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Transcription factors *lhx1/5-1* and *pitx* are required for the maintenance and regeneration of serotonergic neurons in planarians

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SUMMARY

In contrast to most adult organisms, freshwater planarians can regenerate any injured body part, including their entire nervous system. This allows for the analysis of genes required for both the maintenance and regeneration of specific neural subtypes. In addition, the loss of specific neural subtypes may uncover previously unknown behavioral roles for that neural population in the context of the adult animal. Here we show that two homeodomain transcription factor homologs, *Smed-lhx1/5-1* and *Smed-pitx*, are required for the maintenance and regeneration of serotonergic neurons in planarians. When either *lhx1/5-1* or *pitx* was knocked down by RNA interference, the expression of multiple canonical markers for serotonergic neurons was lost. Surprisingly, the loss of serotonergic function uncovered a role for these neurons in the coordination of motile cilia on the ventral epidermis of planarians that are required for their nonmuscular gliding locomotion. Finally, we show that in addition to its requirement in serotonergic neurons, *Smed-pitx* is required for proper midline patterning during regeneration, when it is required for the expression of the midline-organizing molecules *Smed-slit* in the anterior and *Smed-wnt1* in the posterior.

KEY WORDS: Serotonergic neurons, lhx1/5, *pitx*, Regeneration, Locomotion, Cilia, Metachronal synchrony, Lophotrochozoan, Planarian, *Schmidtea mediterranea*

INTRODUCTION

The form and function of any individual neuron is determined by its very specific gene-expression signature: the combinatorial expression of particular enzymes, receptors and structural proteins defines its unique neural identity (Hobert, 2011; Hobert et al., 2010). This neural identity is coordinated and maintained throughout the lifespan of the neuron by the expression of terminal selector transcription factors, and in some cases a single terminal selector gene can regulate the expression of hundreds of downstream effector genes (Eastman et al., 1999; Etchberger et al., 2007; Flames and Hobert, 2009; Hobert, 2011; Liu et al., 2010; Uchida et al., 2003). The instructive power of these regulatory factors has become increasingly clear with recent studies showing that the ectopic expression of terminal selector genes can directly reprogram germ cells and fibroblasts into fully differentiated neurons (Patel et al., 2012; Tursun et al., 2011; Vierbuchen et al., 2010). Considering the relatively poor regenerative abilities of the human central nervous system (CNS), there is a great therapeutic interest in the ability to convert a patient's own skin cells into missing neural cell types using specific combinations of transcription factors. However, before such practices are possible, we require greater understanding of how terminal selectors function during neural regeneration and how these regenerating neurons integrate themselves into the existing neuronal circuitry. To this end, highly regenerative model systems excel to allow dissection of the molecular mechanisms underpinning regeneration and cell-fate specification.

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The freshwater planarian *Schmidtea mediterranea* (*S. med*), is a free-living flatworm of the lophotrochozoan superphylum and is well known for its ability to regenerate any missing body part as an adult, including the entire CNS and peripheral nervous system (PNS) (Cebrià, 2007; Cebrià, 2008; Cebrià et al., 2002; Gentile et al., 2011; Reddien and Sánchez Alvarado, 2004). This regenerative ability is made possible by a large population of pluripotent adult stem cells (ASCs) (Sánchez Alvarado and Kang, 2005; Wagner et al., 2011). *S. med* is a constitutive asexual adult and is amenable to gene-function studies by use of RNA interference (RNAi), which is fed to adult animals and subsequently spreads to all tissues, including neurons (Sánchez Alvarado and Newmark, 1999). Therefore, planarians are an ideal model system to study the molecular mechanisms of neuronal function, specification and regeneration, all in an adult animal *in vivo*. Multiple studies have previously shown that the planarian nervous system contains common invertebrate neuronal subtypes, which exist in specific ratios in the brain and PNS (Agata et al., 1998; Cebrià et al., 2002; Nishimura et al., 2007; Nishimura et al., 2008b; Nishimura et al., 2008c). However, it remains unknown what factors specify each neuronal subtype, how patterning mechanisms during regeneration generate precise subtype ratios, or what the function of each neuronal subtype may be in the larger context of the animal's behavior.

Here we have described the function of two homeodomain transcription factors, *Smed-lhx1/5-1* and *Smed-pitx* (henceforth referred to as *lhx1/5-1* and *pitx*), which were required to regulate the maintenance and regeneration of planarian serotonergic neurons. We found that *lhx1/5-1* and *pitx* were expressed in all fully differentiated serotonergic neurons, where they act to maintain the expression of multiple canonical serotonergic marker genes that designate the unique identity and function of this neural cell type. We also show that specifically during regeneration, *lhx1/5-1* and *pitx* were co-expressed with the stem cell marker *smedwi-1* in a

novel progenitor cell. When either *lhx1/5-1* or *pitx* was knocked-down by RNAi, we observed the rapid loss of functional serotonergic neurons. Surprisingly, we observed that the loss of serotonergic neurons blocked the ability of ventral motile cilia to drive planarian locomotion. We further showed that the locomotion defect was characterized not by the loss of motile cilia, but instead by the loss of metachronal synchrony across the ventral epidermis. Together, these results showed that serotonergic neuronal inputs can be used across large distances to coordinate and tune the beating of cilia in order to produce directed movement. Finally, we demonstrated that in addition to its role in serotonergic neurons, *pitx* was also required during regeneration to specify the planarian midline by controlling *Smed-slit* expression in the anterior and *Smed-wnt1* in the posterior.

MATERIALS AND METHODS

Phylogenetics and cloning

Lhx and Pitx gene family homologs were found by homology in the sequenced and assembled planarian genome, as previously described (Pearson and Sánchez Alvarado, 2010; Robb et al., 2008; Sánchez Alvarado et al., 2002; Sánchez Alvarado et al., 2003). Primers were designed and full-length genes were cloned by 3′ rapid amplification of cDNA ends (RACE). Cloned open reading frames (ORFs) were then converted to predicted proteins and subjected to Bayesian phylogenetic analyses for Lhx family members. Protein sequences used in phylogenies were obtained from the NCBI Entrez protein database or directly from the genome sequencing projects of included organisms. The program Geneious (www.geneious.com) was used with the MUSCLE alignment plugin and two tree building plugins for Geneious were used as independent analyses. Both maximum likelihood and Bayesian analyses were performed with the following settings: maximum likelihood – 10,000 bootstrap replicates, WAG substitution model, estimated distances; Bayesian – 1.1 million replicates, WAG substitution model, 4 heated chains, 25% burn-in, subsample frequency of 1000. Consensus tree images were saved through Geneious, which were then manipulated in Adobe Photoshop. Alignment of the Pitx homeodomain is shown in supplementary material Fig. S3. *Smed-lhx1/5-1*, *Smed-pitx*, *Smed-tph*, *Smed-aadc*, *Smed-vmat*, *Smed-sert*, *Smed-kchan*, *Smed-hmctn*, *Smed-upps*, *Smed-asic* and *Smed-sh3bp2* sequences were deposited in GenBank as accession numbers: KF134112-KF134122. Transcripts listed in supplementary material Tables S1-S3 can be found in the transcriptome database associated with the NCBI GEO project: GSE47348.

Animal husbandry and RNAi

Asexual individuals of *S. med* CIW4 strain were reared as previously described (Sánchez Alvarado et al., 2002). RNAi experiments were performed using previously described expression constructs and HT115 bacteria (Newmark et al., 2003). Briefly, bacteria were grown to an OD_{600} of 0.8 and induced with 1 mM IPTG for 2 hours. Bacteria were pelleted and mixed with liver paste at a ratio of 500 µl liver per 100 ml original culture volume. Bacterial pellets were thoroughly mixed into the liver paste and frozen as aliquots. The negative control, *control*(*RNAi*), was the *unc22* sequence from *C. elegans* as previously described (Reddien et al., 2005a). All RNAi food was fed to 7-day starved experimental worms every third day for five total feedings. Nomenclature denotes time of staining and number of feeds (e.g. 5fd10 is equal to 10 days after the fifth feed). Amputations were performed 3 days after the final feeding unless noted otherwise. All animals used for immunostaining were 2-3 mm in length and size-matched between experimental and control worms.

