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PBX/extradenticle is required to re-establish axial structures and polarity during planarian regeneration

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SUMMARY

Recent advances in a number of systems suggest many genes involved in orchestrating regeneration are redeployed from similar processes in development, with others being novel to the regeneration process in particular lineages. Of particular importance will be understanding the architecture of regenerative genetic regulatory networks and whether they are conserved across broad phylogenetic distances. Here, we describe the role of the conserved TALE class protein PBX/Extradenticle in planarians, a representative member of the Lophotrocozoa. PBX/Extradenticle proteins play central roles in both embryonic and post-embryonic developmental patterning in both vertebrates and insects, and we demonstrate a broad requirement during planarian regeneration. We observe that *Smed-pbx* has pleiotropic functions during regeneration, with a primary role in patterning the anterior-posterior (AP) axis and AP polarity. *Smed-pbx* is required for expression of polarity determinants *notum* and *wnt1* and for correct patterning of the structures polarized along the AP axis, such as the brain, pharynx and gut. Overall, our data suggest that *Smed-pbx* functions as a central integrator of positional information to drive patterning of regeneration along the body axis.

KEY WORDS: TALE class, Planarian, Polarity, Regeneration, Stem cells

INTRODUCTION

The ability of some extant taxa to regenerate adult tissues and organs after injury remains poorly understood at the molecular level. Among the Bilateria, planarian flatworms arguably have the most prodigious capacity for regeneration and are potentially immortal as a result (Aboobaker, 2011; Tan et al., 2012). Adult animals consist of many organized differentiated tissues and cell types that are basal to the Bilaterian lineage. These undergo constant replacement and renewal from a pool of totipotent adult stem cells (Wagner et al., 2011) called neoblasts. These life history traits make them suited to investigating the control of stem cell self-renewal and maintenance (Guo et al., 2006; Solana et al., 2009; Wagner et al., 2012), the global control of stem cell differentiation into particular cell types (Scimone et al., 2010), tissues and organs (Lapan and Reddien, 2011; Rink et al., 2011; Scimone et al., 2011), and the signals that underpin the polarity and position of structures along the body axes (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009; Rink et al., 2009; Yazawa et al., 2009; Felix and Aboobaker, 2010; Gaviño and Reddien, 2011; Iglesias et al., 2011; Molina et al., 2011).

Here, we focus on the last of these processes and describe a new component of AP axis regeneration. Previous work has uncovered the central roles of Wnt signaling and Hh signaling in setting posterior polarity. Posterior polarity and regeneration require active Wnt signaling and this, in turn, requires active Hh signaling to be correctly established at posterior wound sites (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Rink et al., 2009; Yazawa et al., 2009). Both ectopic Hh and Wnt signals are able to reprogram anterior wounds such that they produce tails instead of heads (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Rink et al., 2009; Yazawa et al., 2009). Brain regeneration is independent of polarity at early stages of regeneration, as brain tissues differentiate even in the presence of ectopic posteriorizing Wnt and Hh signals (Evans et al., 2011; Iglesias et al., 2011). Abrogation of Wnt signaling by *β-catenin-1(RNAi)* leads to anterior fate being adopted at all wound sites, suggesting that this is a default fate for regeneration in planarians (Iglesias et al., 2008). It has not been convincingly demonstrated that Hh signaling leads to ectopic anterior fates (Rink et al., 2009; Yazawa et al., 2009; Evans et al., 2011), suggesting its primary role with respect to polarity is to correctly establish a second phase of Wnt signaling after wounding (Petersen and Reddien, 2009). During normal regeneration, *notum* normally acts at anterior wounds to inhibit the establishment of Wnt signals and thus promote anterior regeneration (Petersen and Reddien, 2011). In the absence of *notum*, anterior wound sites form tails. Finally, the TALE (three amino acid loop extension) class homeodomain transcription factor *prep* is required for promoting anterior fates, with knockdown leading to a lack of anterior structures in the anterior blastema but without conversion to posterior fates (Felix and Aboobaker, 2010).

Here, we describe the role of another TALE class homeodomain protein, PBX/Extradenticle, in *S. mediterannea*. This transcription factor has been shown to have broad roles in embryonic and post-embryonic patterning events in vertebrates (Karlsson et al., 2010; Capellini et al., 2011; Vitobello et al., 2011) and members of the Ecdysozoa protostome clade (Peifer and Wieschaus, 1990; González-Crespo et al., 1998; Van Auken et al., 2002; Merabet et al., 2005; Yang et al., 2005; Tanaka and Truman, 2007; Prpic and Telford, 2008). Given the growing evidence for roles of other TALE class proteins in regeneration, this gene represented a good candidate for a central role in planarian regeneration (Mercader et al., 2005; Felix and Aboobaker, 2010; Shaikh et al., 2011). We find that *Smed-pbx* is broadly expressed in stem cells and their progeny, and that it is required for AP patterning along the body axis. Our data place PBX as a central regulator that interprets signals along the AP axis, but is not involved in stem cell maintenance, renewal or pluripotency/differentiation. Overall, we describe a key function for

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this important conserved transcription factor during regeneration in a metazoan and in a representative member of the Lophotrocozoan clade of animals.

MATERIALS AND METHODS

Planarian culture and irradiation

A clonal asexual line of *Schmidtea mediterranea* established from a worm provided by Emili Salo's laboratory in Barcelona was maintained as previously described (Felix and Aboobaker, 2010). The animals were starved for 7 days prior to experiments and not fed for the duration of the experiments. Animals were irradiated as previously described (González-Estévez et al., 2012).

Cloning Smed-pbx/extradenticle

Smed-*pbx* (*pbx* for short) was identified previously in a screen for orthologs of TALE class proteins (Felix and Aboobaker, 2010). The complete ORF was assembled and cloned by combining this genomic data with transcriptomic data (Blythe et al., 2010) and confirmed by sequence data generated by RACE (Ambion RLM RACE Kit). Two non-overlapping fragments were amplified by the primer pairs Fwd1-AATAATCATCGATTGAAGCCTGCG and Rev1- CCTTATGCGCTT-ATTGCCAAACCA, and Fwd2-GCACAGGAAGAAGCTAAT and Rev2-GCTATCAAGGATCAAACAC, and cloned as templates for the synthesis of dsRNA and *in situ* hybridization probes. Both fragments produced identical RNAi phenotypes and staining patterns by *in situ* hybridization (Fig. 1; Fig. 2; supplementary material Fig. S1). The complete *pbx* sequence has been submitted to GenBank with Accession Number KC353351.

RNAi

Template DNA was generated by PCR from plasmid templates and this was used as a template for synthesis of dsRNA by reverse transcription using T7 and Sp6 polymerase (New England Biolabs). DsRNA was diluted to the appropriate concentration and microinjected (Sánchez Alvarado and Newmark, 1999). In single and double RNAi experiments, a concentration of 2 µg/µl dsRNA was maintained for each gene injected (supplementary material Fig. S2).

In situ hybridization

Whole mount *in situ* hybridization and *in situ* hybridization on paraffin sections were performed as described previously (Umesono et al., 1997; Cardona et al., 2005; González-Estévez et al., 2009). The following probes were used: *Smed-laminin-*, *Smed-sFRP*, *Smed-FZ4*, *Smed-GPAS*, *Smed-NB.21.11.e*, *Smed-AGAT1*, *Smed-septin*, *Smed-porcn1*, *Smed-Wnt1*, *Smed-Wnt11-2*, *Smed-slit*, *Smed-H2B* and *Smed-notum* (Cebrià and Newmark, 2005; Cebrià et al., 2007; Cebrià and Newmark, 2007; Eisenhoffer et al., 2008; Gurley et al., 2008; Iglesias et al., 2011; Petersen and Reddien, 2011; Solana et al., 2012). RNA *in situ* probes were generated as described previously (González-Estévez et al., 2009). Bright-field images were taken on a Zeiss Discovery V8 using an Axiocam MRC (Carl Zeiss). Bright-field images of adjacent sections were false colored and overlaid to demonstrate colocalization of *pbx* and *Smed-H2B* in neoblasts. For quantification of *Smed-NB.21.11.e*- and *Smed-AGAT1*-expressing cells by fluorescent *in situ* hybridization, maximum projections were created from ~20 1 µm optical sections from the anterior-dorsal domain. ImageJ was used to quantify the number of cells. Confocal laser scanning microscopy was performed using a Leica SP2 confocal microscope (CLSM, Leica Lasertechnik).

Immunohistochemistry

Intact and regenerating planarians were fixed and processed as previously described (Cebrià and Newmark, 2005). Primary antibodies used were: 3C11 (anti-SYNORF1) (Developmental Studies Hybridoma Bank, dilution 1:50), anti-phospho-serine 10 Histone H3 (H3P) (Upstate, dilution 1:500) and anti-acetylated tubulin (Sigma, dilution 1:200). Secondary antibodies used were: Alexa 488-conjugated goat anti-mouse (Molecular Probes, used at 1:400) and Alexa 568-conjugated goat anti-rabbit (Molecular Probes, used at 1:1000). Fluorescent images were acquired using a Leica MZ16F fluorescent stereomicroscope and DFC 300Fx camera (Leica Lasertechnik).

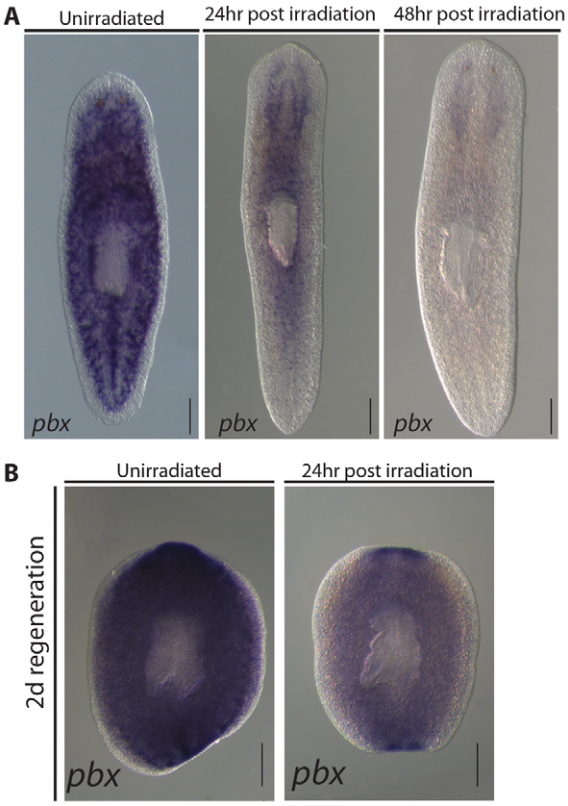

Fig. 1. *pbx* is expressed in irradiation-sensitive cells, the cephalic ganglia and regenerating blastemas. (**A**) *pbx* was expressed throughout the parenchyma of whole worms and resembles the pattern observed for neoblast-expressed genes. Twenty-four hours after a 100 Gy dose of γ-irradiation, most *pbx*-expressing cells are lost, revealing clear expression in the CG. By 48 hours post-irradiation, the distinctive parenchymal expression pattern of *pbx* was completely lost. (**B**) *pbx* expression was broadly observed in both anterior and posterior blastemas of regenerating pieces. Regenerating fragments were γ-irradiated with a dose of 100 Gy at 1 dpa to aid visualization of *pbx* in cells other than neoblasts. Expression was bilateral in both anterior and posterior blastemas, suggesting expression in the regenerating CNS.

