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pbx is required for pole and eye regeneration in planarians

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SUMMARY

Planarian regeneration involves regionalized gene expression that specifies the body plan. After amputation, planarians are capable of regenerating new anterior and posterior poles, as well as tissues polarized along the anterior-posterior, dorsal-ventral and medial-lateral axes. Wnt and several Hox genes are expressed at the posterior pole, whereas Wnt inhibitory genes, Fgf inhibitory genes, and *prep*, which encodes a TALE-family homeodomain protein, are expressed at the anterior pole. We found that *Smed-pbx* (*pbx* for short), which encodes a second planarian TALE-family homeodomain transcription factor, is required for restored expression of these genes at anterior and posterior poles during regeneration. Moreover, *pbx(RNAi)* animals gradually lose pole gene expression during homeostasis. By contrast, *pbx* was not required for initial anterior-posterior polarized responses to wounds, indicating that *pbx* is required after wound responses for development and maintenance of poles during regeneration and homeostatic tissue turnover. Independently of the requirement for *pbx* in pole regeneration, *pbx* is required for eye precursor formation and, consequently, eye regeneration and eye replacement in homeostasis. Together, these data indicate that *pbx* promotes pole formation of body axes and formation of regenerative progenitors for eyes.

KEY WORDS: Axis formation, Eye, Homeodomain, pbx, Planarians, Regeneration

INTRODUCTION

Planarians are capable of regenerating any missing body part and are an emerging system for investigation of cellular and molecular mechanisms underlying regeneration. Regeneration requires production of new cells and instructions that specify the identity of cell types to be regenerated. The planarian *Schmidtea mediterranea* utilizes a population of dividing regenerative cells called neoblasts (Reddien and Sánchez Alvarado, 2004), which includes pluripotent stem cells (cNeoblasts) (Wagner et al., 2011), to regenerate any missing body part. Robust regenerative mechanisms exist for restoration of the body plan, involving genes that regulate anterior-posterior (AP), medial-lateral (ML) and dorsal-ventral (DV) polarization of tissues (Reddien and Sánchez Alvarado, 2004; Reddien, 2011).

Several signaling pathways and transcription factors are essential for regulation of planarian regeneration. Wnt signaling controls AP regeneration polarity (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Adell et al., 2009; Petersen and Reddien, 2009b; Gurley et al., 2010; Petersen and Reddien, 2011), which is the decision to regenerate a head or tail at transverse amputation planes (Morgan, 1898; Morgan, 1905). Multiple Wnt genes and genes encoding candidate secreted inhibitors of Wnt signaling are expressed in distinct spatial domains along the AP axis (Reddien, 2011; Almuedo-Castillo et al., 2012). Several members of the Hox family, such as *DjAbd-Ba* and *Plox4-Dj*, are expressed in the planarian posterior (Orii et al., 1999; Nogi and Watanabe, 2001). *Smed-prep*, which encodes a TALE (three amino acid loop extension) family homeodomain protein, is expressed at the tip of planarian heads and is required for anterior pole marker expression in regeneration (Felix and Aboobaker, 2010). A LIM-homeobox gene, *Djislet*, is expressed in the posterior and is required for posterior pole marker expression in regeneration (Hayashi et al., 2011). Regeneration of the DV and ML axes requires Bmp signaling, with Bmp signaling components differentially expressed along the DV and/or ML axes (Orii et al., 1998; Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007; Molina et al., 2009; Gaviño and Reddien, 2011; Molina et al., 2011). In addition to their functions in regeneration, these signaling pathways and transcription factors also display constitutive expression in the adult planarian body with several being required for homeostatic maintenance of the body plan during natural tissue turnover (Reddien, 2011). For example, RNAi of the Wnt signaling component *β-catenin-1* results in ectopic head appearance around the periphery of intact animals (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). These observations indicate that actively maintained expression of genes regulating body position instructs tissue turnover, but how these regional expression patterns are maintained and regenerated is poorly understood.