Deep sequencing and statistical analysis

RNA deep sequencing (RNA-seq) was performed on whole animals 12 days following RNAi feedings (5fd12) for: *control*(*RNAi*), *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*). Experiments were performed in experimental triplicate and sequenced to a depth of ~50 million reads per sample, and multiplexed on an Illumina HiSeq2000 with 50 bp single-end reads. Sequences were then aligned to a previously described planarian transcriptome with the program Bowtie2 (Labbé et al., 2012; Langmead et al., 2009). To determine significantly up- or downregulated transcripts between experimental animals and controls, the freely available baySeq software was used (http://www.bioconductor.org/packages/2.11/bioc/html/baySeq.html) (Hardcastle and Kelly, 2010). Full sequences and results were submitted to the NCBI bioproject PRJNA205281.

Immunolabeling and *in situ* hybridizations

Whole-mount *in situ* hybridization (WISH), double-fluorescence *in situ* hybridization (dFISH) and immunostaining were performed as previously described (Lauter et al., 2011; Pearson et al., 2009). Colorimetric WISH stains were imaged on a Leica M165 fluorescent dissecting microscope. dFISH and fluorescent anti-α-tubulin (Iowa Hybridoma Bank; 12G10) stains were imaged on a Leica DMIRE2 inverted fluorescence microscope with a Hamamatsu Back-Thinned EM-CCD camera and spinning disc confocal scan head. Cell counts and colocalizations were quantified using freely available ImageJ software (NIH). Significance was determined by a two-tailed Student's *t*-test. All experiments were, at minimum, triplicated and at least five worms were used per stain and per time point. All labeling images were post-processed using Adobe Photoshop.

High-speed video microscopy

To visualize beating cilia, live worms were first placed on a microscope slide with a small amount of planarian culture water, and a coverslip with Vaseline on each corner was placed directly onto the worm to immobilize the specimen during video recording. A Leica DMRA2 compound microscope was used to visualize beating cilia along the lateral body edge of the planarian head region under DIC optics at 60× magnification. Videos were captured at 180 frames per second using a Flare 4M180CCL digital camera (IO Industries, www.ioindustries.com) and were edited with Virtualdub (www.virtualdub.org) to adjust for brightness/contrast, crop the specific region of interest and decompile videos for the isolation of individual jpeg images. In order to determine ciliary beat frequency, a small rectangular region of interest perpendicular to the lateral body wall was selected from each video, and this was used to generate kymographs using ImageJ software (NIH) and methods as previously described (Dentler et al., 2009). From the kymograph images, the number of cycles that individual motile cilia are able to complete during the selected time frame of the video segment were counted, and the beat frequency was calculated for that particular specimen.

Serotonin antagonists and agonists

In order to disrupt serotonin signaling, the serotonin antagonist mianserin hydrochloride (Sigma) was added to wild-type worms at a final concentration of 20 µM. Mianserin-treated worms began displaying defective cilia-based locomotion within seconds after exposure (supplementary material Movie 7). Additionally, the addition of ectopic serotonin hydrochloride (Sigma) at a final concentration of 100 µM was able to interrupt normal cilia-based locomotion within minutes after exposure. This same concentration of serotonin hydrochloride was used in the RNAi rescue experiments to intermittently restore cilia-driven locomotion in *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms.

RESULTS

Planarians express canonical markers of serotonergic neurons in *tph*$^+$ cells

Planarian serotonergic neurons have been described as spatially restricted to the ventral side of the worm with their axonal projections extending across the mediolateral body axis (Fraguas et al., 2012; Nishimura et al., 2007). In order to confirm that this neuronal population is truly serotonergic and conserved at a molecular level with other species, the planarian homologs of four canonical genes required for the biosynthesis, packaging and reuptake of the serotonin neurotransmitter were cloned. *Smed-tryptophan hydroxylase* (*tph*) and *Smed-amino acid decarboxylase* (*aadc*) are the enzymes that catalyze the conversion of the amino acid tryptophan to the intermediate 5-hydroxytryptophan (5-HTP),

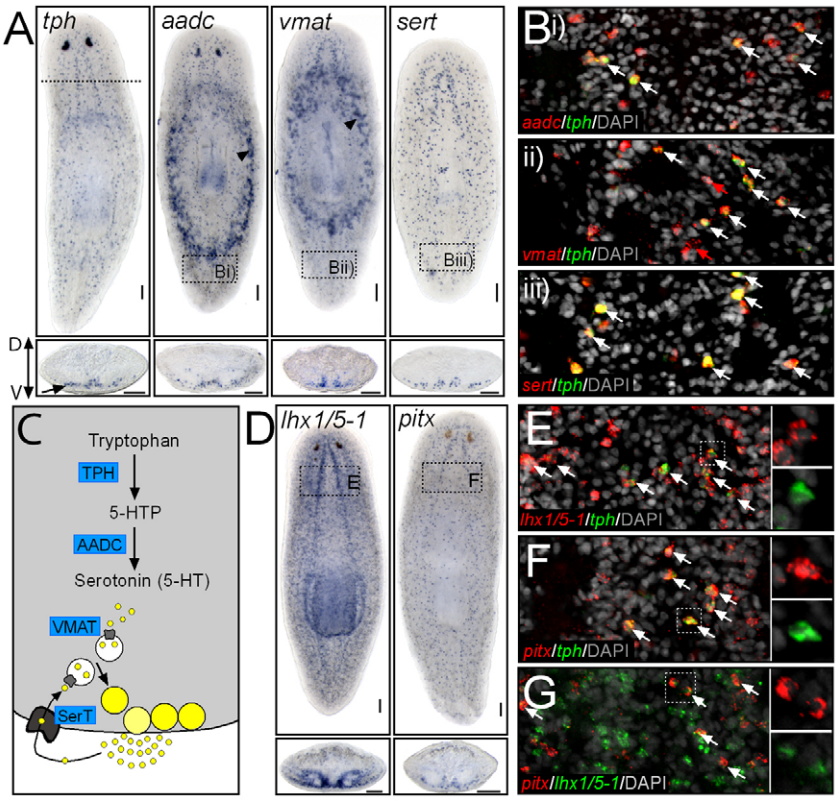

Fig. 1. Expression patterns of serotonergic neural markers, *lhx1/5-1*, and *pitx*. (**A**) WISH for *tph*, *aadc*, *vmat* and *sert*. All whole-mount stained images are ventral views with the anterior of the worm at the top. Bottom panels are transverse sections of the worm at the site indicated by the dashed line, with the ventral surface at the bottom. Ventrally located neurons are indicated by the black arrow. (**B**) dFISH images showing colocalization (white arrows) between (i) *aadc* and *tph*, (ii) *vmat* and *tph* and (iii) *sert* and *tph*. Images are single confocal slices of the ventral surface taken from the tail region as indicated by the dashed boxes in A. Red arrows in ii highlight *vmat*$^{+}$ cells that are not serotonergic neurons. (**C**) Cartoon depicting the canonical cellular functions of *tph*, *aadc*, *vmat* and *sert* in the context of the axon terminal of a serotonergic neuron. (**D**) WISH images of *lhx1/5-1* and *pitx*, with transverse sections in bottom panels. (**E-G**) dFISH images showing colocalization between: (E) *lhx1/5-1* and *tph*, (F) *pitx* and *tph*, and; (G) *pitx* and *lhx1/5-1*. Inset panels depict single channel images from dashed boxes in E-G. Scale bars: 100 µm.

and ultimately into serotonin (5-HT) (Deneris and Wyler, 2012; Nishimura et al., 2007). *Smed-vesicular monoamine transporter* (*vmat*) packages these serotonin molecules into presynaptic vesicles that are ready for release into the synaptic cleft, and the *Smed-serotonin transporter* (*sert*) recycles these released serotonin molecules back into the presynaptic terminal to be repackaged (Fig. 1C) (Deneris and Wyler, 2012).