Pharynx amputation

Removal of the pharynx was performed using sharpened tungsten dissection needles. Animals were placed ventral upwards on glass slides on a bed of ice. A needle with a hooked end was used to pull the pharynx out of the body. The pharynx was then amputated using a second needle.

Analysis of proliferation

Mitotic figures were visualized by anti-H3P immunostaining and counted. Adobe Photoshop CS4 was used to determine the area of each sample analyzed and the number of mitoses/mm^2 was calculated.

RESULTS

pbx is expressed in neoblasts, the regenerating blastema and the central nervous system

A single ortholog of the *extradenticle/pbx* family was identified and named *Smed-pbx* (*pbx* for short). *pbx* expression in multiple RNAseq experiments was reduced by irradiation or loss of neoblasts induced by genetic means, and enriched in stem cells and stem cell progeny in FACS sorted populations (Blythe et al., 2010; Labbé et al., 2012; Onal et al., 2012; Solana et al., 2012). *In situ* hybridization demonstrated that *pbx* is expressed broadly in intact planarians in a

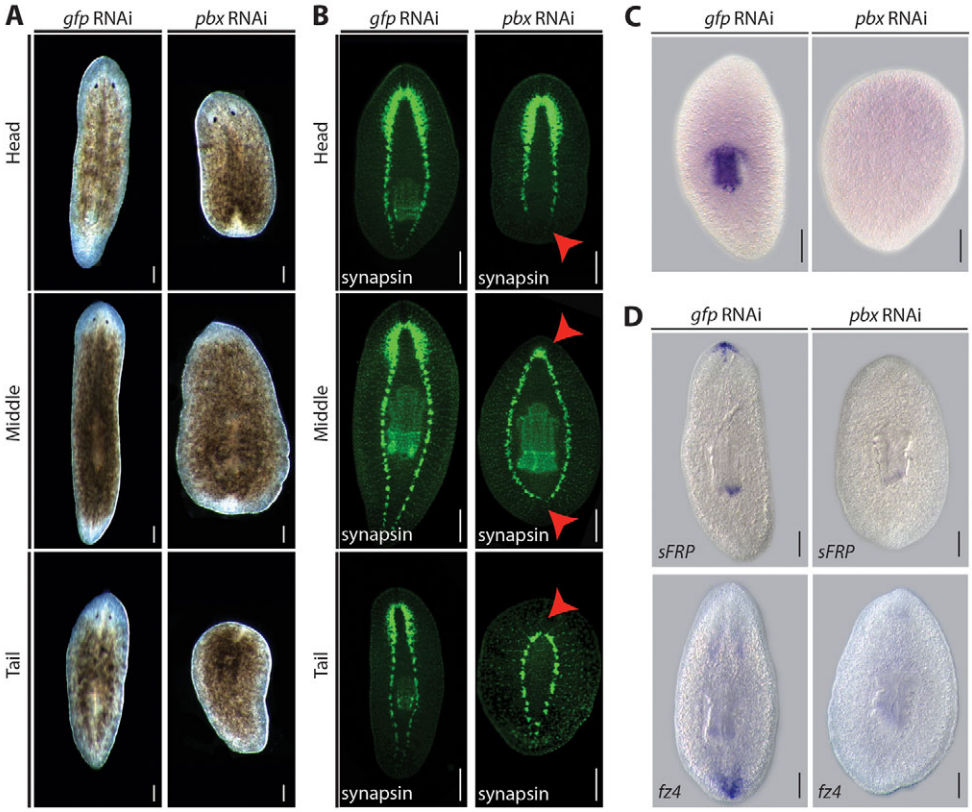

Fig. 2. *pbx(RNAi)* disrupts regeneration. (**A**) Posterior blastemas of control head and trunk pieces regenerated tails, whereas those of *pbx(RNAi)* head and trunk pieces failed to do so (75/75, across three experiments). Anterior blastemas of control trunk and tail pieces regenerated a head and photoreceptors, whereas those of *pbx(RNAi)* trunk and tail pieces failed to do so (75/75, across three experiments). In addition, all *pbx(RNAi)* head and tail pieces failed to regenerate the pharynx correctly . (**B**) Staining of the nervous system shows that the VNCs of regenerated tails meet at an acute angle in control head (35/35 across three experiments) and trunk pieces (35/35), whereas those of *pbx(RNAi)* pieces regenerated aberrantly (35/35 in heads, 35/35 in tails, across three experiments, red arrowheads). Staining of the nervous system also showed that the CG of control trunk and tail pieces regenerated normally when compared with the intact CG of head pieces (35/35, across three experiments). CG regeneration was greatly reduced in *pbx(RNAi)* trunk pieces (35/35, across three experiments, shown by red arrows) and failed completely in tail pieces (33/35, across three experiments, red arrowheads). (**C**) The expression of pharynx-specific *smed-laminin* observed in control regenerated tail pieces (15/5) was absent in *pbx(RNAi)* regenerates (15/15). (**D**) Control regenerated trunk pieces expressed *sFRP-1* (10/10) at the anterior margin and *FZ4* at the posterior (10/10), whereas regenerated *pbx(RNAi)* trunk pieces failed to express either marker (10/10 for both markers). Scale bars: 200 μm.

pattern that resembled that exhibited by neoblasts (Fig. 1A; supplementary material Fig. S1A). We found *pbx* expression was depleted from the parenchyma 24 hours after γ-irradiation to remove neoblasts, revealing the remaining expression of *pbx* in the cephalic ganglia (CG) (Fig. 1A). *pbx* was expressed throughout the parenchyma of regenerating pieces amputated anterior and posterior to the pharynx (Fig. 1B), and within and adjacent to the regenerating blastemas (Fig. 1B). As regeneration proceeded, *pbx* expression was broadly observed throughout anterior and posterior blastemas, indicative of expression in neoblast progeny forming this structure (supplementary material Fig. S1B).

To reveal expression in post-mitotic differentiated cells, regenerating pieces were irradiated 24 hours prior to fixing. In these animals at 2 days of regeneration (dR), bilateral expression was observed within both anterior and posterior blastemas, indicative of expression in the regenerating central nervous system (CNS) (Fig. 1B). We also observed expression in the regenerating pharynx region in head and tail fragments from 3 dR (supplementary material Fig. S1B). This pattern of expression continued to be evident through to 5 dR (supplementary material Fig. S1B).

Taken together, these data suggest that *pbx* has a complex pattern of expression that includes neoblasts, neoblast progeny and the CG. In addition, during regeneration *pbx* is also expressed in the regenerating CNS in both anterior and posterior blastemas, and in the region of the regenerating pharynx.

pbx is required for regeneration along the AP axis

RNA interference (RNAi) was used to knock down *pbx* expression (see supplementary material Fig. S2A). We observed clear defects in the ability of *pbx(RNAi)* animals to regenerate correctly. Animals formed regeneration blastemas but head pieces failed to regenerate a tail or a pharynx, tail pieces failed to regenerate a head or a pharynx, and trunk pieces failed to regenerate a head or a tail (Fig. 2A). Using a pan-neural marker against synapsin, we observed that anterior blastemas had highly reduced CG labeling, and head and trunk pieces failed to correctly regenerate ventral nerve cords (VNCs) posteriorly after 14 days post amputation (dpa) (Fig. 2B). VNCs failed to extend into the posterior blastema in head pieces and were prematurely rounded and fused behind the pre-existing pharynx in trunk pieces (Fig. 2B). To confirm failure in pharynx regeneration, we used the pharynx-specific marker *Smed-laminin*

