pluripotent (Wagner et al., 2011).

Positional information during stem cell-based regeneration is given through the localized expression of secreted signaling molecules. For instance, a planarian homolog of the midline repellent Slit is expressed along the body midline and is absent from lateral regions. RNAi against *Schmidtea mediterranea* (*Smed*)-*slit* leads to cyclopia due to a collapse of the midline (Cebrià et al., 2007). In the tail blastema, posterior identity is marked by the expression of *Smed-wnt1* (Adell et al., 2009; Petersen and Reddien, 2009), a Wnt family member whose expression depends on the LIM homeobox transcription factor *islet1* (Hayashi et al., 2011). Both *Smed-wnt1* and *Smed-islet1* RNAi animals lose the ability to regenerate a tail (Adell et al., 2009; Hayashi et al., 2011; Petersen and Reddien, 2009).

At first sight the planarian nervous system appears relatively simple, with two cephalic ganglia connected to two parallel ventral nerve cords (reviewed by Umesono and Agata, 2009). However, it contains a complex network of different neuronal subtypes comparable to the mammalian repertoire (Nishimura et al., 2007a; Nishimura et al., 2007b; Nishimura et al., 2008a; Nishimura et al., 2008b; Nishimura et al., 2010). Learning more about the differentiation processes in planarians could therefore provide valuable insights into the basic mechanisms of disease and might reveal new conserved drug targets. However, it is currently unclear whether a distinct population of adult neural stem cells exists, or whether all neoblasts have a similar potential to regenerate neurons.

Here we identify a planarian Pitx transcription factor, encoded by *Smed-pitx*, as an essential regulator of the formation and maintenance of the serotonergic lineage. Our data suggest that the basic concept of terminal selection also applies to planarians, strengthening their role as a model organism for neuroscience. In addition, our study provides novel downstream targets of Pitx through an RNA-seq approach, and demonstrates an additional, Serotonin-independent role for *Smed-pitx* and *Smed-islet1* in

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regenerative patterning through the modulation of secreted signaling molecules.

## RESULTS

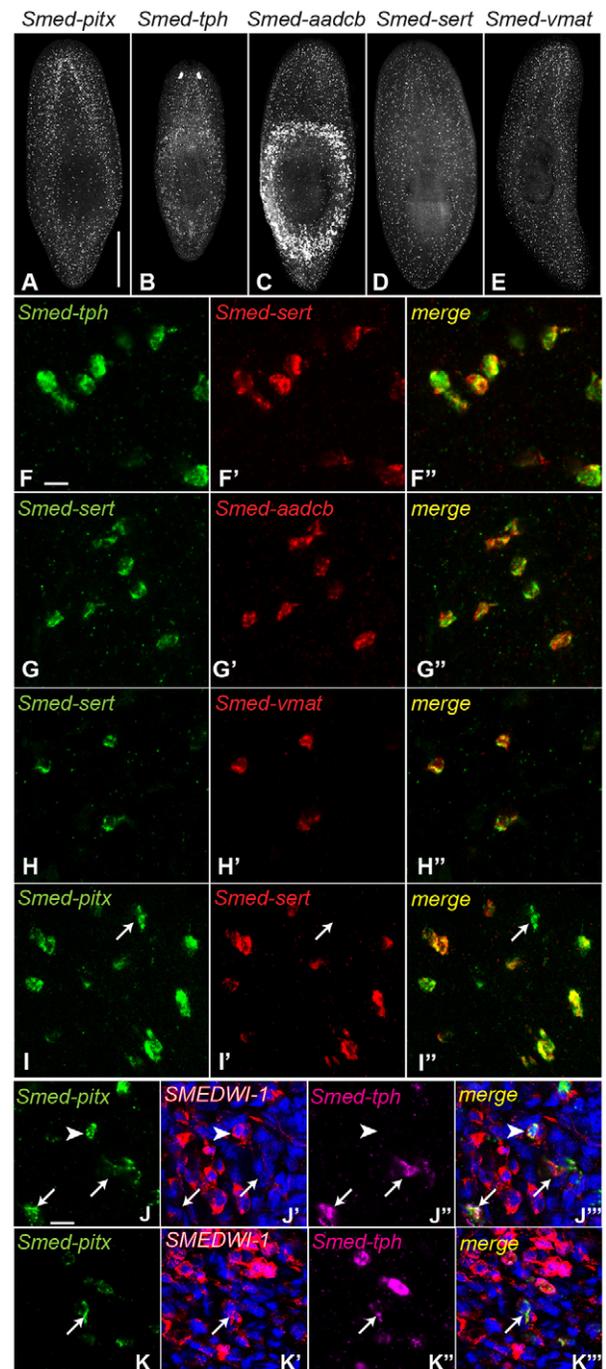
### *Smed-pitx* is expressed in mature serotonergic neurons

In contrast to the existence of three Pitx gene family members (Pitx1-3) in vertebrates (Gage et al., 1999), we found only a single *pitx* gene in the genome (Cantarel et al., 2008) and different transcriptomes of *S. mediterranea* (Abril et al., 2010; Adamidi et al., 2011; Blythe et al., 2010; Sandmann et al., 2011). *In silico* translation of the mRNA sequence and subsequent protein alignment showed that the homeobox is highly conserved between planarian Pitx and the Pitx proteins from other species (supplementary material Fig. S1), indicating that DNA binding is crucial for its function.

We analyzed the expression pattern of *Smed-pitx* in intact animals using whole-mount fluorescent *in situ* hybridization (FISH). *Smed-pitx* is expressed mainly in cells on the ventral side of the animal. This pattern is reminiscent of serotonergic marker genes such as tryptophan hydroxylase (*tph*), an enzyme catalyzing the rate-limiting step in Serotonin synthesis (Nakamura and Hasegawa, 2007; Nishimura et al., 2007b). To reveal whether *Smed-pitx* is expressed in serotonergic neurons, we cloned the putative planarian homologs of known components of the terminal differentiation gene battery of serotonergic neurons, and compared their expression patterns by double FISH (DFISH). In addition to *tph*, this battery consists of amino acid decarboxylase (*aadc*), vesicular monoamine transporter (*vmat*) and Serotonin transporter (*sert*) (Flames and Hobert, 2011). *Smed-pitx* and all serotonergic terminal differentiation genes were expressed in a similar pattern (Fig. 1A-E). *Smed-tph* and *Smed-aadc* displayed additional expression in the eyes and in large cells around the pharynx (Fig. 1B,C). DFISH of *Smed-tph* and *Smed-sert* illustrated that *Smed-sert* is an exclusive marker for serotonergic neurons (Fig. 1F-F''). The same cells further co-expressed *Smed-aadc* (Fig. 1G-G'') and *Smed-vmat* (Fig. 1H-H''). In vertebrates, Aadc (also known as Ddc) and Vmat (also known as Slc18a1) are also expressed in dopaminergic neurons, another monoaminergic cell type (Flames and Hobert, 2011). Similarly, both *Smed-aadca*, a planarian paralog of *aadc* (Nishimura et al., 2007a), and *Smed-vmat* are expressed in dopaminergic neurons in planarians (supplementary material Fig. S2), indicating that the gene batteries that define vertebrate neuronal cell types are conserved in these animals.