tph, *aadc*, *vmat* and *sert* displayed similar gene expression patterns to each other by WISH, characterized by punctate staining throughout the body of the worm. It should be noted that large secretory peripharyngeal cells also expressed *tph*, *aadc* and *vmat* in the middle of the animal (Fig. 1A). However, the ventral restriction of these markers was easily visible when viewed in transverse sections taken through the medial region of the planarian brain (Fig. 1A). dFISH demonstrated the overlapping expression of *tph* with *aadc*, *vmat* and *sert*, confirming that these four genes were all expressed in mature, *tph*$^{+}$, serotonergic neurons (Fig. 1B). Whereas *tph*, *aadc* and *sert* specifically marked serotonergic neurons, *vmat* was also expressed by dopaminergic and octopaminergic neurons, as expected (supplementary material Fig. S1) (Nishimura et al., 2008a; Nishimura et al., 2007).

lhx1/5-1 and *pitx* are co-expressed in ventrally located serotonergic neurons

The Lhx and Pitx homeodomain transcription factors have well-established roles in vertebrate neuronal differentiation, and therefore represented good candidates for having a similar function in the planarian nervous system (Miquelajáuregui et al., 2010; Pillai et al., 2007; Smidt et al., 2004; Zhao et al., 2007). Predicted homologs to these gene families were first identified in the *S. med* genome based on homology to the homeodomain sequence for Pitx and the homeodomain plus LIM domains that are characteristic of Lhx proteins. Eleven planarian *lhx* genes were identified and, based on phylogenetic analysis, representatives of all six major subfamilies were found to exist in planarians (supplementary material Fig. S2). Only one planarian homolog of the *pitx* gene family was identified based on the characteristic lysine residue at position 50 of the homeodomain, which is a hallmark of the *pitx* and *bicoid*-like transcription factors (supplementary material Fig. S3) (Eastman et al., 1999; Lamonerie et al., 1996).

WISH analyses showed that *lhx1/5-1* and *pitx* both exhibited a punctate expression pattern in ventrally located cells, similar to the distribution of serotonergic neurons (Fig. 1D). dFISH analyses for the serotonergic marker *tph* and either *lhx1/5-1* or *pitx* confirmed their expression in this mature neural cell type (245/246 *tph*$^{+}$ neurons also expressed *lhx1/5-1* (99.59%); 193/194 *tph*$^{+}$ neurons also expressed *pitx* (99.48%) (Fig. 1E,F). As expected, dFISH for both *lhx1/5-1* and *pitx* showed that the two transcription factors also had overlapping expression patterns with each other, in which 79.9% of all *pitx*$^{+}$ cells were *lhx1/5-1*$^{+}$ and 39.5% of all *lhx1/5-1*$^{+}$ cells were *pitx*$^{+}$ when quantified in the posterior region of the animal (Fig. 1G; supplementary material Fig. S9). It should be noted that a significant amount of *lhx1/5-1* and *pitx* expression was outside of the serotonergic cell compartment. It is unknown what these additional, nonserotonergic cells represent, but at least some are cholinergic and GABAergic neurons (supplementary material Fig. S4). Nonetheless, these results showed that virtually all serotonergic neurons expressed both *lhx1/5-1* and *pitx* transcription factors, making these ideal candidates to regulate aspects of serotonergic identity or function.

lhx1/5-1 and *pitx* are required for the maintenance of serotonergic neural identity in uninjured animals

To understand perturbations in the serotonergic neuronal population, it was first important to establish the amount of normal

cell turnover (homeostasis) for this cell type. When planarians are irradiated with a lethal dose of γ-irradiation (60 Gray), all stem cells are eliminated within 48 hours and no more differentiated cell types can be produced (Bardeen and Baetjer, 1904; Eisenhoffer et al., 2008; Reddien et al., 2005b). Consequently, irradiated worms are unable to survive much longer than 2 weeks. Following irradiation, we observed no detectable changes in the serotonergic neuron population over the course of 2 weeks (supplementary material Fig. S5). This result showed that planarian serotonergic neurons are a stable, long-lived cell type, and that any changes to this cell population within 2 weeks would not be the result of physiological cell turnover.

To determine whether RNAi to *lhx1/5-1* or *pitx* would disrupt the serotonergic neuron population, uninjured worms were fed RNAi five times and then were examined 10 days later (5fd10) (Fig. 2A) for any changes in their expression of the four described markers of serotonergic neurons (Fig. 1A). Following RNAi, the expression of *tph* and *aadc* in the ventrally located serotonergic neurons was completely lost in *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms, whereas the changes in expression of *vmat* and *sert* were less dramatic but still significantly reduced (Fig. 2B). In order to quantify the reduced expression of *vmat* and *sert* in RNAi-treated worms, dFISH experiments were performed to accurately count the number of cells expressing these two markers in relation to *tph*. In the post-pharyngeal tail region of *control*(*RNAi*) worms, an average of 54.8±4.6 *vmat*$^{+}$ cells were observed, with the majority being *vmat*$^{+}$*tph*$^{+}$ serotonergic neurons (35/54.8) and the remaining ~20 cells being *vmat*$^{+}$*tph*$^{-}$. However, in the post-pharyngeal tail region of *lhx1/5-1*(*RNAi*) or *pitx*(*RNAi*) worms we observed a significant drop in the average number of total *vmat*$^{+}$ cells to 26.5±2.1 and 22±5.7 cells respectively, all of which were *vmat*$^{+}$*tph*$^{-}$ (supplementary material Fig. S6). In the tail region of *control*(*RNAi*) worms we observed on average 48 *sert*$^{+}$ cells, all of which were *tph*$^{+}$. By contrast, in *lhx1/5-1*(*RNAi*) worms we only observed ~12 *sert*$^{+}$ cells on average, all of which were *tph*$^{-}$, and in *pitx*(*RNAi*) worms *sert* expression was completely lost (Fig. 2B; supplementary material Fig. S7). It should be noted that markers for other neural subtypes were not affected in either RNAi condition with the exception of a ventral GABAergic subpopulation in *lhx1/5-1*(*RNAi*) worms (supplementary material Fig. S8). These data showed that *lhx1/5-1* and *pitx* transcription factors were required in serotonergic neurons to maintain the identity of this particular neural subtype by coordinating the expression of multiple genes.

Considering that *lhx1/5-1* and *pitx* were responsible for the expression of several serotonergic neural marker genes, we next tested whether these transcription factors were also required for the maintenance of generic neuronal identity or survival. We took advantage of the fact that *lhx1/5-1*(*RNAi*) worms displayed an incomplete knockdown of *sert*, which allowed us to examine the state of the remaining RNAi-treated serotonergic neurons. In the tail region of *control*(*RNAi*) worms, every serotonergic neuron also expressed the pan-neural marker *Smed-prohormone convertase 2* (*pc2*) (46/46), and 41/46 of the *sert*$^{+}$*pc2*$^{+}$ neurons displayed visible α-tubulin based axon tracts (Fig. 2C) (Collins et al., 2010; Robb and Sánchez Alvarado, 2002). Similarly in *lhx1/5-1*(*RNAi*) worms, all of the *sert*$^{+}$ RNAi-treated neurons that we observed (28/28) maintained the expression of *pc2*, and had visible axons (Fig. 2C). These data showed that *lhx1/5-1* and *pitx* function to maintain the specific identity and function of serotonergic neurons, whereas the maintenance of generic neuronal properties are likely to be under the control of other transcription factors.