pbx encodes a TALE-class homeodomain protein and can regulate gene expression in a variety of developmental contexts (Moens and Selleri, 2006; Laurent et al., 2008). There are four mammalian Pbx genes (Kamps et al., 1990; Nourse et al., 1990; Monica et al., 1991; Wagner et al., 2001), five zebrafish Pbx genes (Pöpperl et al., 2000; Vlachakis et al., 2000), one *Drosophila pbx* homolog, *extradenticle* (*exd*) (Rauskolb et al., 1993) and three *Caenorhabditis elegans pbx* homologs, *ceh-20* (*C. elegans* homeobox), *ceh-40* and *ceh-60* (Bürglin and Ruvkun, 1992; Bürglin, 1997; Mukherjee and Bürglin, 2007). *pbx* was first characterized for regulating antero-posterior patterning during embryonic development as a co-factor of the Hox genes. In *Drosophila*, mutations in the *exd* gene cause homeotic transformations without affecting the expression of corresponding Hox genes and Exd controls anterior-posterior patterning in the fly embryo by acting together with Hox proteins (Peifer and Wieschaus, 1990; Rauskolb et al., 1993; Rauskolb et al., 1995). Studies of the zebrafish *lzr* (*pbx4*) gene in AP hindbrain patterning suggest that it promotes the action of multiple Hox genes in vertebrates (Pöpperl et al., 2000). In *C. elegans*, Hox

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genes, including *lin-39*, *mab-5* and *nob-1*, act with *ceh-20* in a variety of AP regionalized processes, including postembryonic mesodermal differentiation, cell migration, vulval development and programmed cell death (Liu and Fire, 2000; Shemer and Podbilewicz, 2002; Van Auken et al., 2002; Arata et al., 2006; Takács-Vellai et al., 2007). Pbx genes can interact with additional transcription factors to control expression of various signaling factors. *exd* is required for proper expression of *wingless* and *dpp* in parasegments of the *Drosophila* midgut (Rauskolb and Wieschaus, 1994). Induction of *Fgf* during zebrafish hindbrain and fin development requires *lzr* (*pbx4*) and *pbx2* (Pöpperl et al., 2000; Waskiewicz et al., 2002). Mouse Pbx proteins, together with Prep1 (also known as Pknox1) and Meis, can regulate expression of *Wnt9b* and *Wnt3* for face morphogenesis (Ferretti et al., 2011).

We found that *pbx* in planarians is required for proper expression of genes implicated in control of AP or DV axis patterning. *pbx* is the first gene reported to be important for regeneration/formation of both poles of the AP axis. *pbx* is also required for eye regeneration and formation of eye progenitors. Our results suggest that *pbx* has an essential role in cell fate specification and pole formation during regeneration in planarians.

MATERIALS AND METHODS

Animal culture and radiation treatment

Asexual *Schmidtea mediterranea* strain (CIW4) animals were starved 7-14 days prior to experiments. Animals were exposed to a dose of 6000 rads of radiation using a dual Gammacell-40 137 cesium source and amputated 4 days after irradiation.

Molecular biology and RNAi

Primer sequences for constructs generated are listed in supplementary material Table S1. Clone H.110.1c was used for *pbx* RNAi feeding. The negative RNAi control was *C. elegans unc-22*. Bacteria were mixed with 67% liver paste at a 1:300 ratio to culture volume. Worms were fed on days 0, 3, 6 and 9 and amputated on day 10. RNAi feeding (Fig. 1C; Fig. 3; Fig. 4C; Figs 5-7) strongly reduced *pbx* expression (supplementary material Fig. S1). For long-term RNAi, animals were fed twice every week. *prep* RNAi by feeding utilized coding nucleotides 135-2002 and RNAi efficacy was comparable to a previous study (Felix and Aboobaker, 2010). In Fig. 1C, animals were injected with 600 μg/ml of dsRNA to create weak *pbx* and *smedwi-2* RNAi phenotypes. RNAi by injection (Fig. 1A,B; Fig. 2B,C,E; Fig. 4A,B) of 3-5 mg/ml *pbx* dsRNA yielded a stronger phenotype than did RNAi by feeding. Transversely amputated fragments were injected with dsRNA within 30 minutes to 4 hours after amputation, and were injected again the next day. This procedure (amputation and injection, followed by booster injection the next day) was performed again on days 4/5 and 7/8 after the initial amputation. For assessing the neoblast wound response, amputation and injection, followed by booster injection the next day was repeated on days 3/4, injections were performed on days 6/7, and amputation was carried out on day 9.