### *Smed-pitx* is expressed in differentiating serotonergic neurons

DFISH of *Smed-pitx* with the serotonergic marker *Smed-sert* revealed that all serotonergic neurons express *Smed-pitx* but not all *Smed-pitx*-positive cells express terminal differentiation genes of the serotonergic lineage (Fig. 1I-I''). Terminal selector genes are known to be expressed not only in terminally differentiated neurons, where they regulate the expression of terminal differentiation genes throughout the lifespan of a neuron, but also in late progenitors to activate the terminal differentiation program. Planarians undergo continuous cell turnover in which differentiated cells are replaced by differentiating neoblast progeny (Eisenhoffer et al., 2008). Thus, we assumed that serotonergic neurons would also be constantly renewed during adult homeostasis. We hypothesized that *Smed-pitx*-positive cells that did not express serotonergic markers could be in a late progenitor state that had not yet activated the terminal differentiation program. To test this hypothesis we performed a triple staining consisting of a DFISH for *Smed-pitx* and *Smed-tph* (as a



**Fig. 1. *Smed-pitx* is expressed in serotonergic neurons and stem cell progeny.** All images are fluorescent *in situ* hybridizations (FISH) on intact planarians. (A-E) FISH against the serotonergic markers *Smed-pitx*, *Smed-tph*, *Smed-aadc*, *Smed-sert* and against *Smed-vmat*. (F-F'') High-magnification view of *Smed-tph* and *Smed-sert* co-expression in serotonergic neurons. (G-G'') Co-expression of *Smed-sert* and *Smed-aadc*. (H-H'') Co-expression of *Smed-sert* and *Smed-vmat*. (I-I'') Double FISH (DFISH) of *Smed-sert* and *Smed-pitx*. Note that *Smed-pitx* is expressed in all *Smed-sert*-positive cells, whereas not all *Smed-pitx*-positive cells express *Smed-sert* (arrows). (J-J'') DFISH combined with immunostaining against SMEDWI-1 protein demonstrates expression of *Smed-pitx* in *Smed-tph*<sup>-</sup> SMEDWI-1<sup>+</sup> cells (arrowheads) and in *Smed-tph*<sup>+</sup> SMEDWI-1<sup>-</sup> cells (arrows). (K-K'') Few *Smed-pitx*<sup>+</sup> *Smed-tph*<sup>+</sup> cells co-express SMEDWI-1 (arrows). Minimum number of animals analyzed: 40 in A-E, 30 in F-I'', 25 in J-K. Scale bars: 250  $\mu$ m in A; 10  $\mu$ m in F; 10  $\mu$ m in J.

marker for differentiated serotonergic neurons) and an immunostaining against SMEDWI-1 protein, which labels neoblasts and is retained in neoblast progeny (Guo et al., 2006). Most cells that expressed *Smed-pitx* and were not positive for serotonergic markers displayed SMEDWI-1 expression, whereas most cells that co-expressed *Smed-pitx* and *Smed-tph* lacked this protein (Fig. 1J-J'''). However, we found a few cells that were positive for all three markers, indicating a stage in which a cell is in the process of terminal differentiation (Fig. 1K-K'''). We never detected co-expression of *Smed-pitx* with *smedwi-1* mRNA in DFISH experiments in homeostatic animals (not shown), suggesting that *Smed-pitx* is only expressed in stem cell progeny. *Smed-pitx* could thus act as a terminal selector gene that is expressed in late progenitor cells of the serotonergic lineage, activating terminal differentiation genes and maintaining their expression in differentiated cells (see also supplementary material Fig. S8). This observation is in concert with the concept that cell types might differentiate directly from neoblast progeny (Lapan and Reddien, 2011; Reddien, 2013).

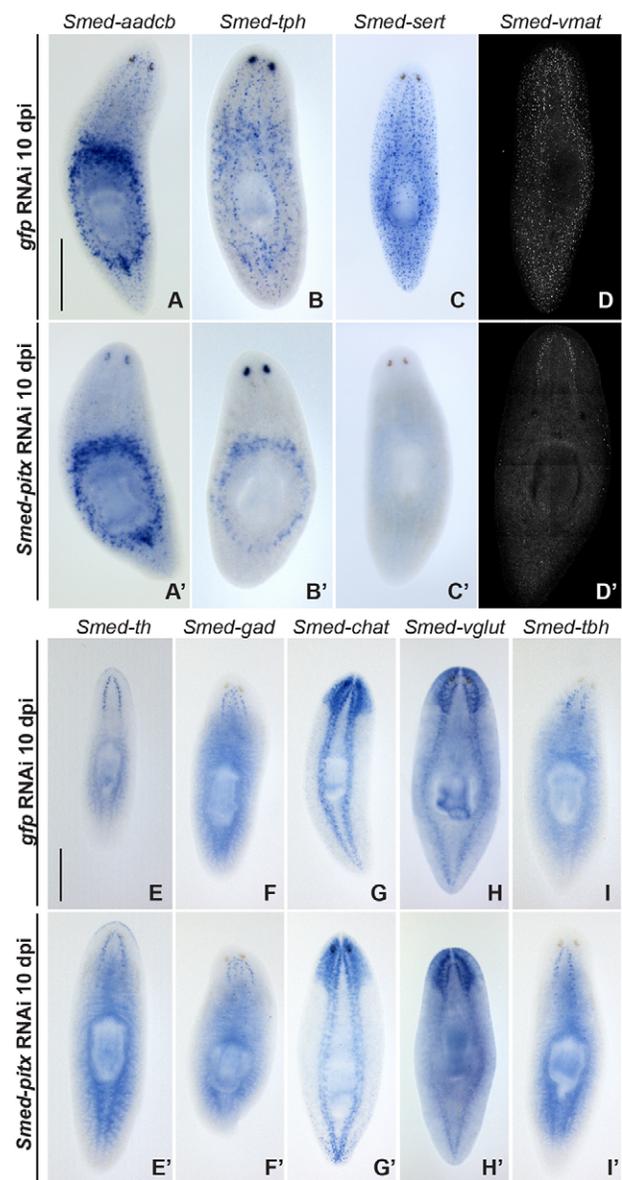
### ***Smed-pitx* maintains the expression of terminal differentiation genes in serotonergic neurons**

To test whether *Smed-pitx* is required to maintain the expression of terminal differentiation genes in serotonergic neurons during homeostasis, we performed RNAi-mediated knockdown of *Smed-pitx* and analyzed serotonergic marker expression. Ten days after three consecutive injections of *Smed-pitx* dsRNA we performed whole-mount *in situ* hybridization (WISH) of four serotonergic markers (Fig. 2A-D'). We observed that, after *Smed-pitx* RNAi, the expression of *Smed-aadcb*, *Smed-tph*, *Smed-sert* and *Smed-vmat* was abolished in serotonergic neurons, whereas it was retained in tissues negative for *Smed-pitx*. These tissues included eyes (*Smed-aadcb*, *Smed-tph*), large cells around the pharynx (*Smed-aadcb*, *Smed-tph*) and dopaminergic neurons (*Smed-vmat*) (Fig. 2A',B',D'; supplementary material Fig S2). In addition, RNAi animals showed impaired movement (supplementary material Movies 1, 2), a phenotype that can result from defective motility of ventral cilia (Rompolas et al., 2013). However, the arrangement of cilia on the ventral surface seemed normal based on  $\alpha$ -tubulin staining (data not shown).

Other neuronal subpopulations, such as dopaminergic (*Smed-th*), GABAergic (*Smed-gad*), cholinergic (*Smed-chat*), octopaminergic (*Smed-tbh*) and glutamatergic (*Smed-vglut*) neurons, were not affected by *Smed-pitx* knockdown (Fig. 2E-I'). Similar results were obtained for regenerating planarians that lacked new serotonergic neurons entirely. These animals regenerated other neuronal cell types in a manner comparable to the control animals (supplementary material Fig. S3), suggesting that *Smed-pitx* function is specific for the serotonergic lineage.

### **Loss of serotonergic neurons is not due to transdifferentiation, increased apoptosis or defects in stem cell-based turnover**

What happens to serotonergic neurons after *Smed-pitx* RNAi? Possibilities include the transdifferentiation of serotonergic neurons into other neuronal subtypes, death of serotonergic neurons, defects in stem cell-based turnover, or the maintenance of serotonergic neurons in an undifferentiated state in which they retain pan-neuronal characteristics but lack serotonergic-specific gene expression. The latter is the case for mouse serotonergic neurons mutant for *Pet1*, the terminal selector gene responsible for the terminal differentiation of mouse serotonergic neurons (reviewed by Deneris, 2011).