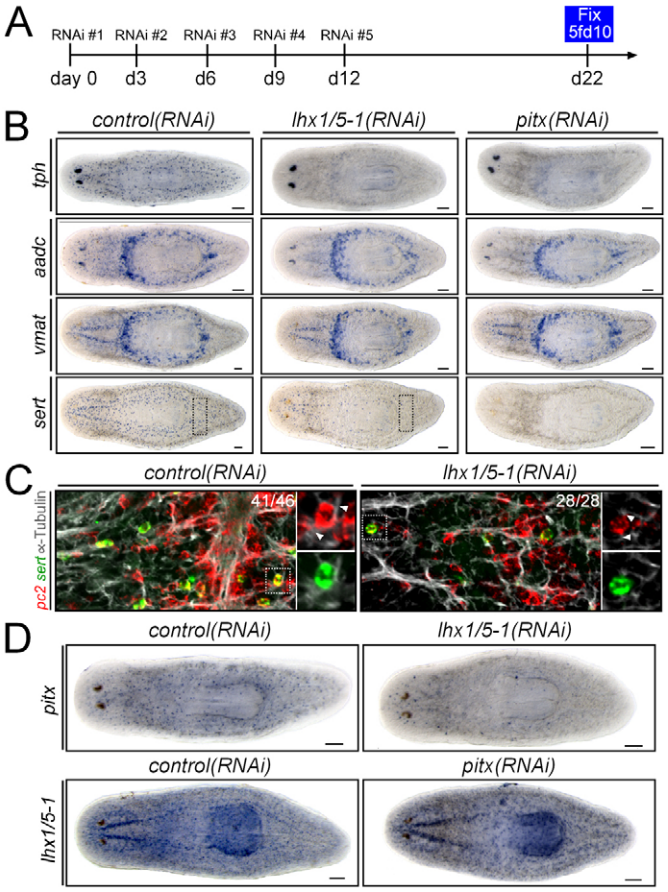

Fig. 2. Molecular analysis of *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms. (**A**) Outline of RNAi experiments, showing schedule of RNAi feedings and the time point at which all *in situ* experiments were performed (5fd10). (**B**) WISH images of *tph*, *aadc*, *vmat* and *sert*, showing the decreased expression of these serotonergic neural markers in *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms compared with *control*(*RNAi*) worms. Note that remaining *tph*, *aadc* and *vmat* staining in RNAi-treated worms is in other tissues besides serotonergic neurons (peripharyngeal cells and eyes). (**C**) dFISH images show *pc2* and *sert* expression along with antibody staining of α-tubulin demonstrate that serotonergic neurons in *control*(*RNAi*) and *lhx1/5-1*(*RNAi*) worms both retain pan-neuronal properties. Images are confocal slices taken from tail region highlighted by the dashed boxes in B. Inset panels depict single channel images from the dashed boxes. Axon tracts for each neuron are highlighted with white arrowheads. (**D**) WISH images showing the loss of expression of *pitx* in lhx1/5-1(RNAi) worms, whereas *lhx1/5-1* expression is largely unaffected in *pitx*(*RNAi*) worms compared to *control*(*RNAi*) worms. All WISH images are ventral views, with the anterior facing to the left. Scale bars: 100 μm.

lhx1/5-1 is required for the expression of *pitx* in serotonergic neurons

Because *lhx1/5-1* and *pitx* were both expressed by planarian serotonergic neurons where they functioned to maintain the identity of this neural cell type (Fig. 1G; supplementary material Fig. 2B), we next tested whether a hierarchy exists between these two transcription factors or whether they function independently. In order to test the regulatory relationship between *lhx1/5-1* and *pitx*, *lhx1/5-1*(*RNAi*) or *pitx*(*RNAi*) worms were examined for any changes to the expression level of the other transcription factor. Although we did not observe any significant change to *lhx1/5-1* expression in *pitx*(*RNAi*) worms, we observed that the expression

level of *pitx* was dramatically downregulated in *lhx1/5-1*(*RNAi*) worms (Fig. 2D). To quantify this result, in the tail region of *control*(*RNAi*) worms, we observed an average of 90.6±9.8 *pitx*$^+$ cells, whereas in *lhx1/5-1*(*RNAi*) worms this number dropped by 75.93% to 21.8±2.7 *pitx*$^+$ cells on average (supplementary material Fig. S9). By contrast, there was no significant decrease in the number of *lhx1/5-1*$^+$ cells in *pitx*(*RNAi*) worms compared to the *control*(*RNAi*) condition (supplementary material Fig. S9). It should be noted that complete elimination of *pitx* expression was not expected because ~20% of its expression does not overlap with *lhx1/5-1* (supplementary material Fig. S9). These data showed that *lhx1/5-1* is required for proper *pitx* expression in serotonergic neurons.

lhx1/5-1 and *pitx* are expressed in the earliest stages of serotonergic neuron regeneration

lhx1/5-1 and *pitx* were required for the long-term maintenance of planarian serotonergic neurons, but it was unclear whether these transcription factors were also involved in the early specification or regeneration of this neuronal subtype. During head regeneration, *lhx1/5-1* and *pitx* expression were observed in head blastemas at 1.5 days after head amputation. By contrast, terminal differentiation markers of mature serotonergic neurons, such as *tph*, were expressed later and became more pronounced ~24 hours after *lhx1/5-1* and *pitx* during regeneration (regeneration day 2.5; Fig. 3B). This time difference between the early expression of *lhx1/5-1* and *pitx* and the appearance of terminal differentiation markers suggested that during regeneration, these transcription factors may be turned on in precursor cells that later give rise to mature serotonergic neurons.

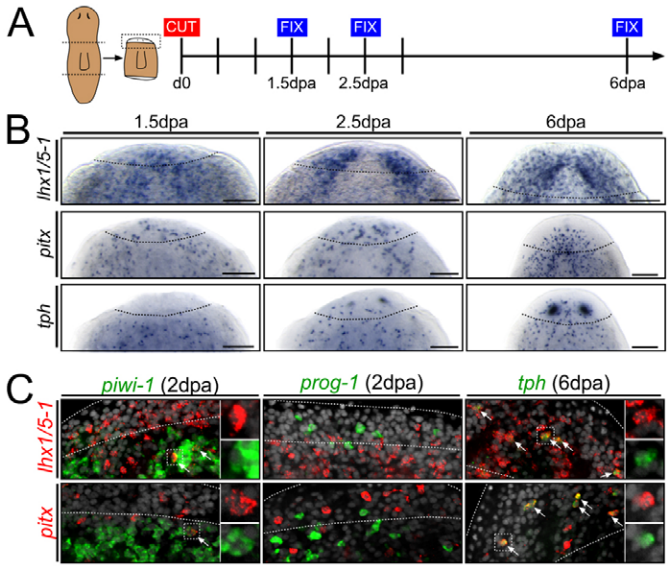

Fig. 3. *lhx1/5-1* and *pitx* are expressed in early serotonergic precursor cells. (**A**) Outline of regeneration series experiment seen in B. The heads and tails of wild-type worms were amputated (day 0) and regenerating 'trunk' fragments were stained at 1.5, 2.5 and 6 dpa. (**B**) WISH images showing the expression of *lhx1/5-1*, *pitx* and *tph* in the regenerating head blastema (as highlighted by the dashed box in A), at the indicated time points. The dashed black line represents the approximate amputation plane. All images are ventral views with the anterior end at the top. (**C**) dFISH images following the expression of *lhx1/5-1* and *pitx*, illustrating colocalization (white arrows) of these two genes with stem cells (*piwi-1*), early progeny (*prog-1*) and mature serotonergic neurons (*tph*). For all dFISH images, dashed white lines represent the body edge of head blastema (top) and the approximate amputation plane (bottom). Inset panels depict single channel images from dashed boxes. Scale bars: 100 µm.