In situ hybridization, immunostaining and imaging

In situ hybridizations (ISH) and fluoresence ISH (FISH) were performed as described (Pearson et al., 2009) with two additional 30-minute prehybridization buffer washes after hybridization. For immunostaining, animals were fixed as for *in situ* hybridization, then treated as described (Newmark and Sánchez Alvarado, 2000). α-Myosin heavy chain, TMUS-13 antibody (Romero et al., 1991), rabbit α-tryptophan hydroxylase (α-TH) antibody against peptide HPSDPFYTPEPDCC, and α-ARRESTIN (α-ARR) were used. Fluorescent images were taken with a Zeiss LSM710 Confocal Microscope. For blastema measurements, animals were imaged with a Zeiss Discovery microscope and the head blastema area (unpigmented area) was determined using the measurement tool in Axiovision software. Numbers of cells labeled with RNA probes were determined by manual inspection and Axiovision software.

RESULTS

pbx is required for normal regeneration and locomotion

We identified a single *Schmidtea mediterranea* gene predicted to encode a protein containing PBC (PBX-containing) homeodomains similar to PBX proteins in other species (supplementary material Fig. S2); we named this gene *Smed-pbx* (‘*pbx*’ hereafter). RNAi of *pbx* resulted in multiple regeneration defects. *pbx(RNAi)* animals exhibited variable anterior blastema size, depending upon RNAi conditions (see Materials and methods), ranging from normal to 50% smaller than the control. These animals were capable of regenerating interior tissues following removal of a lateral tissue wedge, and lateral tissue following parasagittal amputation (Fig. 1A; supplementary material Fig. S3A). *pbx(RNAi)* animals failed to regenerate eyes and displayed uncoordinated movement, involving flat body posture and little cilia-mediated propulsion, while twisting in place (Fig. 1A; supplementary material Movie 2).

Despite slightly reduced blastema size and failed eye regeneration, *pbx(RNAi)* blastemas generated other differentiated cell types (Fig. 1B). For example, the nervous system (*choline acetyltransferase*, *chat*$^{+}$) (Wagner et al., 2011), the protonephridia system (*cubilin*$^{+}$) (Scimone et al., 2011), muscle fibers (myosin heavy chain^{+}) (Cebrià and Vispo, 1997), subepidermal marginal adhesive gland cells (*mag-1*$^{+}$) (Sánchez Alvarado et al., 2002; Zayas et al., 2010), *laminB*$^{+}$ lateral tissue (Kato et al., 1999), *cintillo*$^{+}$ sensory neurons (Oviedo et al., 2003) and *mat*$^{+}$ (Wenemoser and Reddien, 2010) intestine were all regenerated. The central nervous system of *pbx(RNAi)* animals displayed morphological defects: whereas animals normally have separate cephalic ganglia, there were no separate ganglia in *pbx(RNAi)* head blastemas. This phenotype is similar to that caused by RNAi of *Smed-prep*, which is required for anterior pole formation during head regeneration (Felix and Aboobaker, 2010). However, *prep* RNAi only results in partial loss of photoreceptors during regeneration and does not cause overt locomotion abnormalities, indicating that *pbx* has distinct roles from *prep*. The abnormalities in differentiated tissue pattern in *pbx(RNAi)* blastemas (e.g. the cephalic ganglia) were also present at later time points following amputation, suggesting that the defects observed do not simply reflect delayed regeneration (supplementary material Fig. S3B). Neoblasts respond to wounds with a body-wide increase in proliferation, followed by a second increase in proliferation near wounds (Wenemoser and Reddien, 2010). This neoblast wound response pattern still occurred in *pbx(RNAi)* animals, with a slightly lower second increase in proliferation (supplementary material Fig. S3C,D). Therefore, *pbx(RNAi)* regeneration abnormalities are not associated with robust neoblast loss or a gross defect in neoblast capacity to respond to wounds.

Planarian eyes, which contain photoreceptive neurons and pigmented optic cup cells, failed to regenerate in *pbx(RNAi)* animals (Fig. 1C). To determine whether eye absence is simply a consequence of reduced *pbx(RNAi)* blastema size, we compared eye formation in *pbx(RNAi)* and *smedwi-2(RNAi)* animals following partial inhibition by RNAi. Weak *smedwi-2* inhibition resulted in head blastemas smaller than *pbx(RNAi)* head blastemas, but with more prominent eye regeneration, indicating simply reduced blastema size does not explain the *pbx* RNAi eye phenotype (Fig. 1C; supplementary material Fig. S4). Planarian eye regeneration involves migratory precursor cells that express transcription factors promoting eye formation (Lapan and Reddien, 2011). *otxA*$^{+}$ *eya*$^{+}$ eye precursor cells were significantly reduced in *pbx(RNAi)* animals at day 6 of regeneration, indicating that *pbx* is required for eye progenitor formation (Fig. 1C).