**Fig. 2. *Smed-pitx* RNAi leads to specific loss of serotonergic marker expression.** Images are whole-mount *in situ* hybridizations (WISH) (A-C', E-I') or FISH (D, D') on intact planarians. (A-D) Expression of serotonergic marker genes in control animals. (A'-D') Serotonergic marker expression is lost in *Smed-pitx* RNAi animals (30/30 animals per marker) at 10 days post-injection (dpi). Note that *Smed-vmat* expression is always retained in dopaminergic neurons (D'; see also supplementary material Fig. S2). (E-I) Expression patterns of the dopaminergic marker *Smed-th* (A), the GABAergic marker *Smed-gad* (B), the cholinergic marker *Smed-chat* (C), the glutamatergic marker *Smed-vglut* (D) and the octopaminergic marker *Smed-tbh* (E) in control RNAi animals at 10 dpi. (E'-I') *Smed-pitx* RNAi does not affect the expression of these markers at 10 dpi (20/20 animals per marker). Scale bars: 500  $\mu$ m.

As we had already tested available markers for different neuronal subpopulations after *Smed-pitx* RNAi during homeostasis and regeneration (Fig. 2; supplementary material Fig. S3), a transdifferentiation scenario seemed highly unlikely because we did not observe any ectopic expression of other neuronal markers in areas where serotonergic neurons are located.

To investigate the possibility that serotonergic neurons undergo apoptosis, we first performed a WISH timecourse to identify the

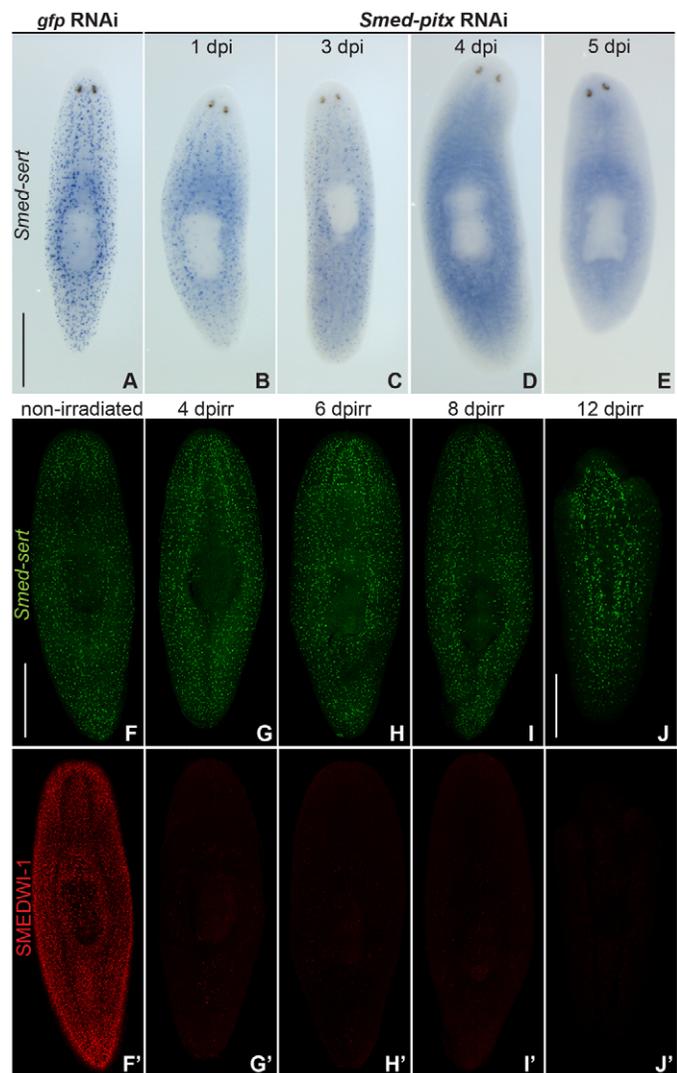
dynamics of serotonergic marker loss after *Smed-pitx* RNAi. *Smed-pitx* dsRNA was injected on three consecutive days and animals were fixed at 1 to 5 days after the last injection. Subsequently, we performed WISH for *Smed-sert* as a serotonergic marker. One day after the last injection *Smed-sert* expression was already reduced. Expression decreased gradually until 4 days after injection, when *Smed-sert* mRNA remained detectable in only a few cells. Serotonergic neurons were completely lost after one additional day (Fig. 3A-E). To test whether the loss of expression was accompanied by an increase in apoptosis we performed TUNEL stainings of intact planarians at time points at which a clear loss of *Smed-sert* expression was observed and counted the TUNEL-positive cells in a total of five animals per time point. An increase in apoptosis could not be detected after *Smed-pitx* RNAi compared with control animals (supplementary material Fig. S4A-C), excluding this type of cell death as the major cause of serotonergic neuron loss.

A role of *Smed-pitx* in the regulation of differentiation combined with a fast turnover of serotonergic neurons might be a reason for the dramatic loss of serotonergic neurons after *Smed-pitx* RNAi. To test this we depleted stem cells by  $\gamma$ -irradiation (Reddien and Sánchez Alvarado, 2004) and observed the expression of SMEDWI-1 and serotonergic marker genes for 12 days, until the first animals died with head regression and ventral curling due to stem cell loss (Reddien et al., 2005). Whereas SMEDWI-1<sup>+</sup> cells were entirely lost 3 days post-irradiation (dpi), *Smed-sert* expression was unchanged until at least 12 dpi (Fig. 3F-J'). As, in addition, CldU labeling of proliferating stem cells did not result in CldU-positive serotonergic neurons after 4 days of labeling, our data suggest that the turnover of serotonergic neurons is far slower than marker gene dynamics after *Smed-pitx* RNAi. Thus, we can exclude the possibility that stem cell-based turnover accounts for the rapid loss of serotonergic markers after *Smed-pitx* RNAi. In summary, our results point to a model in which *Smed-pitx* acts as a terminal selector gene for the expression of the serotonergic gene battery in differentiating and mature serotonergic neurons.