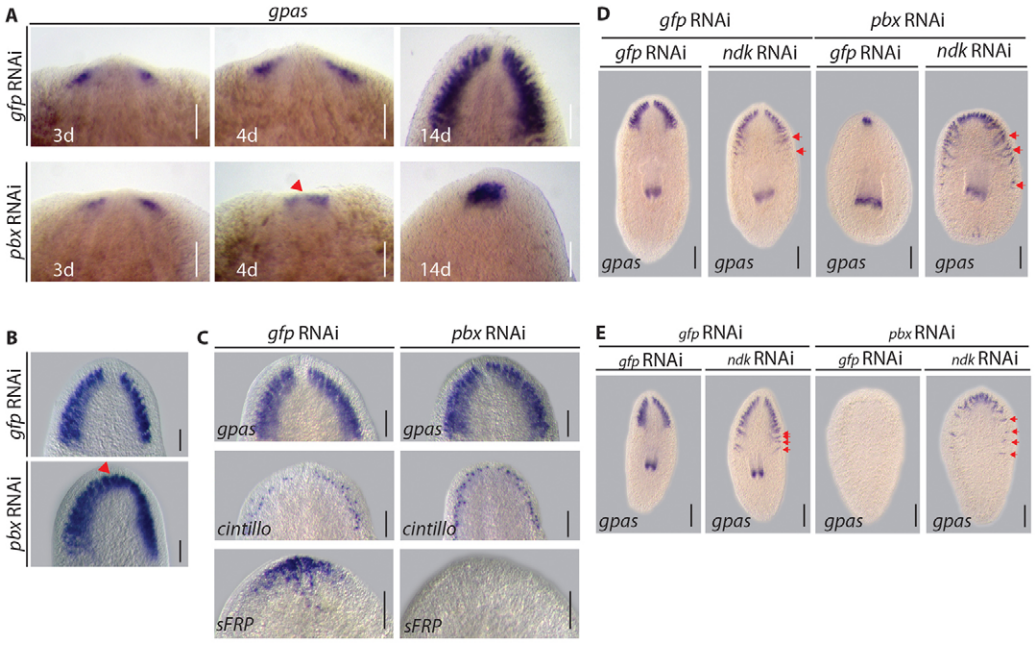

Fig. 3. *pbx* is required for CG patterning. (**A**) By 3 dpa, CG primordia expressed the neural marker *GPAS* in the anterior blastema of regenerating trunk pieces in control (10/10) and *pbx(RNAi)* (10/10). At 4 dpa, these structures remained separate in control regenerates (8/8), whereas they started to coalesce in *pbx(RNAi)* (8/8) (red arrowhead). By 14 dpa, the CG was completely regenerated in controls (10/10), whereas in *pbx(RNAi)* regenerates the fused CG did not enlarge significantly (10/10). (**B**) Following longitudinal amputation the structure of the CG was correctly regenerated by 14 dpa in control lateral regenerates (12/12). Following *pbx(RNAi)*, CG tissue was regenerated to the same extent as in controls; however, the structure was fused at the midline (17/17 fused, red arrowhead). (**C**) The CG structure was comparable in intact control (12/12) and *pbx(RNAi)* (14/14) planarians, as was the presence of *cintillo*-expressing sensory cells (8/8 and 8/8, respectively). In control planarians, *sFRP was* expressed at the anterior margin (13/13), whereas it was lost following *pbx(RNAi)* within 3 weeks of homeostasis (9/13). (**D**,**E**) In control *gfp(RNAi)* background, *ndk(RNAi)* resulted in expansion of the regenerated CG in 14 dpa tail and trunk pieces (17/17 control animals normal, 18/18 tails and 20/20 trunks in *ndk(RNAi)* expanded, red arrows). CG regeneration was not observed following *pbx/gfp(RNAi)* in tails (22/22) and was greatly reduced in trunks (20/20). Tail and trunk pieces regenerated mis-patterned CG at the anterior following combined *pbx/ndk(RNAi)* (20/20 animals). Scale bars: 100 µm.

(Cebrià and Newmark, 2007) and observed that characteristic expression in the pharynx is absent in *pbx(RNAi)*-regenerating animals (Fig. 2C). Regeneration in all control *gfp(RNAi)* animals was normal.

Taken together, these data indicate that *pbx(RNAi)* leads to regenerative defects along the planarian body axis. Supporting this hypothesis, *pbx(RNAi)* led to loss of *sFRP-1* and *Fz4* (Gurley et al., 2008), which are markers of anterior and posterior fate, respectively (Fig. 2D).

We assessed the maintenance and proliferative capacity of neoblasts by monitoring cell proliferation and the characteristic peaks of neoblast proliferation associated with amputation (supplementary material Fig. S3A) (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). Proliferation was also unaffected after the completion of regeneration. We also observed no significant difference in the generation of *nb.21.11.e*- or *agat-1*-expressing neoblast progeny (Eisenhoffer et al., 2008) between *gfp(RNAi)* and *pbx(RNAi)* animals (supplementary material Fig. S3B,C).

Together, these data suggest that defects in stem cell maintenance, proliferation and differentiation do not contribute to the *pbx* phenotype. Instead our data suggest that the observed phenotype results from a failure to regenerate the head/brain and tail/VNCs correctly.

Smed-pbx is required to pattern the anterior compartment and regenerating CG

The *pbx(RNAi)* phenotype highlights a role in CG regeneration and anterior regeneration. Previously, it has been demonstrated that CG regeneration consists of early and late phases, with the early phase being independent of Wnt signaling-mediated polarity cues (Evans et al., 2011; Iglesias et al., 2011). Early CG regeneration was investigated in *pbx(RNAi)* trunk pieces (Fig. 3A), which exhibit a less comprehensive failure of CG regeneration than tail pieces (see Fig. 2B). At 3 days post-amputation, discrete bilateral brain primordia were observed in anterior blastemas (Fig. 3A). Whereas these structures remained discrete in *gfp(RNAi)* regenerates, by 4 days they had started to coalesce in *pbx(RNAi)* regenerates (Fig. 3A). Compared with *gfp(RNAi)* regenerates the fused CG of *pbx(RNAi)* regenerates did not enlarge significantly by 14 dpa (Fig. 3A).

A similar encroachment of CG tissue into midline territory is observed following *Smed-slit RNAi* (Cebrià et al., 2007), prompting us to assess the regeneration of the midline following *pbx RNAi*. Consistent with a secondary role for *pbx* in the re-establishment of the midline, the characteristic stripe of *Smed-slit* expressing cells did not extend into either the anterior or posterior blastemas following *pbx(RNAi)* (supplementary material Fig. S4A).

These data suggest that subsequent to the early initiation of CG differentiation, *pbx* is required for later expansion and patterning of the CG, as well as having an effect on midline establishment.

The defect in midline patterning was also observed when assessing the regeneration of the dorsal cilia. On the dorsal surface of *pbx(RNAi)* animals, the stripe of prominent cilia along the midline is absent (supplementary material Fig. S4B). Cilia are regenerated normally on the ventral surface of *pbx(RNAi)* animals and on the dorsal surface away from the midline (supplementary

material Fig. S4B), suggesting that defects observed in the regeneration of cilia on the dorsal surface result from a failure to re-establish the pattern of the dorsal midline stripe rather than from a general defect in ciliogenesis.

When amputated longitudinally, lateral regeneration re-established the bilateral organization of the CG following *gfp(RNAi)* (Fig. 3B). By contrast, whereas the CG of *pbx(RNAi)* animals regenerated to a comparable extent, the ganglia were fused at the midline (Fig. 3B). *Smed-slit* expression was observed along the midline in *pbx (RNAi)* lateral regenerates; however, expression did not extend as far anteriorly as in *gfp (RNAi)* controls (supplementary material Fig. S4C). These results suggest that *pbx* is required for the complete anterior regeneration of CG and for correct patterning, but not required for lateral regeneration. The fusion of CG may reflect a requirement for *pbx* to re-establish the midline as reflected by changes in *Smed-slit* expression.

During homeostasis, the CG are maintained following *pbx(RNAi)* (Fig. 3C), as are mechanosensory *cintillo*-positive cells (Oviedo et al., 2003) that are associated with the CG (Fig. 3C), despite loss of the anterior marker *Smed-sFRP-1* (Fig. 3C) (Gurley et al., 2008) and of the dorsal stripe of cilia (supplementary material Fig. S4D). These data suggest that *pbx* activity is required for appropriate regeneration and patterning of the CG during anterior regeneration, rather than being required for CG maintenance or pattern during homeostasis.

To test whether *pbx* was absolutely required for the differentiation of stem cells to CG tissue following decapitation, we performed double RNAi experiments with *noudarake* (*ndk*). The FGF-like receptor (*ndk*) normally restricts CG differentiation to the anterior compartment (Cebrià et al., 2002; Felix and Aboobaker, 2010). We reasoned that if *pbx* were required for stem cell to CG differentiation, *pbx/ndk(RNAi)* would not lead to ectopic CG regeneration. However, we observed that *pbx/ndk(RNAi)* leads to the regeneration of extensive, albeit mis-patterned, CG fused at the anterior midline both in trunks and even in tails, which normally fail to regenerate any detectable CG after *pbx(RNAi)* (Fig. 3D,E).

We next wished to assess the role of *pbx* for other characteristic features of the anterior compartment apart from the CG. To do this, we investigated the expression of markers with characteristic distribution with respect to the anterior. The *septin* gene is expressed by gland cells exclusively on the dorsal surface of the animal (Molina et al., 2011), but is also absent from the anterior compartment (Fig. 4A). However, in *pbx(RNAi)* regenerates, *septin* expression extended into the regenerated anterior region of trunk pieces (Fig. 4A), despite the regeneration of limited CG (Fig. 3). This suggests that patterning of the anterior compartment is disrupted. We found no evidence for ectopic ventral expression of *septin* in *pbx(RNAi)* animals (Fig. 4A). This is consistent with the anterior ectopic expression of *septin* resulting from a failure to define the anterior compartment.

In order to further investigate the defects in the patterning of the regenerating anterior compartment following *pbx(RNAi)*, we investigated the distribution of mitotic cells in the anterior compartment of 14 dpa trunk pieces. *H3p*+ neoblasts are characteristically absent from the most anterior regions of *gfp(RNAi)* blastemas, where the regenerated bi-lobed CG is visualized by 3C11 staining (Fig. 4B). By contrast, H3p+ neoblasts are located in the anterior regions of *pbx(RNAi)* animals, surrounding the reduced and mis-patterned CG (Fig. 4B).

These data support two major conclusions regarding the *pbx* phenotype. First, as previously shown, mitotic neoblasts are maintained. Second, as suggested by the ectopic encroachment of normally excluded cell types (*septin*+) and the loss of structures and markers specific to the anterior compartment, the anterior compartment fails to regenerate appropriate identity so as to exclude mitotic neoblasts following *pbx(RNAi)*.

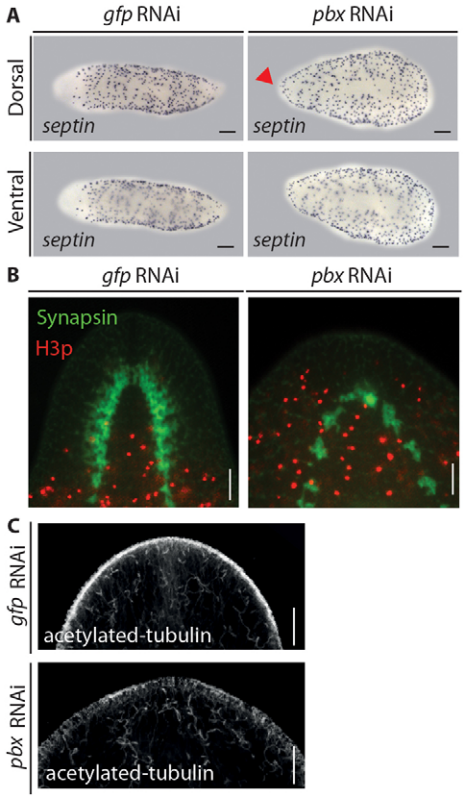

Fig. 4. The anterior compartment is improperly patterned following *pbx(RNAi)*. (**A**) The dorsal marker *septin* was characteristically absent form the anterior compartment of controls (6/6), whereas it was expressed ectopically at the anterior margin of *pbx(RNAi)* regenerates (7/7) (red arrowhead). No ventral expansion of *septin* expression is observed. (**B**) H3p-positive mitotic neoblasts (red) were characteristically absent from the anterior compartment of control regenerates at 14 dpa (15/15). The regenerated CG is shown by 3C11 staining (green). H3p-positive mitotic neoblasts failed to be excluded from the anterior compartment of *pbx(RNAi)* regenerates in which the mis-patterned CG differentiates (15/15). (**C**) Protonephridia labeled by anti-acetylated tubulin immunostaining were observed at the anterior margin of control and *pbx(RNAi)* regenerates (7/7 and 15/15). Scale bars: 100 μm in B,C; 200 μm in A.