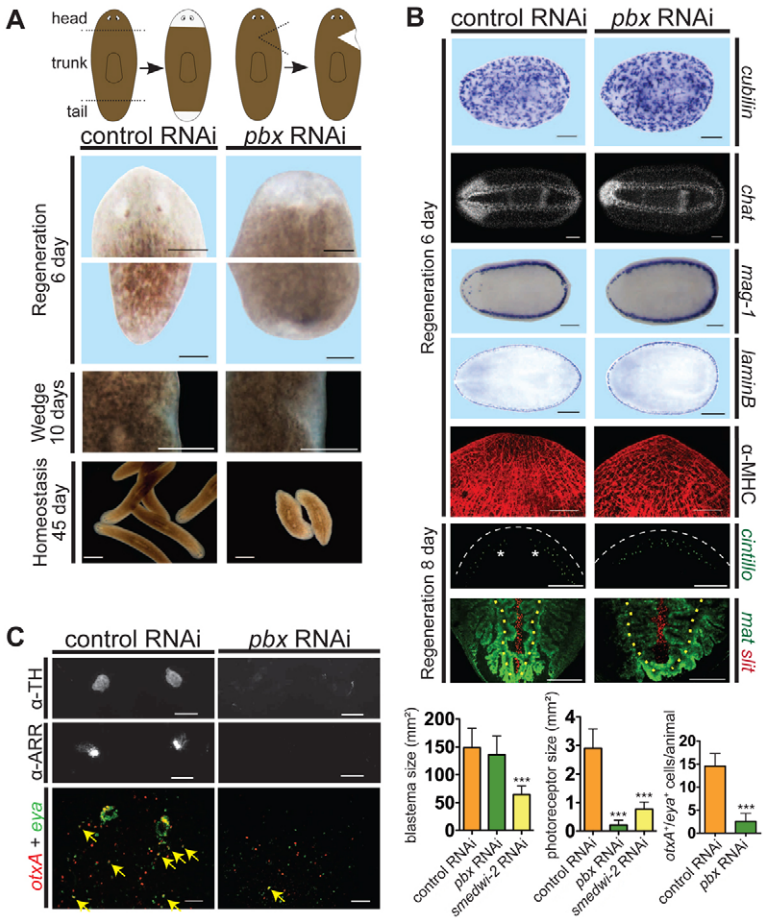

Fig. 1. *pbx(RNAi)* planarians fail to regenerate eyes. (**A**) *pbx(RNAi)* animals regenerated blastemas without eyes 6 days after cutting (n=96) and displayed locomotion defects when exposed to long-term RNAi (n=20). *pbx(RNAi)* animals generated unpigmented tissue at the site of wedge amputations at 10 days after amputation (control, n=8/8; *pbx(RNAi)*, n=8/8). Left cartoon depicts head and tail amputation sites; right cartoon depicts wedge regeneration sites. Scale bars: 200 µm for pole regeneration, 1 mm for wedge regeneration, 1.26 mm for homeostasis. (**B**) Whole-mount ISH 6 days after regeneration demonstrates that *pbx(RNAi)* animals regenerated protonephridia (*cubilin*, n=5/5), the nervous system with morphological defects (*chat*, n=13/13), subepidermal marginal adhesive gland cells (*mag-1*, n=6/6), *lamin B*-positive lateral tissue (n=12/12) and muscle fibers (α-MHC, n=16/16). At 8 days, *pbx(RNAi)* sensory cells (*cintillo*$^+$) regenerated with normal cell number (P=0.325, t-test), but failed to distribute into two lateral domains (n=6/6). *pbx(RNAi)* intestinal branches were generated, but slight defects in medial branch length were present (*mat*, n=2/7), and midline cells failed to extend completely posteriorly (*slit*, n=6/7). Feeding RNAi was used for *lamin B* and α-MHC images; similar results were obtained with dsRNA injection. Anterior is to the left except for α-MHC, *cintillo* and *mat/slit* panels, which are oriented anterior to the top. Asterisks, photoreceptors; white dashed lines, head rim; yellow dotted lines, primary posterior intestinal branches. Scale bars: 200 µm except *chat*, 100 µm; and α-MHC, 50 µm. (**C**) *pbx(RNAi)* animals failed to regenerate pigment cup cells (α-TH immunostaining, n=0/10 were normal) and photoreceptor neurons (α-ARR immunostaining, n=0/10 were normal); eye progenitor numbers (double-labeled with *otxA* and *eya* RNA probes) were greatly reduced (n=13, right-hand graph, ***P<0.0001, t-test). Animals are oriented anterior to the top. Yellow arrows, double-positive cells. The eye regeneration defect in *pbx(RNAi)* animals is not explained only by reduced blastema size (n≥8 each). Left and middle graphs, one-way ANOVA tests followed by a Dunnet post-hoc test; ***P≤0.001 for experimental condition versus control. Graphs show mean±s.e.m. Scale bars: 50 µm.