### Downstream targets of *Smed-pitx*

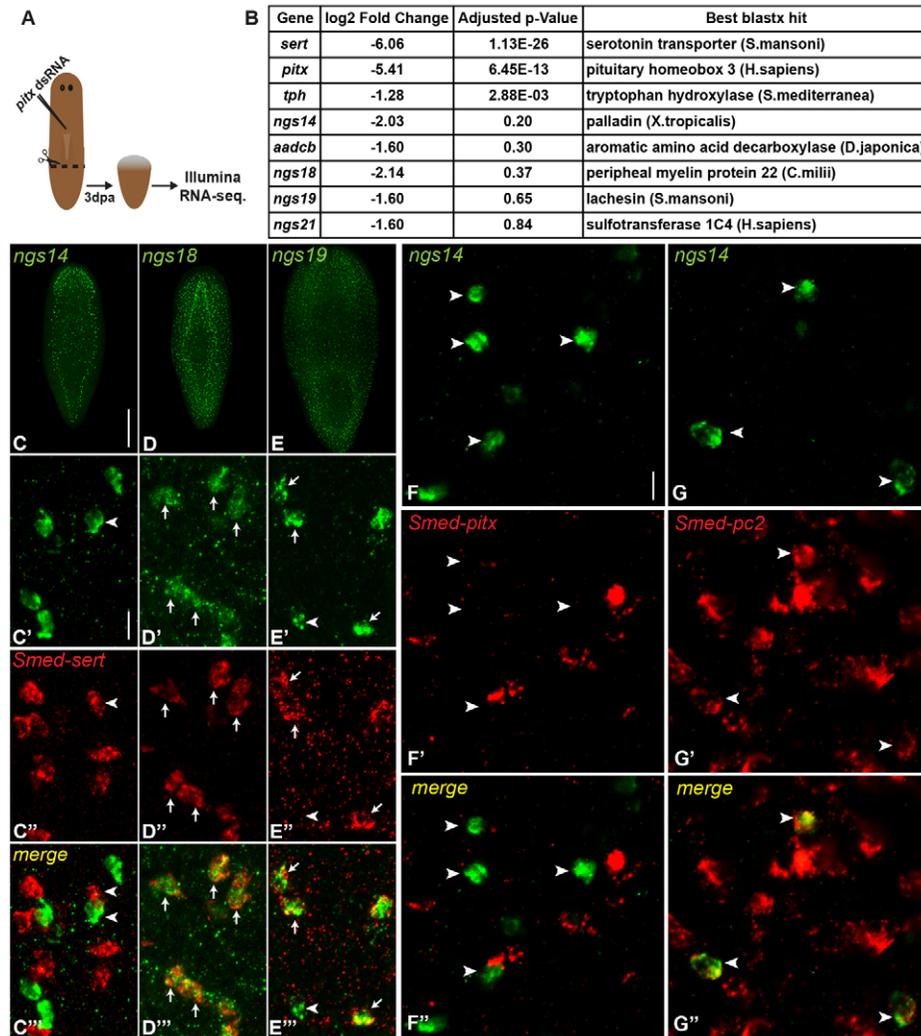
In order to identify downstream targets of *Smed-pitx* during the regeneration and maintenance of serotonergic neurons, we performed Illumina paired-end sequencing of planarian tail fragments regenerating a head (Fig. 4A), and compared the transcriptomes of *Smed-pitx* RNAi and control fragments at 3 days post-amputation (dpa). We identified 11 downregulated genes with  $P < 0.05$  and a  $\log_2$  fold-change smaller than  $-1$  (supplementary material Table S2). Among these were the serotonergic markers *Smed-sert*, *Smed-pitx*, *Smed-tph* and *Smed-aadcb* (Fig. 4B) and four candidates with a neuron-like expression pattern (Fig. 4B-E; supplementary material Fig. S5). *ngs14*, *ngs18*, *ngs19* and *ngs21* encode putative homologs of a *palladin*-like gene, *peripheral myelin protein 22*, *lachesin* and *sulfotransferase 1C4*, respectively. FISH of these candidate genes revealed that knockdown of *Smed-pitx* reduced the expression of *ngs14*, *ngs18* and *ngs19*, validating the RNA-seq results (Fig. 5A-C'). However, expression of *ngs21* was unaffected (supplementary material Fig. S5D-D''), indicating a false-positive candidate gene in our list.

We performed DFISH of candidate genes with *Smed-sert* to test whether they were expressed in serotonergic neurons and could be direct targets of *Smed-pitx*. Interestingly, all *ngs18*-expressing cells were positive for *Smed-sert*, establishing *ngs18* as a new *Smed-pitx*-dependent marker of serotonergic neurons (Fig. 4D-D'''). A majority of *ngs19*-positive cells also co-expressed *Smed-sert*, demonstrating that most *ngs19*-positive cells were serotonergic neurons (Fig. 4E-E'''). Surprisingly, putative homologs of both genes play a role in



**Fig. 3. Loss of serotonergic markers after *Smed-pitx* RNAi is not due to stem cell-based turnover.** WISH (A-E), FISH (F-J) and immunostainings (F'-J') are shown. (A) *Smed-sert* expression in control animals. (B-E) Loss of *Smed-sert* expression after *Smed-pitx* RNAi from 1 to 5 dpi (days after the last of three consecutive injections). (F-J) *Smed-sert* and (F'-J') SMEDWI-1 expression from 4 to 12 days post- $\gamma$ -irradiation (dpi). Note that serotonergic neurons are not affected throughout the timecourse, whereas SMEDWI-1<sup>+</sup> stem cell progeny are entirely lost after irradiation (20/20 animals per time point), indicating a relatively slow turnover of serotonergic neurons. Scale bars: 500  $\mu$ m in A-I'; 250  $\mu$ m in J, J'.

nerve insulation in other organisms (Snipes et al., 1992; Strigini et al., 2006), suggesting that further analysis of *ngs18* and *ngs19* could provide new insights into the role of nerve insulation in planarians. By contrast, *ngs14*-positive cells expressed neither serotonergic markers (Fig. 4C-C''') nor *Smed-pitx* (Fig. 4F-F''') but were closely associated with *Smed-sert*-expressing cells (Fig. 4C''') and positive for the pan-neuronal marker *pc2* (Fig. 4G-G'''). This suggested that, whereas *ngs18* and *ngs19* are regulated within serotonergic neurons, *ngs14* might depend on non-autonomous control, possibly through Serotonin signaling to a neighboring cell type. To test this, we inhibited Serotonin production by depleting planarians of *Smed-tph*. As shown in Fig. 5, RNAi against *Smed-tph* did not affect the expression of *ngs14*, *ngs18* or *ngs19*, pointing towards a Serotonin-independent mechanism.



**Fig. 4. RNA-seq-based transcriptome analysis after *Smed-pitx* RNAi reveals new markers of serotonergic neurons.**

(A) Workflow of the RNA-seq experiment. (B) Gene identifiers, log<sub>2</sub> fold changes, adjusted *P*-values and best BLAST hits for selected candidate genes. (C-E) FISH expression patterns of three candidate genes in intact planarians. (C'-C''') No co-expression of *ngs14* and *Smed-sert*, but a close association of *ngs14* and *Smed-sert*<sup>+</sup> cells (arrowheads). (D'-D''') *ngs18* is expressed in serotonergic neurons (arrows). (E'-E''') *ngs19* is expressed in *Smed-sert*<sup>+</sup> (arrows) and in a few *Smed-sert*<sup>-</sup> (arrowheads) cells. (F-G'') DFISH of *ngs14* with either *Smed-pitx* (F-F'') or the pan-neuronal marker *pc2* (G-G''). Note that *ngs14* is expressed in *Smed-pitx*-negative neurons (arrowheads), suggesting a non-autonomous requirement for *Smed-pitx* for gene expression in this cell type. Number of animals analyzed: 20 per staining. Scale bars: 250 μm (C), 10 μm (C',F).

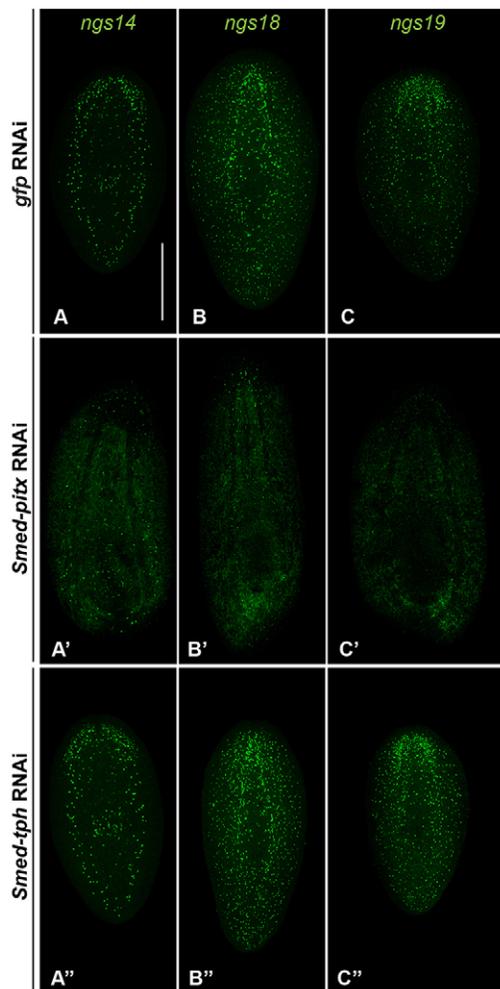
#### An additional role for *Smed-pitx* in regenerative patterning