Protonephridia are also distributed along the AP axis but without any obvious AP differences (Rink et al., 2011; Scimone et al., 2011). We observed normal protonephridia in the regenerative blastemas of *pbx(RNAi)* regenerates (Fig. 4C). Taken together, these data show that despite the failure to regenerate anterior and posterior structures as well as the pharynx, other cells, tissues and organs are regenerated correctly in *pbx(RNAi)* animals.

Smed-pbx is required to pattern the regenerating gut and pharynx

Another planarian tissue that displays polarity along the AP axis is the gut, which in Triclad species has one major anterior branch and two major posterior branches. During regeneration, the gut is coordinately remodeled in existing tissue and regenerated in new tissue to reconstitute this characteristic pattern (Forsthoefel et al.,

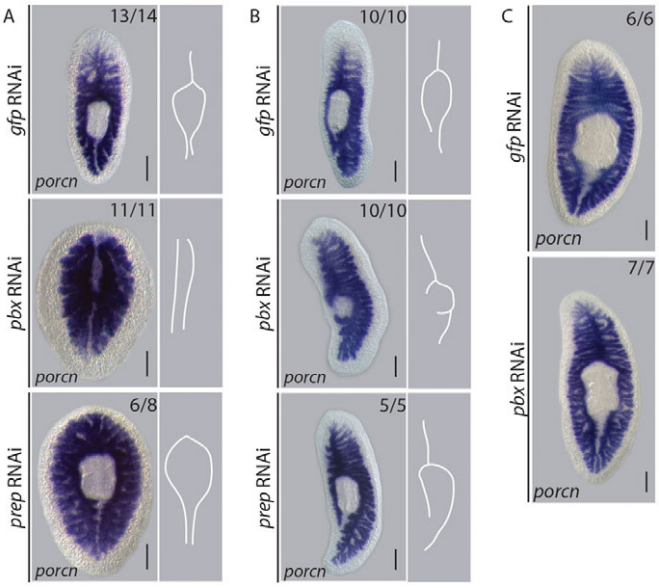

Fig. 5. *pbx* coordinates AP patterning of the regenerating gut. (**A**) The anterior gut branch was regenerated and the existing posterior gut branches re-patterned by 14 dpa in control tail pieces (14/14), revealed by gut-specific Smed-*porcupine* expression. The anterior gut branch failed to regenerate and the existing posterior gut did not re-model following *pbx(RNAi)* (11/11). The posterior gut remodeled in *prep(RNAi)* tail pieces; however, the anterior gut branch did not (8/8). Schematic representations of gut morphology summarize the phenotypes observed. (**B**) By 14 dpa, the posterior gut branch was restored in control lateral regenerates (10/10). The gut extended into the lateral blastema following *pbx(RNAi)*; however, patterning of the posterior branch was not regenerated (10/10). Regeneration of the posterior gut was comparable with controls following *prep(RNAi)* (10/10). Schematic representations of gut morphology summarize the phenotypes observed. (**C**) Gut morphology is maintained during 3 weeks of homeostasis following *pbx(RNAi)* (17/17), resembling controls (16/16). Scale bars: 200 μm.

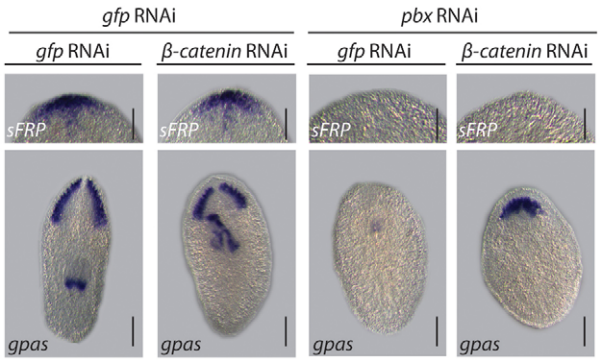

Fig. 6. *pbx* functions independently during CG regeneration and anterior polarity specification. CG regenerated in *gfp/gfp(RNAi)* and *gfp/β-catenin-1(RNAi)* (33/33 and 20/20) 14 dpa tail pieces. These animals also expressed sFRP-1 correctly in the anterior (20/22 and 9/9). CG regeneration was not observed following *pbx/gfp(RNAi)* (38/39). Fused and mis-patterned CG were regenerated following *pbx/β-catenin-1(RNAi)* (18/18). sFRP-1 was not expressed at the anterior margin of 14 dpa *pbx/gfp(RNAi)* (27/27) or *pbx/β-catenin(RNAi)* (21/21) tail pieces. Scale bars: 200 μm (except for those concerning sFRP-1 expression, 100 μm).

2011). We investigated the role of *pbx* in this process, as well as that of *prep*, another member of the TALE-class family previously shown to be required specifically for anterior patterning (Felix and Aboobaker, 2010). Using *Smed-porcupine* as a gut marker (Gurley et al., 2008), we observed regeneration of this structure in tail fragments. Both *prep(RNAi)* and *pbx(RNAi)* animals had defects in gut regeneration. The gut completely fails to remodel in *pbx(RNAi)* animals (Fig. 5A). *prep(RNAi)* regenerating tail pieces join and remodel gut branches correctly around the regenerating pharynx; however, a clearly defined anterior branch and an anterior compartment absent of gut tissue do not form (Fig. 5A). These data demonstrate that *pbx* is required for regeneration and remodeling of the gut along the AP axis, while *prep* is required for this process specifically in the anterior.

Longitudinal amputation requires that regeneration restore tissue identities along the whole AP axis. Following *pbx(RNAi)*, the gut regenerated into the lateral blastema; however, appropriate AP patterning was not restored as the missing posterior gut branch did not form (Fig. 5B). By contrast, the posterior gut branch was restored in *gfp(RNAi)* and *prep(RNAi)* regenerates (Fig. 5B). After 3 weeks of homeostasis, no abnormalities were observed in the organization of the gut in *pbx(RNAi)* animals when compared with *gfp(RNAi)* controls (Fig. 5C). We conclude from these experiments that *pbx* is required to direct the appropriate patterning of the gut structures across the AP axis during regeneration, whereas *prep* is required only to pattern the anterior gut. *Smed-porcupine*-positive gut tissue was regenerated in blastemas of laterally regenerating animals, even though it was incorrectly patterned, suggesting that the capacity for stem cells to form gut cells per se does not require *pbx*.

We next tested this premise for pharynx regeneration. Pharynx regeneration *de novo* in regenerating heads and tails pieces fails entirely (Fig. 2). However, when we removed the pharynx from otherwise intact *pbx(RNAi)* animals we observed the formation of a pharynx anlage (supplementary material Fig. S5A,B). Whereas control animals fully regenerated a normal pharynx, *pbx(RNAi)* animals could generate only a cluster of *Smed-laminin*-expressing cells. This suggests that PBX is likely to be required for both establishment of pharynx regeneration and late patterning and morphogenesis of this key organ.

pbx activity promotes brain regeneration independently of anterior polarity specification

As the Wnt/β-catenin 1 pathway has been clearly demonstrated by a number of studies to play an integral role in AP patterning and CG regeneration, we investigated whether our observations could be explained in the context of this signaling pathway. Wnt signaling inhibits both anterior polarity specification and CG differentiation. *pbx* has the opposite activities: being required to promote anterior polarity and CG differentiation. We performed double *pbx/β-catenin-1(RNAi)* to investigate their relationship in each of these contexts. *β-catenin-1/pbx(RNAi)* did not restore *pbx*-dependent expression of the anterior marker *sFRP-1* and we observed the regeneration of mis-patterned CG tissue, in contrast to *pbx/gfp(RNAi)* regenerates, which failed to differentiate detectable neural tissue (Fig. 6). The anteriors of control *gfp/gfp(RNAi)* and *gfp/β-catenin-1(RNAi)* tails pieces were normal with respect to sFRP expression and CG regeneration.

Taken together, these data suggest that the role of *pbx* in establishing anterior polarity could be separated from its role in promoting CG tissue differentiation. Furthermore, we interpret these data to suggest that *pbx* activity normally promotes CG

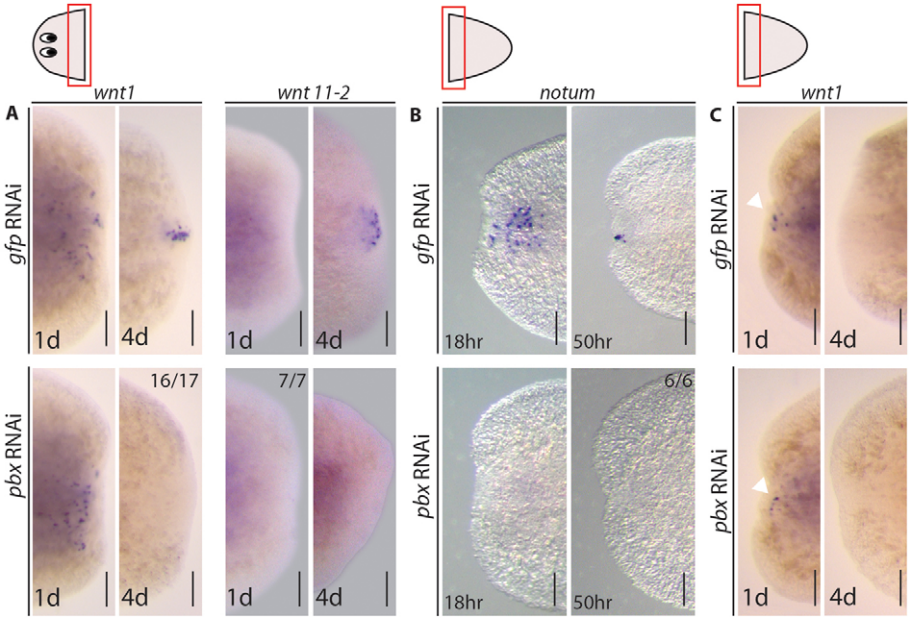

Fig. 7. *pbx* is required for expression of AP polarity determinants. (**A**) *wnt1* was expressed in cells adjacent to the posterior wound in control regenerating head pieces (12/12) and following *pbx(RNAi)* (11/11) at 1 dpa. The sustained posterior *wnt1* expression observed at 4 dpa in control animals (16/18) was absent following *pbx(RNAi)* (16/17). *wnt11-2* expression was not observed at 1 dpa in control or *pbx*(RNAi) animals (8/8 and 7/7), became expressed within the posterior blastema of controls by 4 dpa (17/17) and remained absent from *pbx(RNAi)* animals (17/17). (**B**) *notum* was expressed in cells adjacent to the anterior wound within 18 hours of regeneration in control tail pieces (6/6) and became localized to the anterior tip of the blastema by 50 hours (6/6), whereas its expression was not observed at either time point following *pbx* RNAi (16/16 and 16/16). (**C**) *wnt1* expression was observed adjacent to the anterior wound in control (6/7) and *pbx(RNAi)* (6/7) regenerating tail pieces at 1 dpa. Anterior *wnt1* expression did not persist to 4 dpa in control (6/6) or *pbx* RNAi (8/8) regenerating tail pieces. Scale bars: 100 μm.

differentiation in the anterior by opposing the inhibitory activities of *β-catenin-1* and *ndk* (Fig. 3D,E).