pbx is required for expression of genes at AP poles in regeneration

Given the known roles for *pbx* genes in tissue patterning, we assessed whether regulatory genes involved in regeneration and adult body plan maintenance were expressed normally in *pbx(RNAi)* blastemas. Genes with candidate roles in regulating planarian positional information will be referred to here as 'patterning genes' and are utilized as markers of body regionalization in the experiments described below (Fig. 2A). Patterning genes were selected from previously reported data as (1) displaying regionalized expression and (2) either displaying a patterning-abnormal RNAi phenotype or encoding a protein predicted to regulate a pathway (Wnt, Bmp or Fgf signaling) important for patterning planarian body axes. RNAi phenotypes have not been reported for all patterning genes that will be described. The expression of four patterning genes that normally display strong and restricted expression in animal head tips, *sFRP-1* (Gurley et al., 2008; Petersen and Reddien, 2008), *notum* (Petersen and Reddien, 2009b), *ndl-4* (Rink et al., 2009) and *prep* (Felix and Aboobaker, 2010), was strongly reduced or absent in *pbx(RNAi)* anterior blastemas (Fig. 2B). *sFRP-1* encodes a predicted secreted frizzled-related protein, which can antagonize Wnt signaling in other organisms (Leyns et al., 1997). *notum* encodes a secreted hydrolase that antagonizes Wnt signaling in planarian regeneration (Petersen and Reddien, 2011) and in *Drosophila* (Gerlitz and Basler, 2002; Giráldez et al., 2002). *ndl-4* encodes a Nou darake-family FGF-receptor-like protein; the related planarian *nou darake* gene is expressed anteriorly and is required for anterior cephalic ganglia