In addition to the loss of serotonergic markers, RNAi against *Smed-pitx* resulted in anterior and posterior regeneration defects (Fig. 6A',F'; supplementary material Fig. S3). Regenerating heads displayed a cyclopic appearance with fused eyes at the body midline (Fig. 6A'), while head fragments that should regenerate missing posterior structures remained tailless (Fig. 6F'), with fused ventral nerve cords and lack of the tail marker *fz4* (data not shown). We detected *Smed-pitx* expression in regenerating planarians at both regeneration poles from 2 dpa onwards (Fig. 6B,G; supplementary material Fig. S6A). The posterior RNAi phenotype and gene expression pattern were reminiscent of those described for *Smed-wnt1* (Adell et al., 2009; Petersen and Reddien, 2009) and the LIM homeobox transcription factor *Dj-islet1*, which has been shown to be essential for *Dj-wnt1*-mediated tail formation in the planarian *Dugesia japonica* (Hayashi et al., 2011). DFISH experiments with RNA probes against *Smed-pitx* and either *Smed-islet1* or *Smed-wnt1* confirmed the co-expression of *Smed-pitx* with either gene in a cluster of cells at the posterior-most dorsal midline (Fig. 6H-I'').

To test whether the loss of *Smed-wnt1* or *Smed-islet1* expression could also account for the tailless phenotype of *Smed-pitx* RNAi animals, we performed RNAi against the three genes to establish the hierarchy in the signaling pathway controlling tail regeneration.

After each gene knockdown, the expression of the two other genes was analyzed at 3 dpa (Fig. 6G-G''',J-J''; supplementary material Fig. S6A-B'). *Smed-islet1* RNAi abolished *Smed-pitx* expression in the posterior dorsal midline cluster (Fig. 6G,G'') as well as *Smed-wnt1* expression (Fig. 6J,J'). Interestingly, *Smed-pitx* RNAi had a similar effect on *Smed-islet1* (Fig. 6G',G''') and *Smed-wnt1* (Fig. 6J,J'') expression, and *Smed-wnt1* RNAi depleted both *Smed-pitx* and *Smed-islet1* transcripts (supplementary material Fig. S6A-B'). As neither *Smed-islet1* nor *Smed-pitx* was required for early *Smed-wnt1* expression during the polarity phase of regeneration (Hayashi et al., 2011; Petersen and Reddien, 2009) (supplementary material Fig. S6C-C''), we conclude that *Smed-pitx* and *Smed-islet1* might co-regulate the second phase of *Smed-wnt1* expression, when polarity has been determined and the tail is being formed, and that this interaction accounts for the tailless phenotype of *Smed-pitx* RNAi planarians.

Interestingly, both *Smed-islet1* and *Smed-pitx* RNAi led to a cyclopic regeneration phenotype in tail fragments (Fig. 6A',A''). As cyclopia and the midline collapse of lateral structures have been demonstrated for a planarian homolog of the midline repellent Slit (Cebrià et al., 2007), we examined whether a putative interaction of our transcription factors might regulate midline repulsion through *Smed-slit*. We performed FISH experiments on regenerating tail pieces and found that, similar to their expression in regenerating



**Fig. 5. Loss of candidate gene expression after *Smed-pitx* RNAi is not due to loss of Serotonin production.** (A-C) Expression of *ngs14* (A), *ngs18* (B) and *ngs19* (C) in regenerating trunk fragments at 10 days post-amputation (dpa). (A'-C') *Smed-pitx* RNAi abolishes expression in most cells positive for *ngs14* (A'), *ngs18* (B') and *ngs19* (B'). (A''-C'') RNAi against the Serotonin biosynthesis enzyme tryptophan hydroxylase (*Smed-tph*) does not affect the expression of any of these genes. Number of animals analyzed per knockdown and staining: 30/30. Scale bar: 250  $\mu$ m.

head pieces, *Smed-islet1* and *Smed-pitx* were indeed expressed together in a cluster of cells at the anterior regeneration pole (Fig. 6C-C'') and were essential for each other's expression (Fig. 6B-B''). Strikingly, this cluster was also positive for *Smed-slit* mRNA (Fig. 6D-D''), and RNAi against either transcription factor resulted in loss of *Smed-slit* mainly at the anterior dorsal midline (Fig. 6E-E''). Consistent with the dorsal midline expression of *Smed-pitx* and *Smed-islet1* (Fig. 6B-C''), ventral expression was largely unaffected (supplementary material Fig. S7C'',D''), explaining the relatively mild midline patterning phenotype of these genes compared with *Smed-slit* (Cebrià et al., 2007).

#### ***Smed-pitx/Smed-islet1* patterning functions are separable from a role of *Smed-pitx* in the serotonergic lineage**

Given the different phenotypes after *Smed-pitx* knockdown, we asked whether loss of serotonergic neuron function could cause patterning defects during regeneration and vice versa. To test this, we depleted planarians of *Smed-tph*, an enzyme required for

Serotonin production, and analyzed regenerating head and tail fragments for patterning defects and *Smed-wnt1* expression (Fig. 7A-B'). Efficient *Smed-tph* knockdown was confirmed by pigmentation loss in regenerating eyes, whereas tail formation and eye patterning were unaffected and *Smed-wnt1* expression at the posterior regeneration pole was normal (Fig. 7B,B'). Similarly, although *Smed-wnt1* and *Smed-islet1* RNAi fragments revealed patterning defects they expressed serotonergic markers normally (Fig. 7C-F'), indicating that *Smed-pitx*-mediated gene expression in serotonergic neurons is independent of *Smed-islet1* and Wnt signaling.