We used the combined knockdown of *pbx* and *β-catenin-1* to further investigate the role of *pbx* during eye regeneration. By observing eye regeneration in different scenarios using combinations of *β-catenin-1(RNAi)*, *prep(RNAi)* and *pbx(RNAi)* we found that that *pbx*, but not *prep,* has a role in the regeneration and maintenance of the eye that is independent of its role in the regeneration of CG tissue (see supplementary material Fig. S6).

Smed-pbx is required for the expression of polarity determinants

The range of defects observed following *pbx(RNAi)* are indicative of a broad axis wide role for *pbx* in AP patterning, and possibly subsequent establishment of the midline. We wished to determine whether these defects could be explained in terms of known AP axis polarity determination events that occur early in regeneration. *wnt1* is expressed by differentiated cells at early wound sites, independent of orientation (Petersen and Reddien, 2009; Gurley et al., 2010), and later, through the activity of *notum* (Petersen and Reddien, 2011) and Hh signaling (Rink et al., 2009; Yazawa et al., 2009), becomes confined to posterior-facing wounds. Wnt1 activity in the posterior blastema drives *β-catenin*-mediated differentiation of neoblasts into further *wnt1*-expressing cells and cells expressing the posterior determinant *wnt11-2* (Petersen and Reddien, 2009). Following *pbx(RNAi)*, early posterior *wnt1* expression was detected in the blastema 24 hours post-amputation, demonstrating that the early stem cell independent phase of expression of Wnt1 is not affected (Fig. 7A). The later phase of *wnt1* expression observed 96 hours post-amputation was not detected in *pbx(RNAi)* regenerates (Fig. 7A). Correspondingly, the induction of *wnt11-2* was also not detected following *pbx(RNAi)* (Fig. 7A). These data suggest that the failure in tail regeneration observed in *pbx(RNAi)* worms is due to a failure to establish posterior polarity correctly through the previously described Wnt-dependent program (Petersen and Reddien, 2009) and that *pbx* is required to establish the correct posterior program of Wnt expression during regeneration. Indeed, the posterior tailless phenotype of *pbx(RNAi)* mimics that described for *wnt1(RNAi)* (Petersen and Reddien, 2009).

During anterior regeneration, the normal expression of *notum* observed 18 hours and 50 hours post-amputation was not detected following *pbx(RNAi)* (Fig. 7B). This suggests *pbx* activity is required for *notum* expression. *notum(RNAi)* leads to posteriorization of anterior wounds owing to sustained anterior expression of *wnt1* (Petersen and Reddien, 2011). However, as already demonstrated, *pbx(RNAi)* leads to loss of anterior structures, but not posteriorisation. We reasoned that this could be explained if loss of *notum* expression in the anterior was not accompanied by ectopic *wnt1* expression in *pbx(RNAi)* animals. Similar to controls, the normal anterior *wnt1* expression observed at 24 hours post-amputation was not sustained at 96 hours post-amputation in *pbx(RNAi)* blastemas (Fig. 7C). From these data, we conclude that *pbx* is required for a stem-cell dependent phase of *wnt1* expression, irrespective of position in the anterior or posterior, and independently of its requirement for *notum* expression. Loss of expression of these two polarity determinants explains much of the *pbx(RNAi)* phenotype, particularly the failure to regenerate both anterior and posterior structures.

DISCUSSION

In this work, we present a key role in specifying positional identity for a conserved transcription factor expressed in stem cells, stem cell

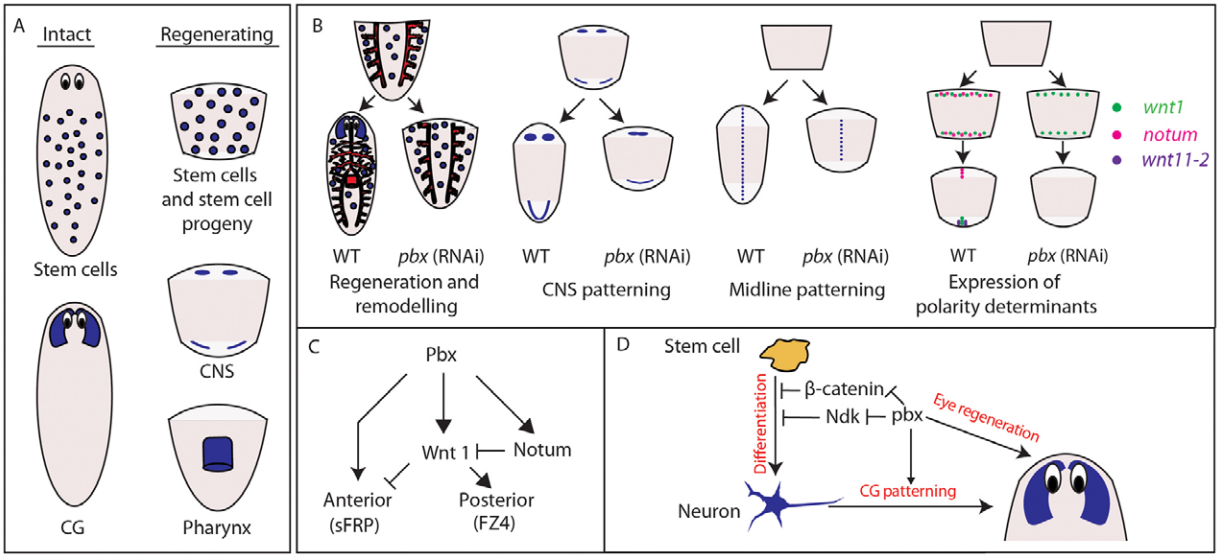

Fig. 8. Summary of *pbx* expression and function. (**A**) *pbx* is expressed in stem cells and the CG of intact worms; and in stem cells, stem cell progeny, the CNS and pharynx of regenerating worms. (**B**) *pbx* is required for correct regeneration and remodeling of the pharynx, gut and anterior compartment; correct patterning of the regenerating CNS, including the CG/brain; correct re-establishment of expression of the midline determinant *slit*; and for the correct expression of previously described anterior and posterior polarity determinants. (**C**) A model of *pbx* function with respect to known regulatory logic of AP polarity specification. *pbx* is required for expression of both *wnt1* and *notum*, and has an instructive role in promoting anterior polarity that is independent of the previously described *wnt1/notum* circuit. (**D**) *pbx* promotes CG/brain regeneration by inhibiting the activities of *β-catenin-1* and *ndk* independently of its role in patterning the growing CG. *pbx* is also required for eye regeneration independently of its roles during CG regeneration and patterning.

progeny and the regenerating CNS (Fig. 1; Fig. 8A for summary). In order for stem cells to replace missing tissues correctly during regeneration, they must be able to self-renew, produce progeny capable of differentiation into all the missing cell types and correctly interpret their position with regard to missing tissue. We have established that the TALE class homeobox gene *pbx/extradenticle* is essential for the last of these processes, demonstrating at the same time that the last of these processes can be separated out from the other two at a regulatory level in planarian stem cells and their progeny. Animals with reduced *pbx* do not show defects in stem cell maintenance or their general capacity to differentiate into missing cell types, rather they fail to restore positional identity across the AP axis. This includes failure to regenerate the anterior, posterior, the pharynx or the gut appropriately. In the case of the gut and pharynx, we demonstrate that cell types associated with these tissues can regenerate per se (Fig. 5; supplementary material Fig. S5), but they are not patterned correctly. In addition, we show that *wnt1* and *notum*, which are early determinants of anterior and posterior polarity, require *pbx* for their expression to be established correctly (Fig. 7). Coupled to this, we also observe that the midline fails to be correctly specified during regeneration, leading to fusion of any remaining CG, loss of the dorsal midline cilia, ectopic fusion of VNCs and failure to re-establish expression of the midline determinant *slit*. Finally, our data also highlight a distinct role for *pbx* in brain/CG differentiation that is independent of its role in specifying AP polarity (all summarized in Fig. 8). This suggests that *pbx* may be a central regulator that is required for both interpreting pre-existing polarity signals and subsequently driving regeneration of cell types expressing polarity determinants (see Fig. 8C for summary). Overall, we propose a model in which PBX is required for the establishment of AP polarity and that failures to establish this at the poles leads to subsequent defects in midline establishment and remodeling along the body axis.

A requirement of *pbx* for polarity determinant expression explains defects in anterior and posterior regeneration

pbx is required for the correct establishment of expression of *notum* and *sFRP-1* in the anterior and *wnt1*, *wnt11-2* and *Fz4* in the posterior. This lack of expression clearly correlates with failures in anterior and posterior regeneration. These data imply a key role for *pbx* in establishing and generating regenerative polarity. Loss of both *wnt1* and *wnt11-2* expression explains the lack of posterior regeneration caused by *pbx(RNAi)*. We also observe that loss of expression of the anterior determinant *notum* correlates with a loss of the anterior compartment caused by *pbx(RNAi)*. *notum* has been previously described as being dependent on early *wnt1* expression and itself is required for inhibition of *wnt1* expression at anterior blastemas to prevent posteriorisation (Petersen and Reddien, 2011). However, despite loss of *notum* in *pbx(RNAi)*, anterior blastemas do not regenerate with anterior or posterior fate. We show that this is because *wnt1* expression is not established in the anterior in the *pbx(RNAi)* background, even though the suppressive effect of *notum* is absent. Thus, the requirement of *pbx/extradenticle* for *wnt1* expression is independent of wound orientation with respect to the AP axis. This suggests a model where *pbx* is normally required for expression of both *notum* and *wnt1*, with anterior *notum* subsequently inhibiting the establishment of *wnt1* in the anterior but not the posterior (Fig. 8C). Simultaneous *wnt1/notum* knockdown has been shown to lead to correct regeneration of the anterior but not the posterior (Petersen and Reddien, 2011). Both *wnt1* and *notum* expression are also lacking following *pbx(RNAi)*, but the anterior fails to regenerate; thus, we uncover a specific requirement for *pbx* in establishing anterior polarity, apart from its role in promoting *notum* expression (Fig. 8C).