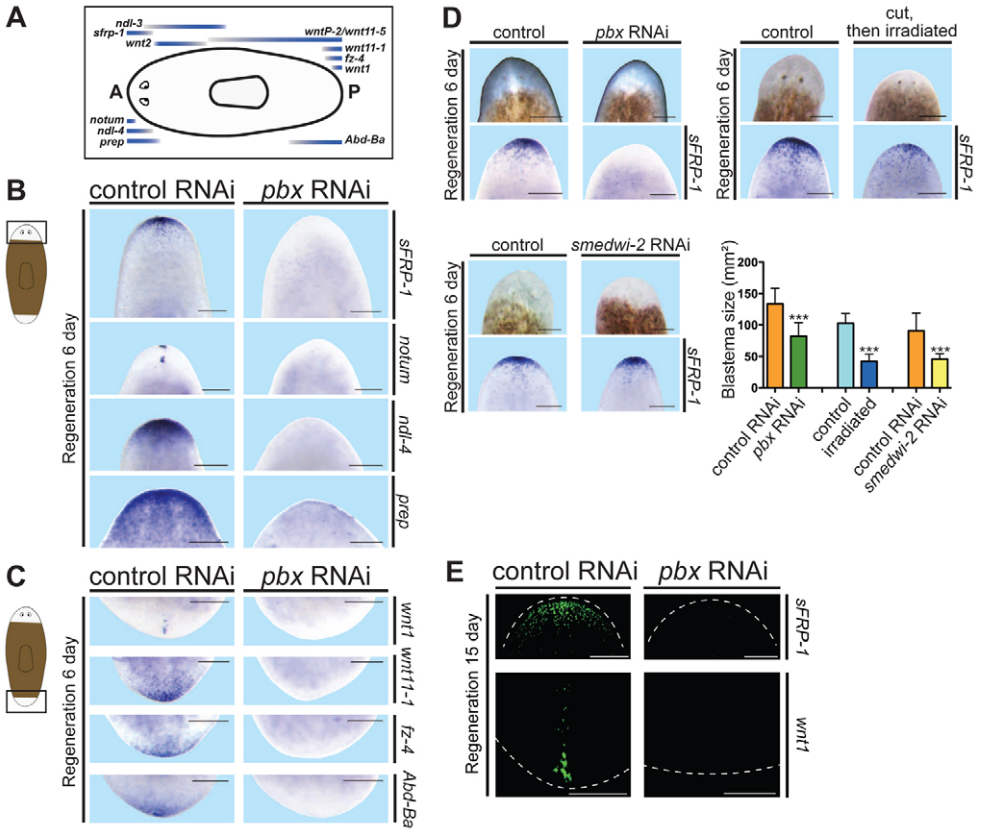

Fig. 2. *pbx* is required for expression of genes at AP poles in regeneration. (**A**) Cartoon shows AP expression locations of genes used in this study to characterize body axis regionalization; eyes (in the head) and the pharynx (centrally located) are depicted. A, anterior; P, posterior. (**B**) Whole-mount ISH showing missing or reduced gene expression in the anterior blastema of *pbx(RNAi)* planarians: *sFRP-1* (control *n*=14/14; *pbx n*=0/15), *notum* (control *n*=6/6; *pbx n*=4/6 greatly reduced and mislocalized, *n*=2/6 not present), *ndl-4* (control *n*=5/6; *pbx n*=0/6) and *prep* (control *n*=12/12; *pbx n*=0/12). Cartoon depicts area shown in images. (**C**) Expression of posterior markers, *wnt1* (control *n*=6/6; *pbx n*=1/6), *wnt11-1* (control *n*=4/6; *pbx n*=0/6), *fz-4* (control *n*=6/6; *pbx n*=1/6) and *Abd-Ba* (control *n*=6/6; *pbx n*=0/5) was absent in *pbx(RNAi)* animals. Cartoon depicts area shown in images. (**D**) Animals with reduced anterior blastema size displayed more *sFRP-1* expression than did *pbx(RNAi)* animals. Upper left panel: *pbx* RNAi involved dsRNA injection with 3-5 mg/ml dsRNA (*n*=10 for both conditions); upper right panel: small blastemas were generated by exposing cut worms to 6000 rads of γ-radiation 24 hours after cutting (control *n*=10; irradiated *n*=8); lower left panel: small blastemas were generated by weak RNAi of *smedwi-2* (one feeding with amputation 3 days later) (*n*=10). Quantification of blastema size is shown in the bottom graph (mean±s.e.m.; ***P=0.0001, left pair; ***P<0.0001, middle pair; ***P=0.0002, right pair; *t*-test for each pair of conditions). (**E**) Pole markers *sFRP-1* (control, *n*=6/6, *pbx* RNAi, *n*=0/6) and *wnt1* (control, *n*=6/6, *pbx* RNAi, *n*=1/6) were absent in *pbx(RNAi)* animals after 15 days of regeneration. All animals are at regeneration day 6 except for panel E and are oriented anterior at the top. Scale bars: in E, 100 μm; in B,C,D, 200 μm.

restriction (Cebrià et al., 2002). *prep* is required for normal head regeneration (Felix and Aboobaker, 2010). These observations suggest that *pbx* is required for anterior pole regeneration.

Similarly, expression of patterning genes normally restricted to the posterior pole was abnormal in *pbx(RNAi)* regenerating tails (Fig. 2C). The posterior pole expression of *wnt1* (Petersen and Reddien, 2008), *wnt11-1* (Petersen and Reddien, 2008), *frizzled-4* (Gurley et al., 2008) and *Abd-Ba* (Nogi and Watanabe, 2001) was absent in *pbx(RNAi)* animals. These data indicate that *pbx* is also required for posterior pole regeneration. The reduced AP pole expression of patterning genes in *pbx(RNAi)* animals is not simply explained by slightly small blastema size. Irradiation after amputation, or partial inhibition with RNAi of 12 genes required for blastema formation (e.g. the *smedwi-2* gene) (Reddien et al., 2005a; Reddien et al., 2005b), caused regeneration of smaller head blastemas than did *pbx* RNAi, but these small blastemas displayed more *sFRP-1* expression than did *pbx(RNAi)* blastemas (Fig. 2D; supplementary material Fig. S5). Furthermore, patterning gene expression at poles was not present even at a late time point after amputation of *pbx(RNAi)* animals, indicating that pole formation is not simply delayed (Fig. 2E). These data suggest that *pbx* is required for expression of polarized markers in anterior and posterior blastemas.