## **DISCUSSION**

### ***Smed-pitx* as a neuronal terminal selector gene**

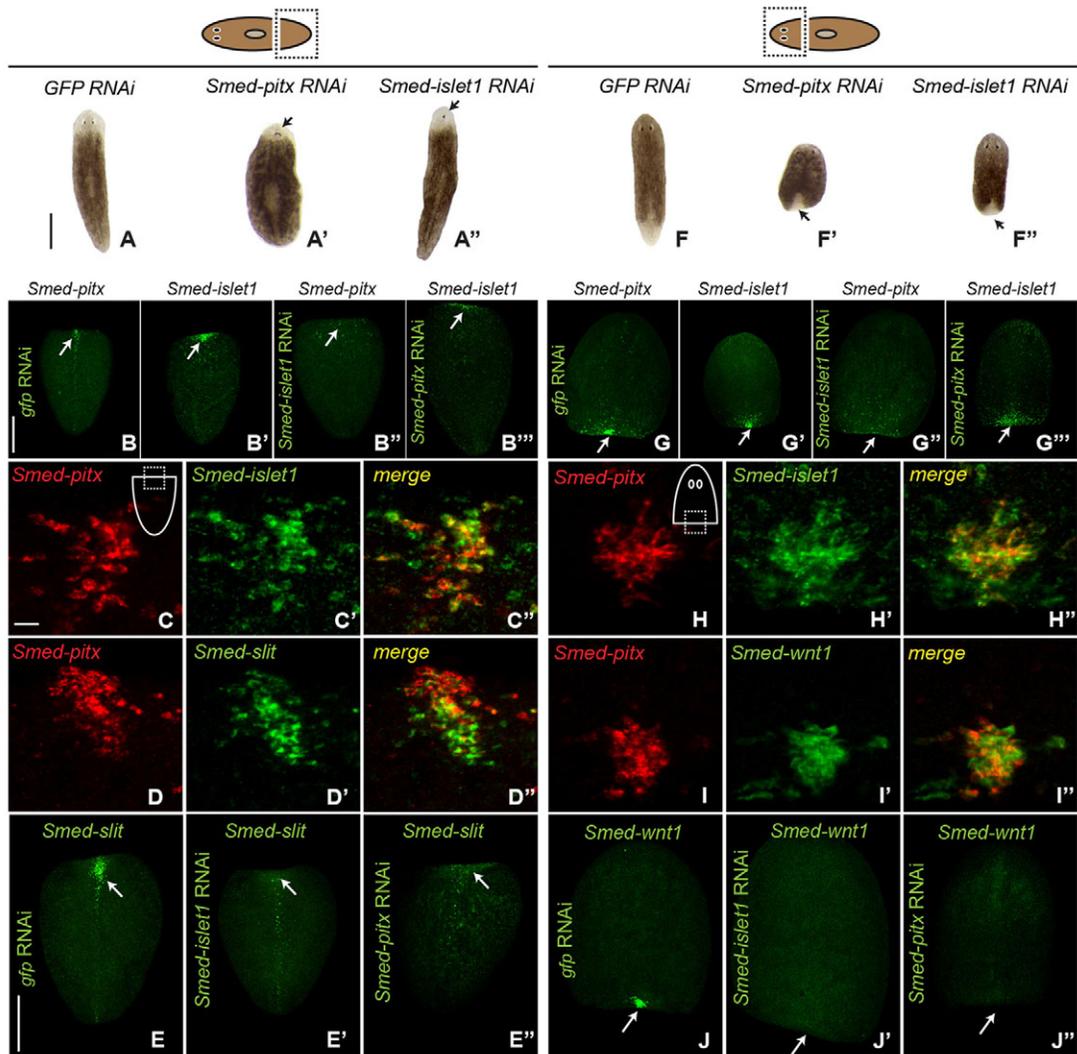
Neuronal terminal selectors are transcription factors that regulate the expression of specific terminal differentiation genes in the last phase of neuronal differentiation and maintain the expression of these genes during the lifetime of a neuron (reviewed by Hobert, 2008). Elimination of a neuronal terminal selector results in loss of identity of the respective neuron. For instance, homologs of *Smed-pitx*, such as *C. elegans unc-30* and mouse *Pitx2*, have been described as terminal selectors for GABAergic neurons (Westmoreland et al., 2001), whereas mouse *Pitx3* is a terminal selector for midbrain dopaminergic neurons (Smidt et al., 2004b).

Interestingly, the terminal differentiation of mammalian serotonergic neurons and transcription of the serotonergic gene battery are controlled by the transcription factor *Pet1*. Ablation of *Pet1* in adult mouse brains leads to a loss of *Tph2* and *Sert* (*Slc6a4* – Mouse Genome Informatics) expression, but cells do not undergo apoptosis and histological features seem normal (Liu et al., 2010). We could not identify a *Pet1* homolog in *S. mediterranea*. However, serotonergic markers are lost in intact animals upon *Smed-pitx* knockdown, a phenotype that is caused neither by increased apoptosis nor transdifferentiation into other neuronal types. Owing to the lack of transgenic and cell culture techniques for planarians we cannot provide definitive evidence for the loss of identity in otherwise intact cells after *Smed-pitx* RNAi. However, we can rule out the possibility that this phenotype is due to defects in stem cell-based turnover, as irradiation experiments and CldU labeling demonstrated that the rapid loss of serotonergic neurons after *Smed-pitx* RNAi is not within the dynamic range of general serotonergic turnover.

In addition, we were able to show that *Smed-pitx* is expressed in stem cell progeny committed to the serotonergic fate and that RNAi resulted in a loss of cell type-specific markers in newly regenerated tissue. Thus, we propose that, similar to *Pet1* in vertebrates, *Smed-pitx* exerts a terminal selector function for the serotonergic lineage in planarians, with a crucial role in their differentiation and maintenance. Even though it seems that different transcription factors have been employed for the terminal selection of the same cell types during evolution, our data suggest that the concept of terminal selection is conserved from planarians to mammals and shed light on a possibly general principle of how planarian neurons obtain and maintain their identity during regeneration and homeostasis.

### **Neuronal target genes of *Smed-pitx***

RNA-seq-based transcriptome analysis of regenerating *Smed-pitx* RNAi animals revealed 11 genes that were significantly downregulated. Three of these genes were known markers of serotonergic neurons (*sert*, *aadcb*, *tph*). WISH experiments revealed four genes with expression patterns that could include serotonergic