In order to test whether immature serotonergic neural precursor cells exist in the head blastemas of wild-type planarians, animals were examined for any *lhx1/5-1*$^+$ or *pitx*$^+$ cells that also expressed known markers of adult stem cells (*smedwi-1*) (Reddien et al., 2005b) or early stem cell progeny (*Smed-prog-1*) at 2 days post-amputation (dpa) (Eisenhoffer et al., 2008) (henceforth referred to as *piwi-1* and *prog-1*). Even though serotonergic neurons represent a very small proportion of all planarian cells, we observed measurable *piwi-1*$^+$ cells that also expressed the transcription factors *lhx1/5-1* (19/1575 total *piwi-1*$^+$ cells counted) or *pitx* (10/1294 total *piwi-1*$^+$ cells counted) (Fig. 3C). By contrast, the expression of *lhx1/5-1* and *pitx* was conspicuously absent in *prog-1*$^+$ early progeny cells. Only 2/648 *prog-1*$^+$ cells expressed *lhx1/5-1*, and 0/517 *prog-1*$^+$ expressed *pitx* (Fig. 3C). Finally, all newly regenerated serotonergic neurons that expressed the terminal differentiation marker *tph*, also expressed *lhx1/5-1* and *pitx* as expected (Fig. 3C). Of note, we never observed any *piwi-1*$^+$ stem cells that also expressed the terminal differentiation marker *tph* (supplementary material Fig. S10), which suggested that the differentiation process from *lhx1/5-1*$^+$*pitx*$^+$*piwi-1*$^+$ precursor cell to *lhx1/5-1*$^+$*pitx*$^+$*tph*$^+$ mature neuron is a distinct process that does not go through a *prog-1*$^+$ progeny stage.

lhx1/5-1 and *pitx* are required for ventral cilia coordination and gliding behavior

The ventral surface of the planarian epidermis is composed of multiciliated epithelial cells, and in order for normal gliding locomotion to occur, these motile cilia must be formed properly as well as coordinate their beating in 'metachronal synchrony' (Glazer et al., 2010; Rink et al., 2009; Rompolas et al., 2010; Salathe, 2007). If adjacent cilia are not coordinated in their beating, they will not produce net forward movement. Previous studies have shown that RNAi to structural components of planarian cilia such as IFT88 or Iguana leads to the loss of cilia and loss of gliding locomotion (Glazer et al., 2010; Rompolas et al., 2010). However, it is largely unknown how large ciliated epithelia in any species are coordinated across large distances, resulting in directional fluid flow, and in the case of planarians, forward gliding.

In addition to the molecular defects in serotonergic maintenance and regeneration, surprisingly, both *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms displayed severe locomotion defects (Fig. 4A). In contrast to *control*(*RNAi*) worms, which were capable of normal gliding locomotion (supplementary material Movie 1), *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms had no gliding ability, yet muscular movements and 'inch worming' behavior was unaffected (Fig. 4A; supplementary material Movies 2, 3). We hypothesized that the loss of gliding behavior could either be due to loss of cilia, loss of cilia beating or loss of cilia coordination. Because the ventral and dorsal epithelia meet at the lateral edge of the animal, cilia beating can be imaged from a dorsal view (Rompolas et al., 2010). Using high-speed imaging on *control*(*RNAi*) worms, the characteristic wave-like beating pattern of these motile cilia was confirmed, and the ciliary beat frequency (CBF) was calculated to be roughly 25 Hz (Fig. 4B,C; supplementary material Movie 4), which was similar to previously reported CBF measurements in wild-type planarians (Rompolas et al., 2010). When *lhx1/5-1*(*RNAi*) or *pitx*(*RNAi*) worms were imaged, both displayed normal numbers and length of motile cilia that were also beating. This showed that the observed locomotion defects were not due to defects in ciliogenesis, nor the

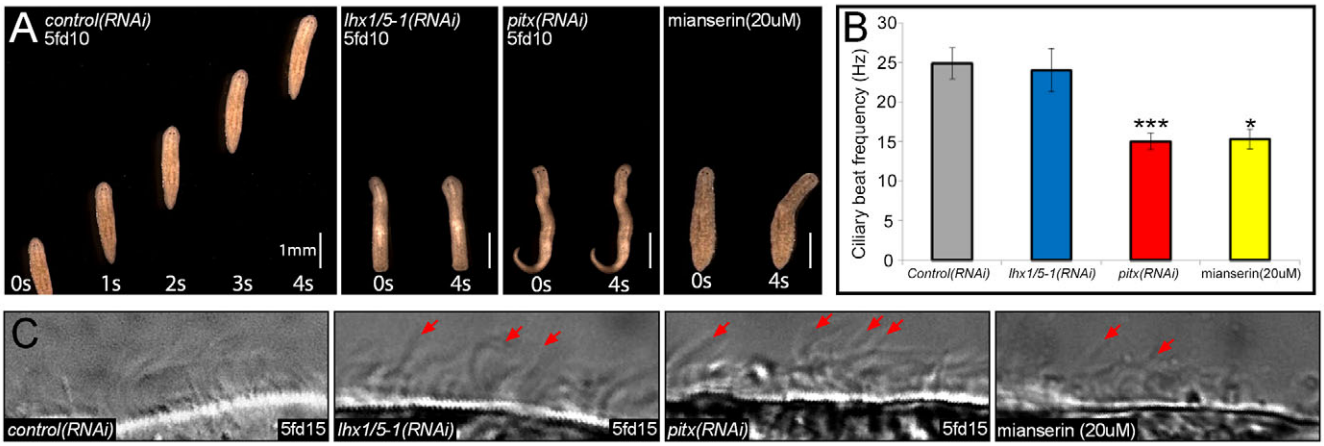

Fig. 4. Analysis of locomotion defects seen in *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms. (**A**) Still images taken from videos depicting the locomotion of *control*(*RNAi*), *lhx1/5-1*(*RNAi*), *pitx*(*RNAi*), and mianserin-treated worms, with corresponding time points indicated at the bottom of each image. (**B**) Quantification of ciliary beat frequency for *control*(*RNAi*) [*n*=4], *lhx1/5-1*(*RNAi*) [*n*=3], *pitx*(*RNAi*) [*n*=3; *P*=0.000484], and mianserin-treated worms [*n*=2; *P*=0.004247]. Statistical differences are measured compared to *control*(*RNAi*) by two-tailed Student's *t*-test and error bars indicate standard deviations. (**C**) Still images from high-speed movies (supplementary material Movies 1-4) of the motile cilia that line the lateral body edge, showing the normal metachronal wave patterns of *control*(*RNAi*) worms, and the uncoordinated beating cilia observed in *lhx1/5-1*(*RNAi*), *pitx*(*RNAi*), and mianserin-treated worms. Individual cilia beating out of phase are highlighted by red arrows. RNAi-treated worms were imaged 15 days after last RNAi feeding (5fd15). Scale bars: 1 mm.

maintenance of ciliary structures (Fig. 4C). However, following RNAi of either transcription factor, we observed significant disruptions to the synchronous beating patterns of motile cilia as characterized by an individual cilium beating out of phase with a neighboring cilium (Fig. 4C, red arrows; supplementary material Movies 5, 6). In addition to the lack of synchrony observed in each RNAi condition, *lhx1/5-1*(*RNAi*) worms did not exhibit any appreciable drop in the CBF (25 Hz), while motile cilia in *pitx*(*RNAi*) worms displayed a significantly lower CBF of 15 Hz (Fig. 4B).

Serotonin signaling is required and sufficient for ciliary locomotion

Considering that both *lhx1/5-1* and *pitx* were expressed in mature serotonergic neurons, which are spatially adjacent to ventral ciliated epithelial cells, the simplest explanation was that these ciliary locomotion defects could be related to dysfunctional serotonin signaling. In order to examine the influence of serotonin on motile cilia dynamics, we exposed wild-type planarians to mianserin, a pharmacological antagonist that has been shown to block serotonin receptors (Kuang et al., 2002). Within seconds, mianserin-treated worms began to exhibit loss of gliding locomotion defects similar to *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms, although muscular inch worming was unaffected (Fig. 4A; supplementary material Movie 7). In addition, mianserin-treated worms displayed uncoordinated motile cilia with a significantly reduced CBF of ~15 Hz (Fig. 2B,C; supplementary material Movie 8). These data strongly suggested that the ciliary locomotion defects observed in *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms were likely to be due to dysfunctional serotonin signaling in neurons rather than defects in cilia or the ciliated epidermis itself. To confirm that ciliary beat frequency coordination depends on serotonin signaling, we hypothesized that too much serotonin would have similar effects on wild-type worms. When serotonin was directly added to wild-type worms, we observed the loss of gliding movement within minutes of addition (supplementary material Movie 9). Surprisingly, when serotonin was added to *lhx1/5-1*(*RNAi*) or *pitx*(*RNAi*) animals that were incapable of gliding, intermittent rescue of gliding was observed (supplementary material Movies 10, 11). Together, these data demonstrated that serotonergic signaling is essential to coordinate cilia beating to produce gliding behavior in planarians.

pitx is expressed and required during the regeneration of the planarian midline

In addition to the serotonergic maintenance and locomotion defects in uninjured worms, *pitx*(*RNAi*) animals displayed dramatic regeneration defects, resulting in the collapse of the planarian midline and the fusion of normally separate bilateral structures. During normal regeneration of the head and tail, bilateral organ systems such as the anterior eyespots, brain ganglia and the posterior gut branches are kept separated by midline mechanisms (Blassberg et al., 2013; Cebrià et al., 2007; Chen et al., 2013; Gurley et al., 2010; Petersen and Reddien, 2009; Rink et al., 2009). As expected, all *control*(*RNAi*) worms regenerated two distinct eyespots (0/33 with cyclopia) in the anterior head blastema, and in the newly formed tail, no cases of gut branch fusion were observed (0/8). By contrast, numerous *pitx*(*RNAi*) worms exhibited regenerating eyespots fused at the midline resulting in 14/43 cyclopic worms, and gut branch fusion events were observed in the majority of *pitx*(*RNAi*) worms (6/7) (Fig. 5A,B). Finally, *pitx*(*RNAi*) worms displayed blunt-end tails with reduced or no apparent posterior blastema (55/56) compared with *control*(*RNAi*) worms, in which only one such case was observed (Fig. 5B).