β-catenin-1 and *pbx* control brain regeneration independently of Wnt expression and AP polarity

Knockdown of *wnt1* expression can sometimes also lead to the ectopic differentiation of neural tissue within posterior blastemas as opposed to a tailless phenotype (Adell et al., 2009; Petersen and Reddien, 2009). However, following *pbx* knockdown, this was never observed despite the loss of *wnt1* expression, suggesting that *pbx* may be required for ectopic neural structures in *wnt1(RNAi)*. *β-catenin-1* is the downstream effector of *wnt1* and is itself required for establishment of the stem cell-dependent expression of *wnt1* (Petersen and Reddien, 2009). Thus, the loss of *wnt1* after *pbx(RNAi)* might also reflect a loss of *β-catenin-1* activity. *β-catenin-1* may also be required for the reduction or absence of brain tissue caused by *pbx(RNAi)*, because, when combined, *pbx/β-catenin-1(RNAi)* enhances brain regeneration (Fig. 6). This indicates that a balance between *pbx* and *β-catenin-1* activity may control this process.

pbx activity is required for appropriate CG patterning either when regenerating CG tissue *de novo* following decapitation or when integrating with existing tissue following longitudinal amputation. In contrast to the reduced CG regeneration observed following decapitation, laterally regenerating CG is restored to the same extent as controls. This indicates in the context of lateral regeneration signals promoting neural regeneration are present that are absent following decapitation. However, similar to anterior regeneration, the CG is fused at the midline. Combined *ndk/pbx(RNAi)* restores CG tissue, but this is also mis-patterned and shows fusion at the midline (Fig. 3). Following combined *pbx/β-catenin-1(RNAi)*, CG regeneration is also enhanced; however, it is fused at the midline similar to lateral regenerates (Fig. 6). Together, these experiments demonstrate that the extent of CG regeneration after decapitation depends on the ability of *pbx* activity to overcome inhibitory effects mediated by ndk and *β-catenin-1*, and is independent of the process governing patterning of CG (Fig. 8D). The observation of fused CG correlates with loss of *slit* expression in both anterior and posterior regenerates, and a failure of *slit* expression to extend to the anterior in lateral regenerates (supplementary material Fig. S4A). Thus, either *pbx* has a further role in establishing the midline or the establishment of anterior and posterior polarity is required as a prerequisite to re-establishment of the midline in regenerating animals. In addition, *pbx/β-catenin-1* RNAi does not restore anterior *sFRP-1* expression, further evidence that the process of anterior polarity specification and the extent of CG regeneration are functionally separable.

Conserved roles for TALEs in axial regeneration?

We report for the first time the crucial role of an ortholog of PBX/Extradenticle during animal regeneration and present evidence that it is essential for appropriate tissue patterning and restoration of organismal integrity. Future work with improved experimental tools, particularly transgenesis, will help uncover which components of *pbx* expression are related to which of the functions we have described. We have added to a limited body of work detailing the activities of other members of the TALE class family during regeneration (Mercader et al., 2005; Felix and Aboobaker, 2010; Shaikh et al., 2011), and further highlight the role these proteins play in the orchestration of pattern formation. The interaction of PBX with other members of the TALE class family, Homothorax/Meis and Prep, regulates their nuclear localization and ability to regulate target gene expression by acting as transcription factors (Berthelsen et al., 1999). Dimers of TALE class proteins also interact with various other homeodomain-containing proteins, particularly Hox proteins, diversifying their regulatory potential (Karlsson et al., 2010; Noro et al., 2011). The planarian ortholog of Prep has been shown to be a crucial determinant of anterior fate during regeneration, being required both for anterior polarity specification and CG regeneration (Felix and Aboobaker, 2010). The salamander Homothorax/Meis ortholog has been show to mediate the specification of proximodistal (PD) identity of blastema cells during limb regeneration, as is the case during limb development (Mercader et al., 1999; Mercader et al., 2005). However, both in the case of limb regeneration and limb development a functional interaction between Homothorax/Meis and Extradenticle/PBX remains to be clarified. Salamander Homothorax/Meis orthologs also directly regulate the expression of the PD determinant Prod1 during regeneration through binding to cis-regulatory elements in the *prod1* promoter, one of which is closely linked to a potential PBX-binding site (Shaikh et al., 2011). These data suggest that regulatory interactions between TALE homeodomains may be key in regenerative patterning. The similarities of anterior regeneration phenotypes following *pbx* and *prep* RNAi make it tempting to envisage that the two proteins interact functionally to coordinate anterior patterning, and this possibility is now open to investigation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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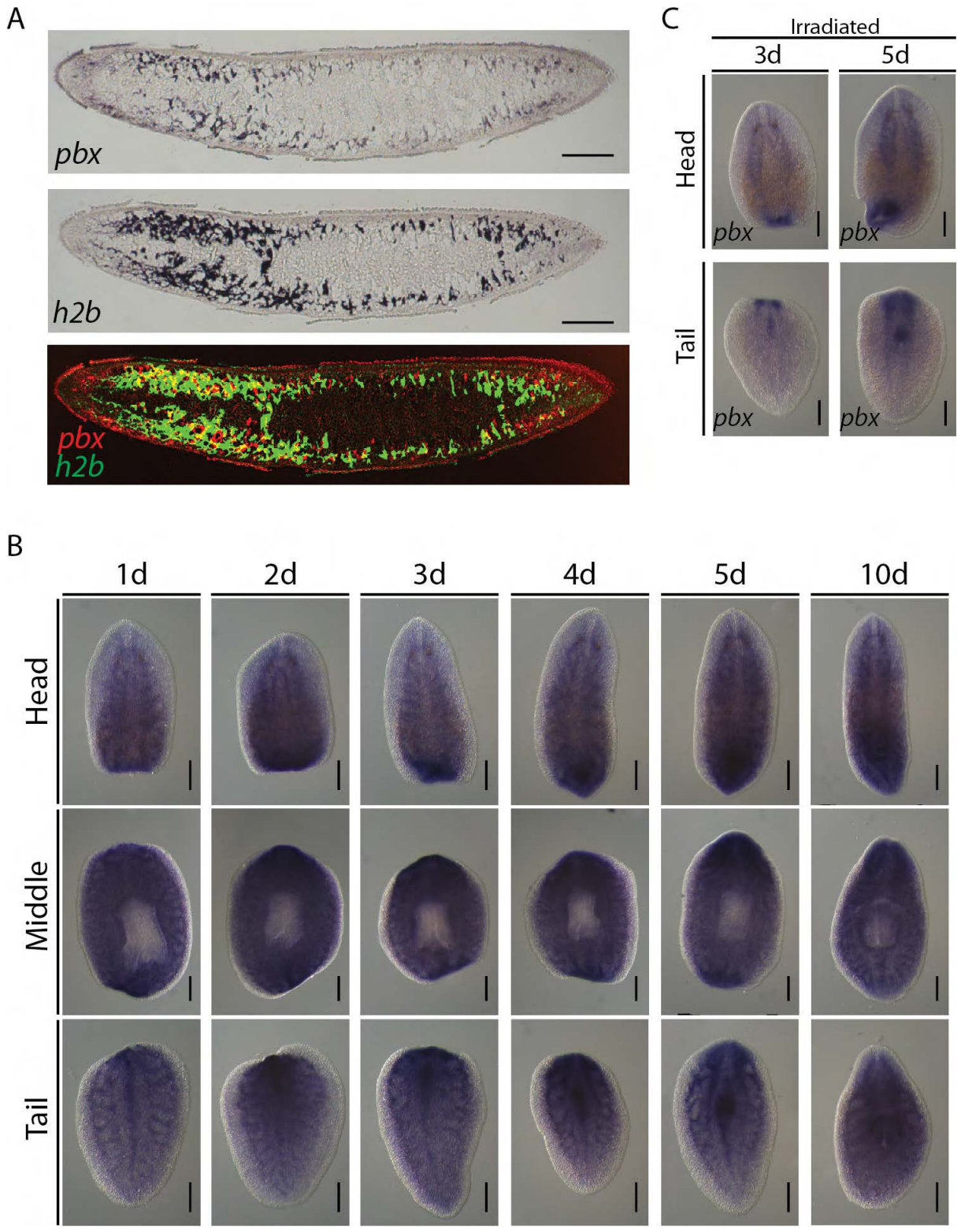

Fig. S1. *pbx* is expresed in cells colocalizing with neoblasts and is expressed in the regenerating central nervous system and the regenerating pharynx. (**A**) Adjacent sagittal sections of whole worms showed that *pbx* is expressed in a pattern resembling that of the neoblast marker *H2B*. Images were false colored and overlaid to illustrate the colocalization of *pbx*- and *H2B*-expressing cells within the parenchyma (*n*=5 section pairs from three animals). (**B**) *pbx* expression is broadly observed in both anterior and posterior blastemas of regenerating pieces. Expression in the CG of head pieces was also clearly visible. By 10 days, expression resembles that seen in intact animals. Expression in the regenerating pharynx is also observed in head and tail pieces from 3 dpa. (**C**) Regenerating fragments were γ-irradiated with a dose of 100 Gy 1 day prior to fixation to aid visualization of *pbx* in cells other than neoblasts, and fixed at the time points indicated. As in B, expression is observed in anterior and posterior blastemas and in the CG of head pieces. Expression in blastemas is bilateral, suggesting expression in the regenerating CNS. Expression in the regenerating pharynx is also clear from 3 dpa. Scale bars: 200 μm. CG, cephalic ganglion; dpa, days post-amputation.