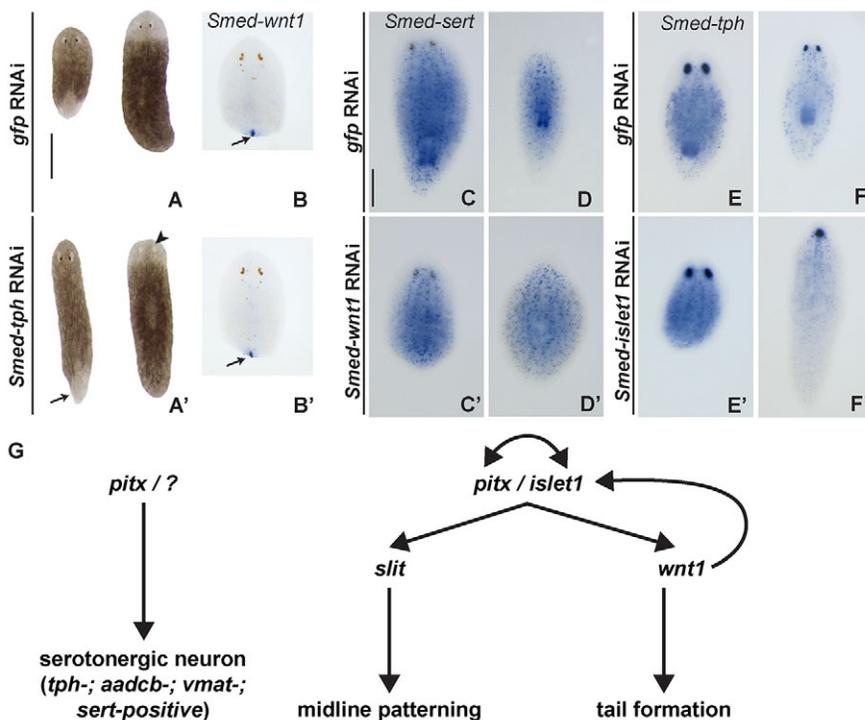


**Fig. 6. *Smed-pitx* and *Smed-islet1* control Wnt-dependent tail formation and Slit-dependent midline patterning during regeneration.** (A-E'') Regenerating tail fragments. (F-J'') Regenerating head fragments. (A-A'') Live images of planarian tail fragments at 10 dpa. Eyes of *Smed-pitx* (A'; 70/100) and *Smed-islet1* (A''; 98/100) RNAi animals fuse at the midline (arrows) whereas control animals are normal (A; 100/100). (B) FISH for *Smed-pitx* (B,B'') and *Smed-islet1* (B',B'') on tail fragments at 3 dpa. Note that in control animals both *Smed-pitx* and *Smed-islet1* are expressed in a cluster of cells at the anterior dorsal midline (arrows) (70/70). Expression of these genes is lost in this cluster after *Smed-islet1* RNAi (B''; 78/80) and *Smed-pitx* RNAi (B'''; 72/80), respectively. (C-D'') DFISH images acquired at high-magnification from the boxed region indicated in C at 3 dpa. (C-C'') Co-expression of *Smed-pitx* and *Smed-islet1* in the anterior dorsal midline cluster was detected in 70/70 animals. (D-D'') Co-expression of *Smed-pitx* and *Smed-slit* in the anterior dorsal midline cluster was detected in 60/60 animals. (E-E'') FISH for *Smed-slit* in the anterior dorsal midline cluster is lost after *Smed-islet1* RNAi (E'; 50/50) and *Smed-pitx* RNAi (E''; 37/50) as compared with control RNAi animals (E). (F-F'') Live images of planarian head fragments at 10 dpa. *Smed-pitx* RNAi (F'; 100/100) and *Smed-islet1* RNAi (F''; 95/100) animals display a tailless phenotype (arrows) whereas control animals regenerate a normal tail (F; 100/100). (G-G'') FISH for *Smed-pitx* (G,G'') and *Smed-islet1* (G',G''') on head fragments at 3 dpa. Note that in control animals both *Smed-pitx* and *Smed-islet1* are expressed in a cluster of cells at the posterior dorsal midline (arrows) (70/70). Expression of these genes is lost in this cluster after *Smed-islet1* RNAi (G''; 79/80) and *Smed-pitx* RNAi (G'''; 76/80), respectively. (H-I'') DFISH images acquired at high magnification from the boxed region indicated in H at 3 dpa. (H-H'') Co-expression of *Smed-pitx* and *Smed-islet1* in the posterior dorsal midline cluster was detected in 70/70 animals. (I-I'') Co-expression of *Smed-pitx* and *Smed-wnt1* in the posterior dorsal midline cluster was detected in 80/80 animals. (J-J'') *Smed-wnt1* expression is lost after *Smed-islet1* RNAi (J'; 75/75) and *Smed-pitx* RNAi (J''; 75/75) as compared with control RNAi animals (J). Scale bars: 250  $\mu$ m in A,B,E; 10  $\mu$ m in C.

neurons. Two of these genes were indeed expressed in serotonergic neurons (*ngs18*, *ngs19*) and constitute new markers of this cell type. Putative homologs of *ngs18* and *ngs19* belong to the immunoglobulin superfamily and display similarity to cell surface proteins acting in nerve insulation. *ngs18*, a putative homolog of peripheral myelin protein 22 (*pmp22*), encodes a component of the myelin sheath produced by glial cells of the peripheral nervous system in vertebrates (Snipes et al., 1992). *ngs19* is a putative homolog of *Drosophila Lachesin*, which plays a role in the

insulation of axons through septate junctions between neurons and ensheathing glia (Strigini et al., 2006). Further analysis of these genes could provide interesting insights into the regulation of nerve insulation in planarians.

Strikingly, *ngs14* and *ngs21*, which encode a putative Palladin-related actin-binding protein and a sulfotransferase, respectively, were expressed in *pc2*-positive neurons that expressed neither *Smed-pitx* nor serotonergic markers. Although we could not reproduce the *Smed-pitx* dependence of *ngs21*, suggesting that this gene was a false-



**Fig. 7. Independent functions of *Smed-pitx*.** (A-B') *Smed-wnt1*-dependent tail formation is independent of Serotonin production. (A,A') Live images of planarian head (left) and tail fragments (right) at 10 dpa. *Smed-tph* RNAi animals display normal tail regeneration (arrow) but lack eye pigmentation in regenerating tail fragments (arrowhead) (90/90). (B,B') *Smed-tph* RNAi animals display normal *Smed-wnt1* expression in head fragments at 3 dpa (arrows) (90/90). (C-F') Serotonergic differentiation and marker gene expression is independent of *Smed-wnt1* and *Smed-islet1*. (C-D') *Smed-sert* expression is similar to that in control head (C) and tail (D) fragments after *Smed-wnt1* RNAi at 10 dpa (45/45). (E-F') Regeneration of *Smed-tph*-positive serotonergic neurons from head (left) and tail (right) fragments is not affected after *Smed-islet1* RNAi at 10 dpa (45/45). WISH images are shown in B-F'. (G) Summary of *Smed-pitx* functions in differentiating serotonergic neurons (left) and at the regeneration poles (right). Note that *pitx* acts independently of the LIM homeodomain transcription factor gene *Smed-islet1* in serotonergic neuron differentiation and maintenance. Scale bars: 250  $\mu$ m.

positive candidate in our list, *ngs14* is a confirmed downstream target of *Smed-pitx*. Interestingly, *ngs14*-positive neurons were located in the proximity of serotonergic neurons, pointing to the possibility that *Smed-pitx* might affect *ngs14* expression through a non-autonomous mechanism. It is unlikely that this mechanism is based on Serotonin signaling, as perturbation of Serotonin production by RNAi against *Smed-tph* did not affect *ngs14* expression. Following these lines of evidence in the future might provide valuable insights into how serotonergic neurons communicate with neighboring cell types through Serotonin-independent mechanisms.

#### ***Smed-islet1*-dependent functions of *Smed-pitx* during regenerative morphogenesis**

Several lines of evidence suggest that *Smed-pitx* and *Smed-islet1* act together in regulating morphogenetic growth and patterning during regeneration. First, both *Smed-islet1* and *Smed-pitx* RNAi planarians are incapable of regenerating a tail and reveal midline defects. Second, both genes are co-expressed in clusters of cells at the anterior and posterior regeneration poles, where they are required for the expression of the secreted signaling molecule encoded by *Smed-slit* (Fig. 6D-E"; supplementary material Fig. S7). In addition, RNAi against either gene results in the loss of expression of the other at anterior and posterior regeneration poles, raising the possibility that they control each other's expression as transcription factors (Fig. 7G).

At the posterior regeneration pole, *Smed-islet1* and *Smed-pitx* are essential for *Smed-wnt1* expression, explaining their tailless RNAi phenotypes. Vertebrate homologs of *Smed-pitx* and *Smed-islet1* play important roles in the development of hindlimbs, where they regulate hindlimb outgrowth and patterning during development in concert with Wnt signaling (reviewed by Rabinowitz and Vokes, 2012). What might be the role of *Smed-pitx* at the posterior pole? RNAi and expression analyses showed that *Smed-pitx*, *Smed-islet1* and *Smed-wnt1* depend on each other's expression. Based on these results, different epistasis models of signaling are possible. One

option is that *Smed-pitx* and *Smed-islet1* act together on the *Smed-wnt1* promoter to activate transcription. *Smed-wnt1* might in turn control the expression of these transcription factors by a positive-feedback loop (Fig. 7G). As already shown for an *islet1* gene in *D. japonica* (Hayashi et al., 2011), *Smed-islet1* and *Smed-pitx* (this study) affect only the second, later phase of *Smed-wnt1* expression, but not the first few hours, when polarity decisions are being made. This is consistent with the observation that *Smed-pitx* and *Smed-islet1* RNAi never led to the formation of an ectopic head at the posterior pole, as it is the case for *Smed-wnt1* RNAi (Gurley et al., 2008; Petersen and Reddien, 2009). As *Smed-pitx* and *Smed-islet1* expression is absent after *Smed-wnt1* RNAi, it is likely that wound-induced expression of *wnt1* is required for the initial activation of *Smed-pitx/Smed-islet1* and other pole genes, which then act to induce and maintain the expression of morphogens at the pole. As proposed for *Dj-islet* (Hayashi et al., 2011), a second explanation for the mutual loss of expression could be an indirect effect of these genes on pole formation, for instance through differentiation control.

What might be the role of *Smed-pitx* and *Smed-islet1* in midline patterning? Knockdown of either gene led to anterior midline collapse of the nervous system as indicated by the presence of fused eyes and anterior brain structures. This phenotype was reminiscent of that of the midline repellent *Smed-slit* (Cebrià et al., 2007), and is most likely due to a loss of dorsal *Smed-slit* expression. However, the phenotype was less severe, probably owing to the remaining ventral expression of *Smed-slit* after *Smed-pitx* knockdown. It is currently unclear whether *Smed-pitx* and *Smed-islet1* regulate *Smed-slit* through direct transcriptional regulation or through the differentiation control of dorsal *Smed-slit*-expressing cells at the regeneration poles.