Cyclopia and gut branch fusion phenotypes observed in *pitx*(*RNAi*) worms appeared similar to defective *slit* signaling, which has been shown to function at the planarian midline to keep these tissues separate (Cebrià et al., 2007). In addition, gut branch fusion events have been observed in cases where the expression of posterior *wnt* genes is disrupted, resulting in abnormal patterns of *slit* expression (Cebrià et al., 2007; Gurley et al., 2010; Petersen and Reddien, 2009). Therefore, *pitx*(*RNAi*) animals were tested for defects in the expression of *Smed-slit* and the midline tail-specific *Smed-wnt1* ligand. During early regeneration (2 dpa), *pitx* was strongly expressed at the dorsal midline of both the anterior and

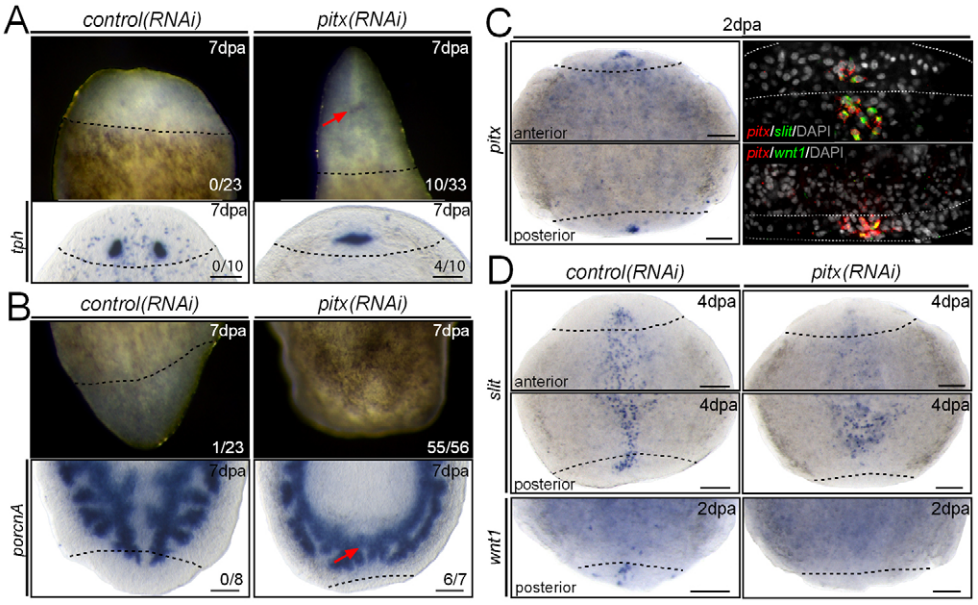

Fig. 5. *pitx* is required for proper midline patterning. (**A**) Top panels are live images of worms 7 days after head amputation, showing the collapse of the regenerating eyespots in *pitx*(*RNAi*) worms (red arrow). Bottom panels are WISH images for *tph*, marking the regenerating eyespots. (**B**) Top panels are live images showing the reduced tail regeneration in *pitx*(*RNAi*) worms. Bottom panels are WISH images for *Smed-porcupine-A* (*porcnA*), demonstrating fusion of the posterior gut branches (red arrow). (**C**) Left panels are WISH images showing the focal expression of *pitx* at the poles of the anterior and posterior blastemas. Right panels are dFISH images showing that the *pitx*-expressing cells at the poles also express *slit* at the anterior pole, and *wnt1* at the posterior pole. (**D**) WISH images showing the reduction of *slit* and *wnt1* expression in *pitx*(*RNAi*) worms compared with *control*(*RNAi*) worms. All images are dorsal views with the anterior at the top. Dashed lines illustrate amputation planes. Scale bars: 100 μm.

posterior blastemas, and dFISH experiments showed that the *pitx*-expressing cells at the anterior pole co-expressed *Smed-slit*, whereas those at the dorsal pole expressed *Smed-wnt1* (Fig. 5C). Consistent with these findings, in *pitx*(*RNAi*) worms, the normal midline expression of *Smed-slit* was significantly decreased in both the anterior and posterior blastemas, and the expression of *Smed-wnt1* at the posterior pole was completely lost (Fig. 5D). These results showed an unexpected role for *pitx* specifically during midline regeneration in planarians, which may represent an ancestral patterning function of the Pitx family of genes, similar to their role in left-right patterning in vertebrates (Campione et al., 1999; Gage et al., 1999).

RNA-seq identified target genes of *lhx1/5-1* and *pitx* and novel serotonergic neural markers

RNA-seq has recently been applied successfully to planarian studies (Abril et al., 2010; Labbé et al., 2012; Sandmann et al., 2011; Solana et al., 2012). However, it is unknown whether RNA-seq has the sensitivity to detect changes in transcript levels in a cell type that represents so few cells in the entire animal (i.e. several hundred serotonergic neurons in an animal that contains ~10^6 total cells). *lhx1/5-1* and *pitx* have both been shown to regulate the identity of planarian serotonergic neurons, but to more broadly understand how they coordinate the expression profile of this neural cell type and to discover additional markers of serotonergic neurons, RNA-seq was performed on *lhx1/5-1*(*RNAi*) or *pitx*(*RNAi*) worms, and then compared to *control*(*RNAi*) worms. From this, 25 transcripts in *lhx1/5-1*(*RNAi*) animals and 14 transcripts in *pitx*(*RNAi*) animals, were found to be significantly downregulated compared with *control*(*RNAi*) worms using baySeq, the most stringent analysis method (Fig. 6A; supplementary material Tables S1-S3). Importantly, *lhx1/5-1* and *pitx* were both 4.5-fold and 14.1-fold downregulated in their own RNAi experiments, respectively. In addition, no known cilia components were significantly changed, which supported the conclusion that cilia were unaffected by *lhx1/5-1* or *pitx* knockdowns. Finally, another positive control for serotonergic neurons, *sert*, was significantly downregulated in both RNAi conditions (Table 1). The other serotonergic markers used in this study were not significantly changed, which we attributed to their remaining expression in peripharyngeal cells or in the optic cups (Fig. 2B). From both RNAi conditions, we cloned and analyzed a total of 13 genes by WISH in wild type and in RNAi animals. We observed that 2/13 genes were co-expressed in tph^+ cells (Fig. 6B), and importantly, were both lost after *lhx1/5-1* and *pitx* RNAi (Fig. 6E). These two genes were homologous to *hemicentin precursor 1* (*hmctn 017595*) and a *voltage-gated potassium channel* (*Kchan 017730*) (Fig. 6E, Table 1). In addition, we found examples of genes that were only regulated by *lhx1/5-1*, such as the *uroporphyrinogen III synthase* (*upps 014402*) homolog that was expressed specifically in peripharyngeal cells or the *acid sensing ion channel 4* (*asic4 009950*) homolog specifically expressed in ventrally located cells that did not express *tph* (Fig. 6C, Table 1). An *SH3 domain-binding protein 2* (*sh3bp2 007007*) homolog was expressed in a few isolated cells within the brain region of wild-type worms and was the only gene in our validated clones to be downregulated specifically in *pitx*(*RNAi*) animals (Fig. 6D, Table 1).