Some patterning gene expression fails to be restricted from *pbx(RNAi)* poles

Because of the anterior and posterior pole defects of *pbx(RNAi)* animals, we assessed patterning gene expression that is normally restricted from poles. *wnt2* and *ndl-3* are expressed in the anterior, but not strongly at the head tip of intact animals (Petersen and Reddien, 2008; Rink et al., 2009). In contrast to other AP polarized markers, *wnt2* and *ndl-3* were expressed in *pbx(RNAi)* anterior blastemas, but expression extended abnormally to the regenerating head tip (Fig. 3A). Therefore, *pbx* is not required for expression of all patterning genes in regeneration. Furthermore, these observations indicate that the wild-type anterior-most gene expression domain is absent in *pbx(RNAi)* heads, with head tips instead displaying expression typical for the posterior region of heads.

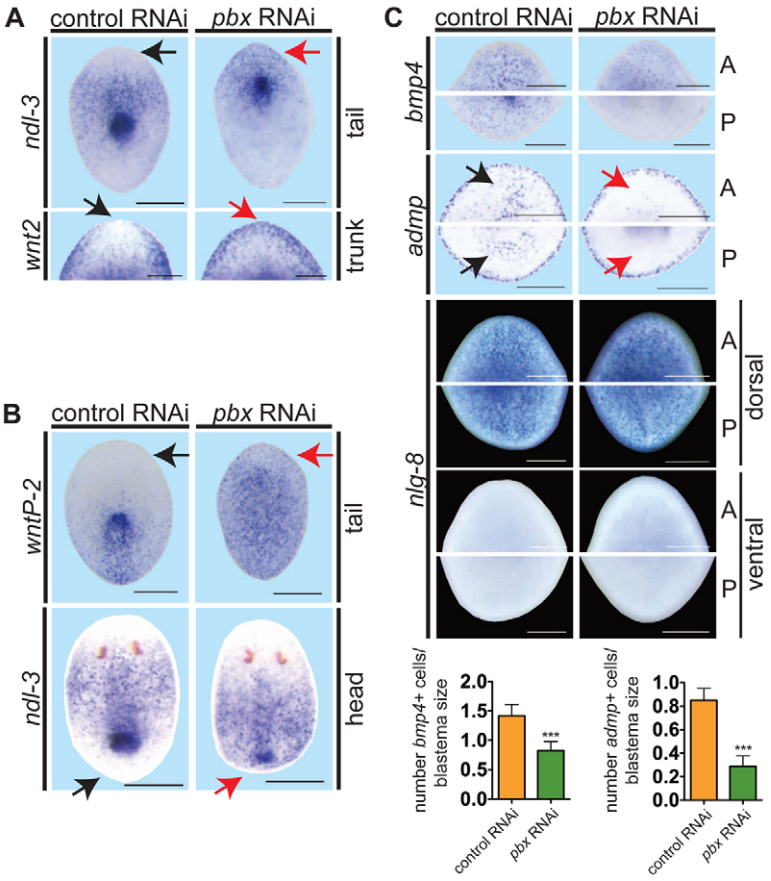

Fig. 3. *pbx(RNAi)* animals display abnormal pole gene expression and fail to re-scale expression gradients during regeneration. (**A-C**) Whole-mount ISH of regenerating pieces of planarians at day 6. Anterior is to the top. Black arrows, normal gene expression location (control); red arrows, aberrant expression in *pbx(RNAi)* animals. (A) *pbx(RNAi)* animals exhibited aberrant anterior blastema gene expression: *ndl-3* (control, *n*=15/15; *pbx* RNAi, *n*=1/16 display restriction from the head tip of tail fragments) and *wnt2* (control, *n*=10/11; *pbx* RNAi, *n*=0/13 display restriction from the head tip; head blastema of trunk fragment shown). (B) *pbx(RNAi)* animals failed to re-scale the *wntP-2* expression gradient (control, *n*=17/18; *pbx* RNAi, *n*=4/20 tail fragments show re-scaling) and *ndl-3* expression (control, *n*=22/25; *pbx* RNAi, *n*=1/22 head fragments show re-scaling). (C) Dorsal *bmp4* expression (control, *n*=18; *pbx* RNAi, *n*=20) and ventral *admp* expression (control, *n*=13; *pbx* RNAi, *n*=10) were reduced in *pbx(RNAi)* animals, but dorsal expression of *nlg-8* was normal in both anterior (A) and posterior (P) blastemas (control, *n*=13/13; *pbx* RNAi, *n*=12/12). 'Head', 'trunk' and 'tail' refer to a head fragment regenerating a tail, a fragment with head and tail amputated transversely, and a tail fragment regenerating a new head after transverse amputation, respectively. Graphs show quantification of $bmp4^+$ and $admp^+$ cells normalized to blastema size (mean±s.e.m.; ***P=0.0001, *t*-test). Scale bars: 200 µm.