#### **Control of tissue-specific functions of *Smed-pitx***

In this study we demonstrate that a single Pitx transcription factor controls independent processes during planarian regeneration and homeostasis. First, in synergy with the LIM homeobox transcription

factor gene *Smed-islet1*, *Smed-pitx* regulates *Smed-wnt1*-dependent tail regeneration in a cluster of cells at the posterior pole of regenerating fragments. Additionally, both genes are required for proper midline patterning through *Smed-slit*. Second, and independently of *Smed-islet1*, *Smed-pitx* is a putative terminal selector gene that is expressed in differentiating and mature serotonergic neurons where it controls their maturation and maintenance through the regulation of cell type-specific genes that generate, package and transport the neurotransmitter Serotonin (Fig. 7G).

By contrast, several functions are distributed among the three Pitx family members (Pitx1-3) in vertebrates. For instance, Pitx2 and Pitx3 act as neuronal terminal selector genes controlling the differentiation of GABAergic and midbrain dopaminergic neurons, respectively (Smidt et al., 2004b; Westmoreland et al., 2001). Furthermore, Pitx transcription factors have been associated with the regulation of developmental processes of anterior (craniofacial, eyes, anterior pituitary) and posterior (hindlimb) structures, and with left-right patterning (Dickinson and Sive, 2007; Duboc and Logan, 2011; Gage et al., 1999; Smidt et al., 2004b; Westmoreland et al., 2001). It is therefore tempting to speculate that the tissue-specific functions inherent in a single planarian *pitx* gene have been distributed among different vertebrate paralogs during evolution.

How could tissue-specific functions of a single *pitx* gene be regulated in planarians? Our data suggest that *Smed-pitx* might interact with *Smed-islet1* in tail formation and midline patterning, but not in serotonergic neurons (Fig. 7). This raises the possibility that tissue-specific co-factors might control spatial and temporal activation of different sets of *Smed-pitx* target genes. Whether *Smed-pitx* relies on a co-factor during transcriptional regulation in the serotonergic lineage remains to be answered.

## MATERIALS AND METHODS

### Planarians

All animals used in this study were asexual planarians of the species *Schmidtea mediterranea* (clonal line BCN-10) provided by E. Saló and maintained as described (Molina et al., 2007). Animals were starved for 7 days prior to experiments.

### Accession numbers

Amino acid sequences of corresponding vertebrate genes were used for tBLASTN searches against internal transcriptome datasets to identify planarian homologs. Accession numbers: *Smed-pitx* (KC568450), *Smed-aadcb* (KC568452), *Smed-sert* (KC568451), *Smed-vmat* (KC568453), *Smed-islet1* (KC568454), *Smed-vglut* (KC568455). Protein alignment was performed with Clustal Omega software.

### WISH

WISH was carried out as previously described (Nogi and Levin, 2005; Umesonu et al., 1999). For FISH, animals were processed as previously described (Cebrià and Newmark, 2005; Pearson et al., 2009) using tyramide signal amplification (Perkin Elmer) according to the manufacturer's instructions. Probes used for DFISH were labeled with digoxigenin (DIG) or dinitrophenyl (DNP) (Mirus DNP Labeling Kit). After incubation in anti-DIG-POD (poly, 1:100; 11633716001, Roche) or anti-DNP-HRP (1:100; FP1129, Perkin Elmer) samples were washed in PBS/0.1% Tween 20 for 2 hours and were developed with FITC-tyramide or Cy3-tyramide (Perkin Elmer). The first color reaction was quenched with 1% H<sub>2</sub>O<sub>2</sub> in PBS/0.1% Tween 20 for 45 minutes, followed by 10 minutes at 56°C in 50% formamide/2×SSC/1% Tween 20. Primers used for probe synthesis are listed in supplementary material Table S1.

### Immunostaining

Immunostainings were performed as described (Cebrià and Newmark, 2005). Antibodies used were anti-SYNORF (1:50; 3C11, Developmental

Studies Hybridoma Bank), rabbit anti-SMEDWI-1 [1:1000; synthesized against a previously published peptide (Guo et al., 2006)] and rat anti-BrdU (cross-reacts with CldU; OB0030G, Serotec). Secondary antibodies were Alexa 488 goat anti-mouse, Alexa 647 goat anti-rabbit and Alexa 568 goat anti-rat (Molecular Probes). Nuclear staining was performed with Hoechst 33342 (Life Technologies).

### CldU labeling

For continuous CldU labeling, animals were injected three times a day (every 6-8 hours) with 96 nl 5 mg/ml CldU (Sigma-Aldrich) solution in water with 0.75% DMSO for 4 days (Newmark and Sánchez Alvarado, 2000). After 4 days, animals were processed and stained as for FISH with a *Smed-sert* DNP-labeled probe. After FISH staining animals were washed in PBS/0.1% Triton X-100 (PBSTx) four times for 10 minutes each. Animals were then incubated in 2 N HCl/PBSTx for 30 minutes at room temperature followed by four washes of 10 minutes in PBSTx. Samples were then blocked for 1 hour at room temperature in 1% BSA/PBSTx and processed for immunostaining against CldU as described above.

### Irradiation

Irradiation was performed in a Gammacell 40 Exactor (Nordion) with two caesium-137 sources delivering ~92 rads/minute for ~65 minutes for lethal irradiation.

### TUNEL staining

TUNEL staining to label apoptotic cells was performed as described (Boutros et al., 2004; Pellettieri et al., 2010; Sánchez Alvarado and Newmark, 1999).

### Microscopy

Live images were taken with a Leica M80 microscope. Image of WISH samples were captured with a Leica M165 FC microscope. Fluorescent images were acquired with a Zeiss LSM700 confocal laser-scanning microscope.

### RNAi

dsRNA microinjection was performed as described previously (Sánchez Alvarado and Newmark, 1999). dsRNAs were synthesized as described (Boutros et al., 2004). Control animals were injected with dsRNA against green fluorescent protein (*gfp*). Injected planarians were either amputated pre- and post-pharyngeally and left to regenerate for the time indicated, or were left uncut for the indicated times for observation of a homeostatic phenotype. Primers for RNAi probes are listed in supplementary material Table S1.

### Illumina paired-end sequencing and differential expression analysis

For RNA-seq analysis, planarians were injected six times with dsRNA during a 2-week period to knockdown *Smed-pitx*. The tails of injected planarians were amputated and left to regenerate for 3 days. Two biological replicates of 20 tail fragments each were analyzed and compared with the same number of *gfp* dsRNA-injected controls. Illumina RNA-seq libraries for paired-end sequencing were prepared according to the TruSeq RNA Sample Preparation Kit v2. RNA was sequenced on an Illumina HiSeq2500. Binary base call files were processed into FASTQ files using the Illumina CASAVA software package (version 1.8.2). Reads were mapped to an in-house assembled transcriptome using Bowtie 2 (version 2.0.0-beta6) in end-to-end mode with default options. Mapped reads were then counted up using a custom Python script that interfaced with HTSeq (version 0.5.3p3) in order to produce a table of counts by experiment and transcript. The table of transcript counts was processed using the R Bioconductor package DESeq. Experiment reads were normalized by DESeq using the 'shorth' method, with biological replicates grouped as the same experiment. Experiments were compared using the negative binomial function of DESeq with default options. Sequencing reads are available from the NCBI Sequence Read Archive (accession number: SRA090982).

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## Competing interests

The authors declare no competing financial interests.

## Author contributions

M.M. and K.B. designed and interpreted the experiments and wrote the manuscript. M.M. and F.S. conducted the experiments.

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## Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.100081/-DC1>

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