DISCUSSION

Due to its remarkable regenerative capabilities, the adult planarian has proven to be an excellent model organism to study the regeneration of specific cell and tissue types from pluripotent somatic stem cells, including the gut, eye and excretory system (Forsthoefel et al., 2011; Lapan and Reddien, 2011; Scimone et al.,

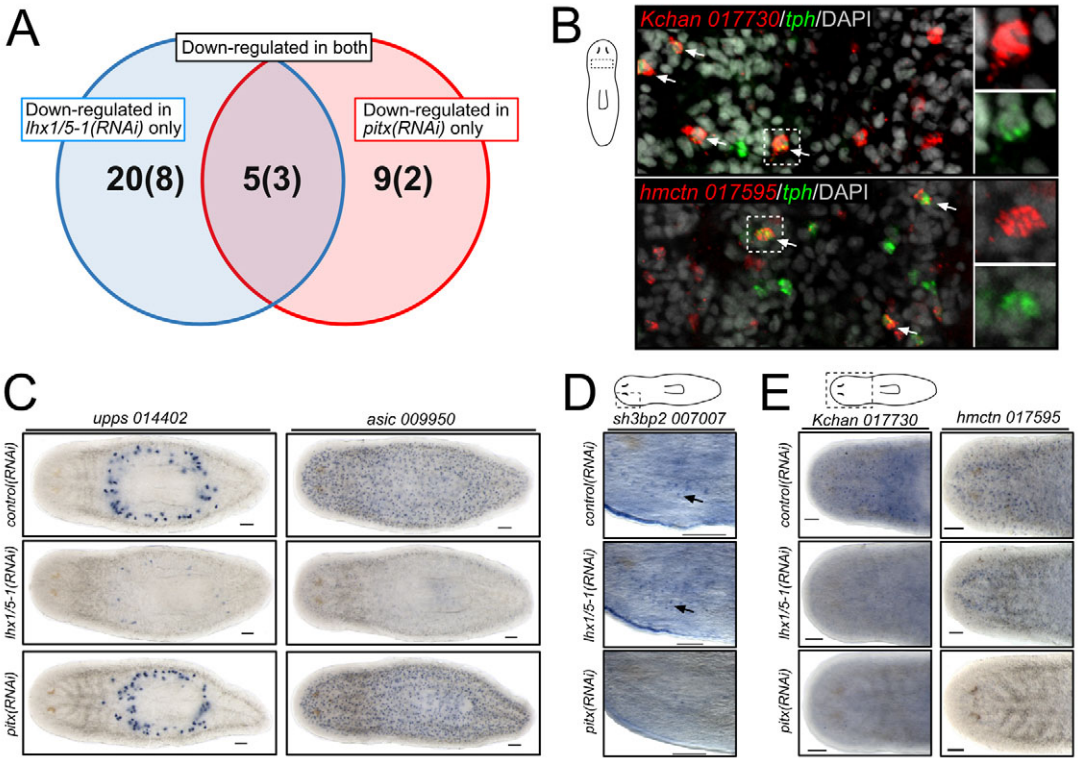

Fig. 6. RNA-seq analysis reveals additional target genes of *lhx1/5-1* and *pitx*. (**A**) Venn diagram demonstrating the number of genes that are found in the RNA-seq analysis to be downregulated in *lhx1/5-1*(*RNAi*) worms (blue), in *pitx*(*RNAi*) worms (red) and in both conditions (middle). The first number represent the predicted number of downregulated genes, while the number in brackets is the number of downregulated that have been validated by WISH. (**B**) dFISH images depicting the expression of two novel gene targets *Kchan 017730* and *hmctn 017595* within *tph*$^{+}$ serotonergic neurons (white arrows). Images are single confocal slices taken from the anterior brain region, and inset panels depict single channel images of dashed boxes. (**C-E**) WISH images depicting decreased expression of novel gene targets in *lhx1/5-1*(*RNAi*) and *pitx*(*RNAi*) worms compared with *control*(*RNAi*) worms. Images in D depict expression of *sh3bp2 007007* in isolated cells within the brain region (black arrows). Images in E highlight the expression of *Kchan 017730* and *hmctn 017595* within the anterior head region. All WISH images are ventral views with the anterior to the left. Scale bars: 100 μm.

2011). However, specific transcription factors that regulate the differentiation of distinct neuronal subtypes in the planarian have not been identified, and thus, it has been difficult to ascribe function to a particular class of neurons. Here we have shown that two homeobox transcription factors, *lhx1/5-1* and *pitx*, are terminal selector genes with important roles in the differentiation and long-term maintenance of planarian serotonergic neurons, which are located throughout the ventral side of the worm adjacent to the ciliated ventral epithelium (Fig. 7A). Similarly to other known terminal selector genes, *lhx1/5-1* and *pitx* function to coordinate the subtype identity and function of serotonergic neurons, but they are not in fact required for their overall pan-neuronal identity (Flames and Hobert, 2009; Hobert et al., 2010; Liu et al., 2010; Uchida et al., 2003). In the absence of either *lhx1/5-1* or *pitx*, serotonergic neurons maintain generic neuronal properties but they lose the expression of important genes required for the biosynthesis and vesicular packaging of the serotonin neurotransmitter (Fig. 7C). Without this crucial input from functional serotonergic neurons, the motile cilia of the ventral epidermis can no longer beat together in metachronal synchrony, and the worms are rendered immobile.

Additional heterogeneity in the *piwi-1*-expressing stem cell population

It is known that at least some planarian stem cells are pluripotent, but whether all *piwi-1*$^{+}$ cells in planarians are true stem cells or restricted progenitor cells has yet to be determined (Guedelhoefer and Sánchez Alvarado, 2012; Wagner et al., 2011). Several studies have recently shown that *piwi-1* does indeed overlap with 'differentiation' markers for the eye and protonephridia, and that this overlap may represent a progenitor cell that has not fully differentiated but is committed to a particular lineage (Lapan and Reddien, 2011; Scimone et al., 2011). Therefore, it is likely that there may be many unappreciated and dedicated *piwi-1*$^{+}$ cells that are restricted to a specific cell fate. Our data support this hypothesis because we showed that *lhx1/5-1* and *pitx* overlap with *piwi-1*$^{+}$ cells specifically when planarians are induced to regenerate serotonergic neurons. Interestingly, we did not observe appreciable co-labeling of *lhx1/5-1* or *pitx* with a known stem cell progeny marker, *prog-1* (Eisenhoffer et al., 2008). This suggests that the specification and differentiation of serotonergic neurons in planarians does not proceed through a *prog-1*$^{+}$ progenitor state (Fig. 7B).

The ancestral Pitx transcription factor may have had roles in both neuronal differentiation and body patterning

Members of the Pitx gene family have wide-ranging functions in vertebrate developmental processes, including the proper patterning of the hindlimb and pituitary gland, and the differentiation of midbrain dopaminergic neurons (Lamonerie et al., 1996; Smidt et al., 2004; Szeto et al., 1999). Interestingly, one family member, *pitx2*, is required

Table 1. Downregulated genes in *lhx1/5-1*(*RNAi*) worms, *pitx*(*RNAi*) worms or in both conditions as validated by WISH analysis

Transcript number	Closest gene homolog in mouse	Fold change in *lhx1/5-1*(*RNAi*)	Fold change in *pitx*(*RNAi*)
Downregulated in *lhx1/5-1*(*RNAi*)			
smed007555	glioma pathogenesis related protein like 1	–12.176	+1.306
smed014402	uroporphyrinogen III synthase (upps 014402)	–10.002	+1.136
smed27634	LON peptidase N-terminal domain and RING finger protein 1	–7.009	–1.079
smed014819	*lhx1/5-1*	–4.471	–1.213
smed009950	acid sensing ion channel 4 (asic4 009950)	–3.421	–1.122
smed015361	acid sensing ion channel 4	–3.001	–1.107
smed013671	immunoglobulin heavy chain	–2.09	–1.004
smed011418	uncharacterized aarF domain protein kinase 2	–4.743	+1.275
Downregulated in both			
smed009244	*Sert*	–1.87	–8.172
smed017730	K^+ voltage-gated channel subfamily A member 2 (Kchan 017730)	–8.702	> –1000
smed017595	hemicentin 1 precursor (hmctn 017595)	+1.02	–2.824
Downregulated in *pitx*(*RNAi*)			
smed006457	*Pitx*	–1.227	–13.972
smed007007	SH3 domain-binding protein 2 (sh3bp2 007007)	–1.218	–1.762