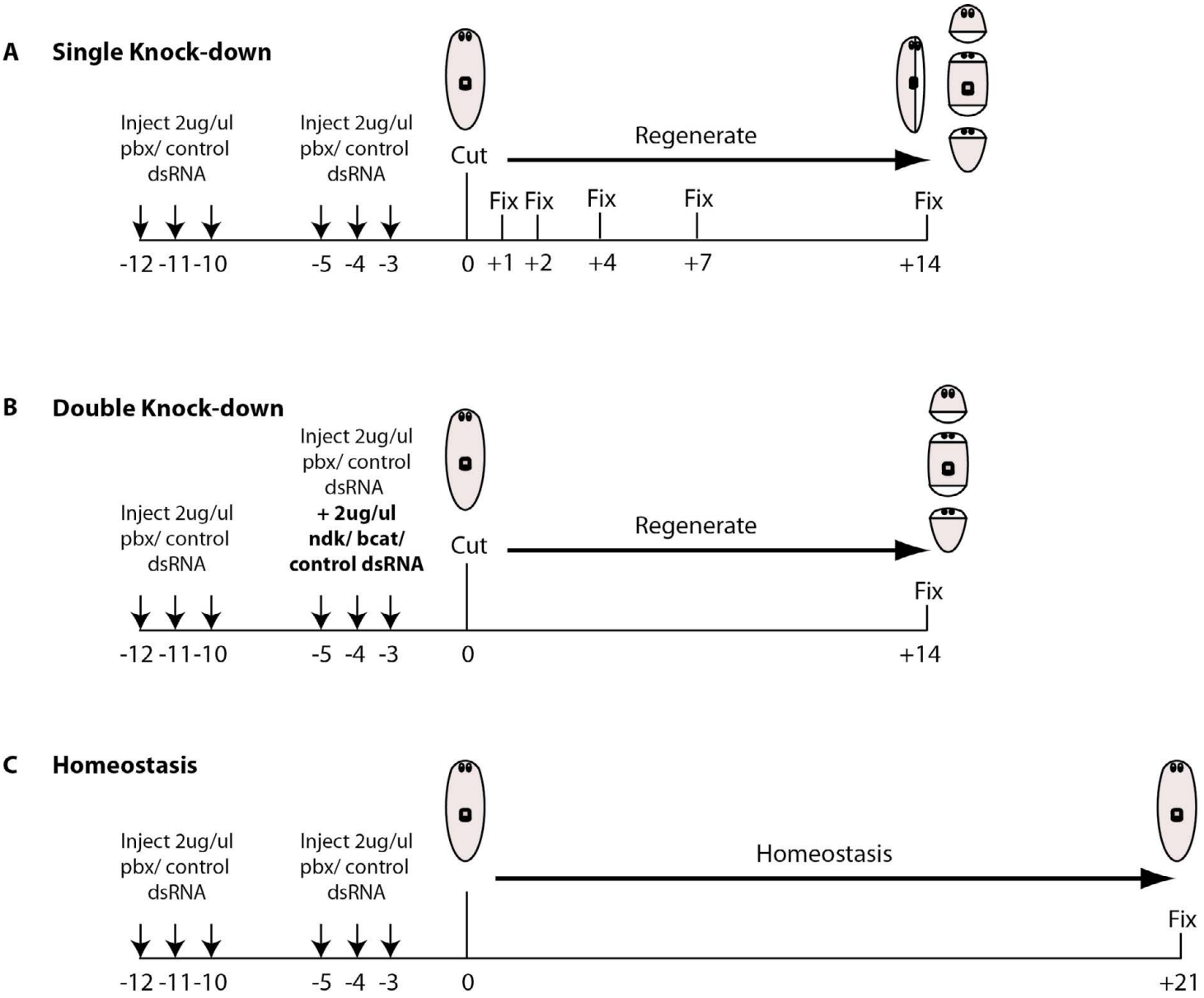

Fig. S2. Experimental procedures for RNAi experiments. (**A**) For single gene RNAi experiments, animals were injected with 2 µg/µl dsRNA with 33 nl injections for each day as shown. Animals were either cut laterally or into head, trunk and tail pieces, and fixed at the appropriate time-point for analysis. (**B**) For double RNAi experiments, animals were injected as for single gene injections, except on the second set of 3 days when dsRNA for the second gene was added so that both genes were at a final concentration of 2 µg/µl dsRNA. (**C**) For homeostasis experiments, the injection protocol was the same as for regeneration experiments as outlined in A. In this case animals were not amputated.

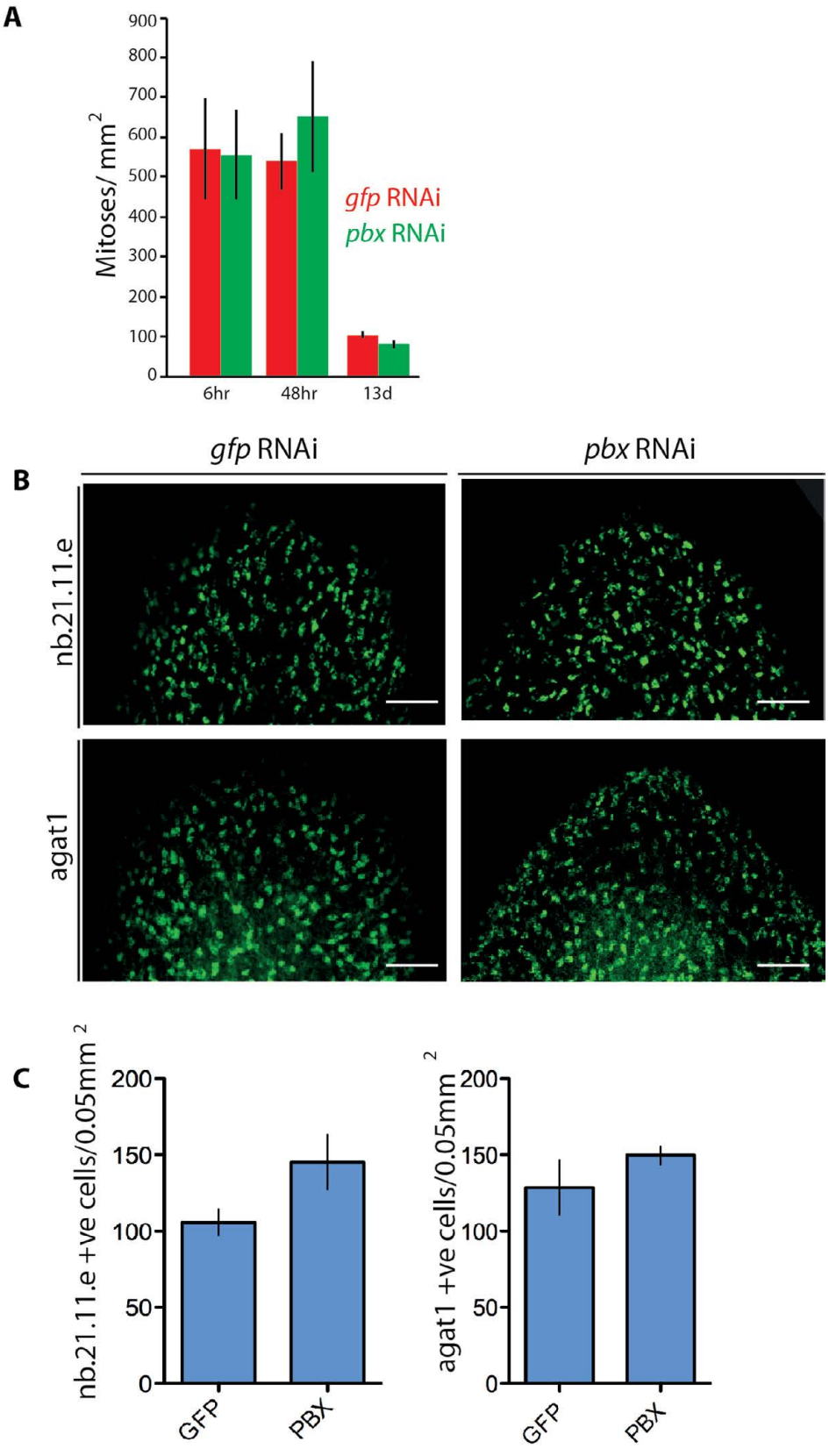

Fig. S3. *pbx* is not required for the neoblast proliferative response or for neoblast differentiation to early and late progeny. (**A**) Neoblast proliferation at 6 hours and 48 hours after amputation is not significantly different from controls in pbx(RNAi) animals, and neoblast proliferation after regeneration is also not affected at 13 days after amputation (P>0.2, two tailed students t-test, n>7 in each of three separate experiments). (**B**) Staining with markers of early (nb.21.11.e) and late (agat1) progeny in anterior blastemas reveal that both these cell populations are reconstituted correctly. Scale bars: 100 µm. (**C**) Cell counts of both nb.21.11.e- and agat1-positive cells confirm that *pbx(RNAi)* animals can reconstitute these cell types normally (P>0.15, two-tailed test, n=4 animals, three different regions).