The relative fold-change in expression is based on the RNA-seq dataset.

for both the differentiation of midbrain GABAergic and glutamatergic neurons and also to the nonautonomous establishment of left/right asymmetry in several organ systems (Campione et al., 1999; Waite et al., 2011). Similarly, we have found that the single planarian member of this gene family, *Smed-pitx*, plays dual roles in the terminal differentiation of neurons as well as the nonautonomous separation of bilateral organ systems during regeneration by regulating the midline patterning genes *slit* and *wnt1* (Fig. 7D). Thus, the multiple functions of *Smed-pitx* suggests that the ancestral Pitx gene had roles in both neuronal differentiation and organ patterning, which over time have undergone gene duplications and subfunctionalizations during vertebrate evolution.

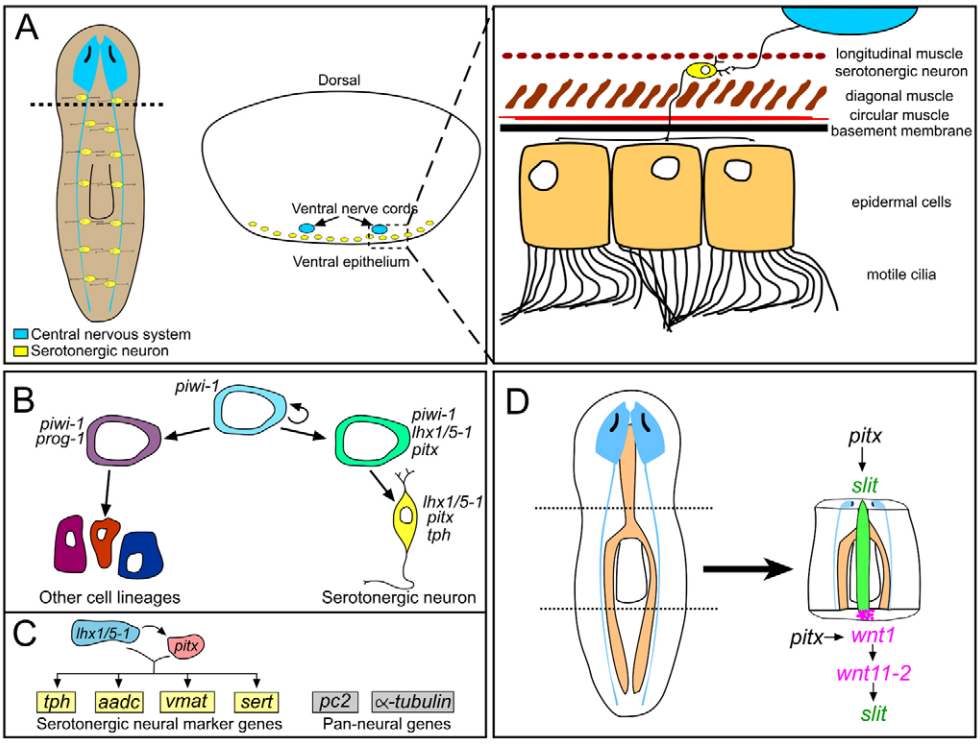

Fig. 7. Models. (**A**) Cartoon image displaying the diffuse distribution of serotonergic neurons throughout the body of the worm (yellow), as well as the basic structures of the central nervous system (blue) including the anterior cephalic ganglia (top) and ventral nerve cords. The next cartoon to the right shows the distribution of serotonergic neurons in relation to the ventral nerve cords in a transverse section and to the right of that, an enlarged depiction of the planarian body wall, taken from the dashed box. Here we see how serotonergic neurons must pass through several outer layers of muscle as well as the basement membrane before it would be able to communicate with the underlying multiciliated epidermal cells. (**B**) Cell lineage diagram as supported by this study, illustrating the heterogeneity observed within the *piwi-1*$^+$ population, as well as the distinct differentiation steps observed along the serotonergic neural lineage. (**C**) Depiction of the hierarchical relationship between *lhx1/5-1* and *pitx*, as well as their regulation of serotonergic neural identity genes, and not pan-neural identity. (**D**) Model showing how *pitx* controls the expression of *slit*, *wnt1* and *wnt11-2*, to ensure the proper separation of bilateral organ systems during regeneration.

Neuronal input is required for metachronal synchrony in planarian multiciliated epithelia

Many examples of epithelia in the animal kingdom rely on synchronously beating motile cilia to generate fluid flow, but the manner in which these vast fields of tiny beating cilia are instructed to beat together has long remained unanswered (Salathe, 2007). Mathematical modeling has suggested that simple hydrodynamic forces acting between neighboring cilia may be sufficient to self-organize metachronal wave patterns (Elgeti and Gompper, 2013; Gueron et al., 1997; Yang et al., 2008). Although it is likely that such hydrodynamic forces may help promote metachronal synchrony, as this represents an energetically favorable state (Gueron et al., 1997; Yang et al., 2008), our data suggest that the coordination of motile cilia requires additional input in the form of serotonergic neurons in order to generate and maintain metachronal synchrony.

Planarian gliding locomotion can be induced in stationary worms by attraction to food sources and avoidance of light (Hyman, 1951). Both of these behavioral responses are initiated in the anterior cephalic ganglia (brain) and then presumably travel via the ventral nerve cords to reach the proper effector organs. Serotonergic neurons are distributed along the ventral surface of the worm, with their axonal projections largely oriented along the mediolateral axis of the worm, creating a neural network capable of transmitting such a message to the ventral epidermis (Fig. 7A) (Fraguas et al., 2012; Nishimura et al., 2007). The precise circuitry of planarian serotonergic neurons has yet to be determined, but in order to communicate with the outer epithelial tissues it is likely that their axon projections traverse through the multiple muscle layers and a basal lamina, before finally communicating with multiciliated epidermal cells (Fig. 7A) (Hyman, 1951). Although it is unknown how serotonin instructs the synchronous beating of motile cilia, we can hypothesize two scenarios: (1) serotonin might be acting as a permissive cue, where at a specific concentration it induces all motile cilia to beat at the same beat frequency; or (2) the finely tuned and rhythmic release of serotonin maintains a constant frequency of ~25 Hz (Rompolas et al., 2010). We believe that serotonin concentration is critical due to the fact that both too much and too little serotonin signaling in planarians results in loss of gliding.

While this is the first example of neuronal input being required for the maintenance of metachronal synchrony, serotonin has been previously reported to influence motile cilia dynamics in both invertebrate and mammalian systems. Similar to planarian locomotion, the pond snail *Lymnea stagnalis* uses a ciliated epidermis to glide across surfaces, and it has been shown that the multiciliated epidermal cells of the snail foot organ are directly innervated with serotonin-containing axon tracts (McKenzie et al., 1998). Direct serotonergic innervation of ciliated epithelial tissues is also observed in the embryo of another pond snail, *Helisoma trivolvis*, in which serotonin release acts to increase the beat frequency of the associated motile cilia (Koss et al., 2003; Kuang et al., 2002). Similarly, when serotonin is applied to cultured sections of mouse trachea, the motile cilia that line the airway epithelium respond by increasing their ciliary beat frequency, resulting in a faster transport of foreign particles (König et al., 2009). This ciliostimulatory role of serotonin observed in several biological contexts points to a conserved role of this neurotransmitter in the regulation of coordinated ciliary behaviors, and opens the possibility that additional neuronal inputs may be functioning to maintain metachronal synchrony in other ciliated epithelia.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
K.W.C. performed all experiments. K.W.C. and B.J.P. interpreted the experimental data and wrote the manuscript.

Supplementary material
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