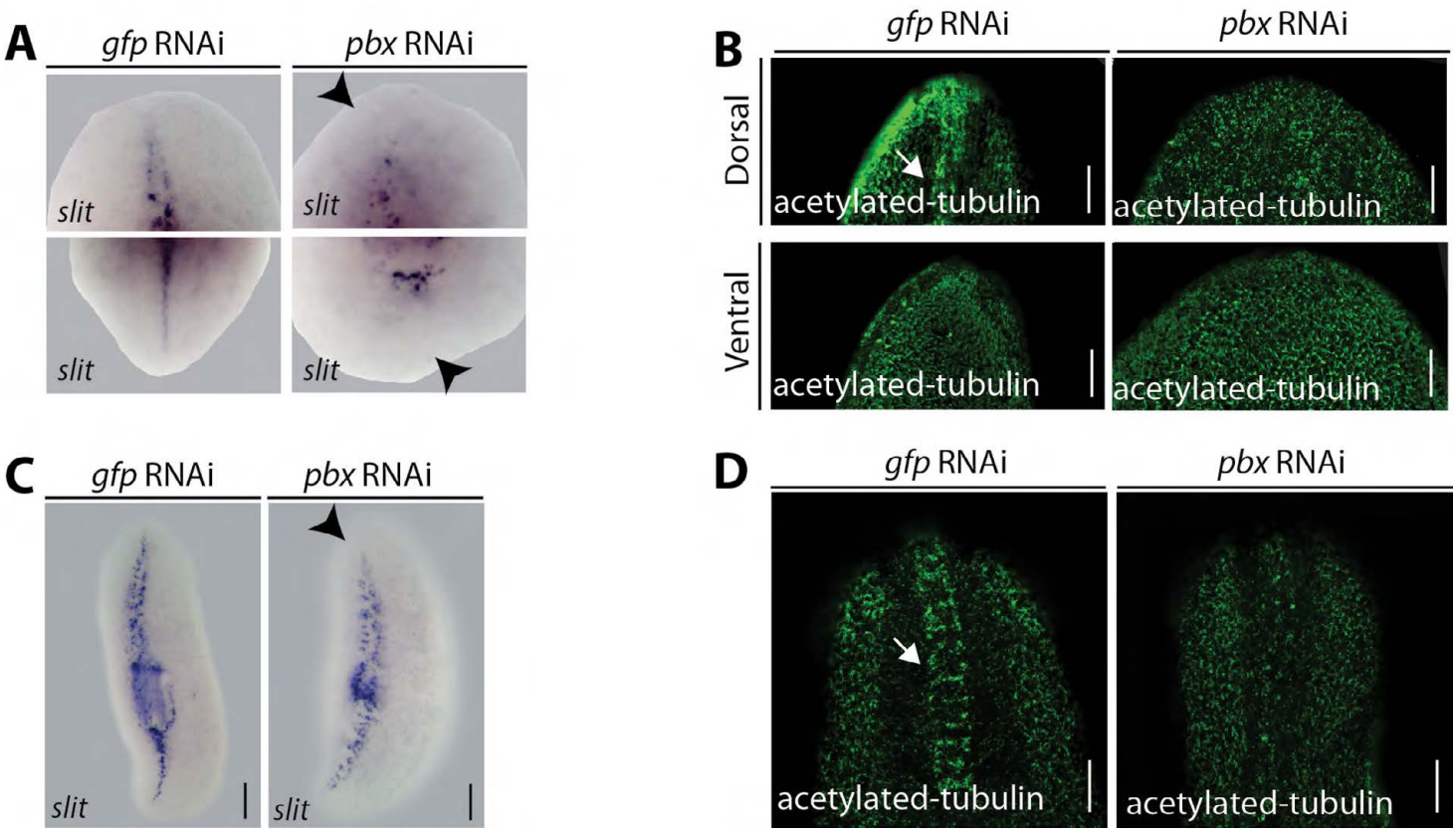

Fig. S4. *pbx* is required for patterning of the midline. (**A**) *slit*-expressing cells are absent from the anterior and posterior compartments of *pbx (RNAi)* regenerates at 12 dpa, whereas the characteristic stripe of cells is observed along the midline of control *gfp(RNAi)* regenerates. (**B**) Anti-acetylated tubulin immunostaining reveals that the dorsal stripe of cilia indicated by a white arrow in the anterior compartment of controls (7/7), but is absent from *pbx(RNAi)* regenerates (14/14). Ventral differentiation of cilia is not affected. (**C**) *slit*-expressing cells are observed along the midline of *pbx(RNAi)* and control *gfp(RNAi)* lateral regenerates; however, are absent from the anterior compartment of *pbx(RNAi)* regenerates. (**D**) The dorsal stripe of cilia observed in the anterior compartment of controls (7/7) was lost within 3 weeks of homeostasis following *pbx(RNAi)* (17/17).

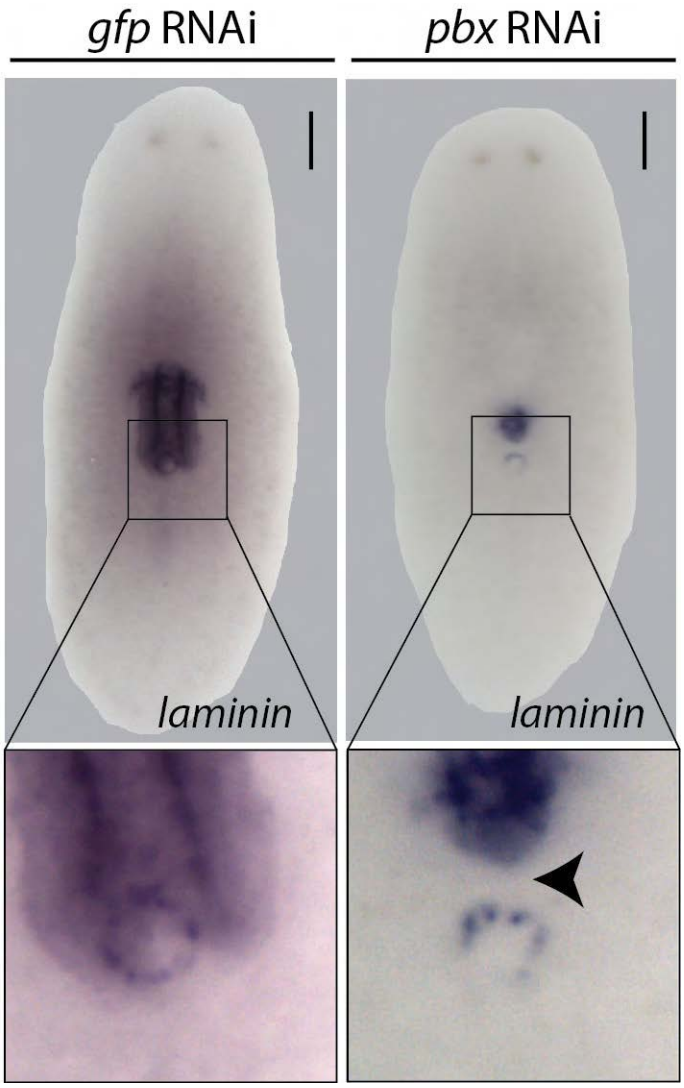

Fig. S5. Pharynx regeneration and patterning requires *pbx*. The pharynx was removed from control *gfp(RNAi)* and *pbx(RNAi)* worms. The complete pharynx was regenerated within 14 days in controls, as shown by *Smed-laminin in situ* hybridization; however, the pharynx was not regenerated properly following *pbx(*RNAi). The mouth of the pharynx does not connect with the aborted pharynx rudiment following *pbx(RNAi)*.

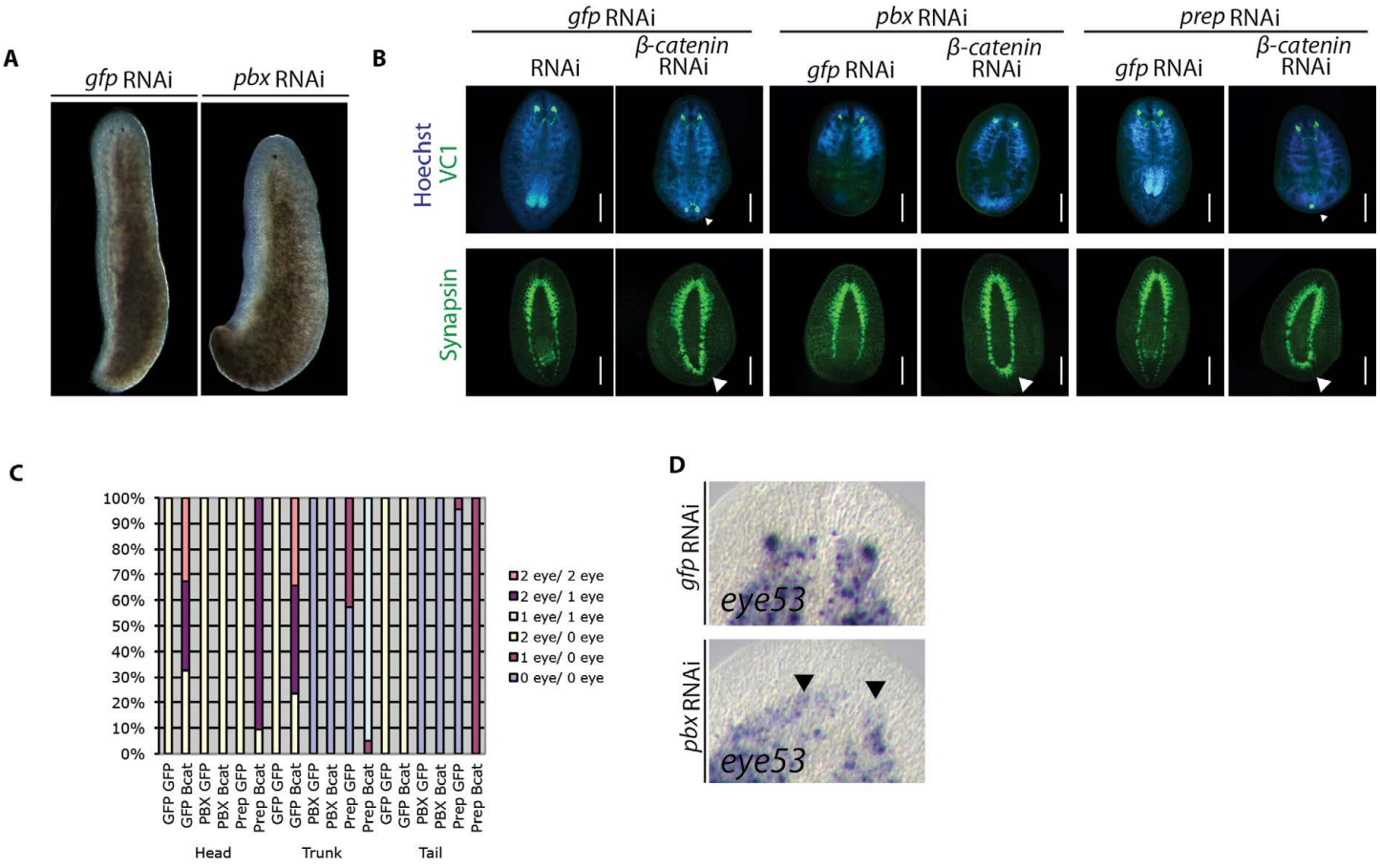

Fig. S6. *pbx* is required for eye regeneration independently of its role during CG regeneration. (**A**) By 14 dpa, regenerated eyes were observed in control *gfp(RNAi)* lateral blastemas, whereas they are absent following *pbx(RNAi)*. (**B**) VC1 anti-arrestin immunolabeling of eye structures in 14 dpa head pieces. Ectopic posterior eye regeneration was observed following combined *gfp/*β-*catenin* RNAi and *prep/*β-*catenin* RNAi (small arrowheads), whereas it was not observed following *pbx/*β-*catenin* RNAi, despite regeneration of a comparable degree of ectopic posterior CG in each case, revealed by 3C11 anti-synapsin immunolabeling (shown by large arrowheads). VC1 labeled the existing eye structures of *pbx(RNAi)* heads. Hoechst staining reveals the outline of the regenerating head. (**C**) Eye scoring of 14 dpa regenerates following combined *pbx /*β-*catenin* and *prep/*β-*catenin* RNAi. Each pool consists of more than 30 regenerated pieces. Eyes were not regenerated in any of the conditions following *pbx(RNAi)*. (**D**) By 3 weeks of homeostasis, *eye53* expression is lost from the eyes following *pbx(RNAi)*. Scale bars: 200 μm.