pbx is required for re-scaling of AP gene expression gradients during regeneration

Blastema formation can be accompanied by re-scaling and re-patterning of pre-existing tissues (a process known as morphallaxis) (Morgan, 1898; Reddien and Sánchez Alvarado, 2004). Accordingly, patterning genes can change expression during regeneration through new expression (in fragments initially lacking expression) or by re-scaling an existing expression domain to accommodate changing dimensions of the regenerating fragment. For example, *wntP-2* (also known as *wnt11-5* as *wntP-2* has been proposed to be a *wnt11*-family member) (Gurley et al., 2010) is normally expressed in a posterior-to-anterior gradient (Petersen and Reddien, 2008) and can be involved in regeneration polarity (Petersen and Reddien, 2009b). *wntP-2* expression is initially uniform in tail fragments and recedes towards the posterior pole during regeneration (Petersen and Reddien, 2009a; Gurley et al., 2010). *pbx(RNAi)* animals failed to rescale *wntP-2* expression 6 days after amputation (Fig. 3B). Irradiation kills neoblasts and consequently blocks regeneration (Bardeen and Baetjer, 1904; Dubois, 1949; Reddien and Sánchez Alvarado, 2004). *pbx(RNAi)* tail fragments are similar to irradiated tail fragments (supplementary material Fig. S6), in that *wntP-2* expression is initially restricted from the wound but returns to the anterior within 6 days (Gurley et al., 2010). Similarly, *ndl-3* expression (normally prepharyngeal) was restricted from the posterior-facing wound of control head fragments, but failed to restrict away from the regenerating posterior blastemas of *pbx(RNAi)* head fragments (Fig. 3B). These results are consistent with the possibility that *pbx*-dependent patterning gene expression at regenerating AP poles is required for stable re-scaling of patterning gene expression gradients.

Reduced *bmp4* and *admp* expression in *pbx(RNAi)* animals

Given the patterning gene expression defects at anterior and posterior poles, we next assessed whether regenerating *pbx(RNAi)* animals had defects in polarized gene expression on the DV axis. *bmp4* expression in unamputated animals is normally strongest medially on the dorsal side and graded laterally; expression is also strong in the dorsal head region (Orii et al., 1998; Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007). The dorsally restricted *bmp4* expression pattern is regenerated in control blastemas. In *pbx(RNAi)* animals, *bmp4* expression in blastemas was reduced but not eliminated (Fig. 3C). Because *bmp4* is normally expressed more strongly in the anterior than the posterior blastema, AP defects might contribute to the *bmp4* expression reduction observed in *pbx(RNAi)* animals. Furthermore, because the effect of *pbx* RNAi on *bmp4* expression was weaker than the effect on AP pole gene expression, it is possible that *pbx* has a more prominent role in AP rather than DV axis regeneration. A second Bmp family-encoding gene, *admp*, is expressed laterally and in a ventral, medial domain (Molina et al., 2009; Gaviño and Reddien, 2011). In transversely amputated *pbx(RNAi)* animals regenerating heads and tails, the ventral domain of *admp* expression was reduced, whereas the lateral domain appeared normal (Fig. 3C). By contrast, *nlg-8*, a noggin-like gene involved in Bmp pathway regulation and normally expressed evenly over the dorsal side of animals and blastemas (Molina et al., 2009), was expressed normally in regenerating *pbx(RNAi)* animals. Therefore, not all patterning genes with DV polarized expression require *pbx* for their expression (Fig. 3C). These data indicate that some DV patterning gene expression levels at the dorsal and ventral midlines also require *pbx*.

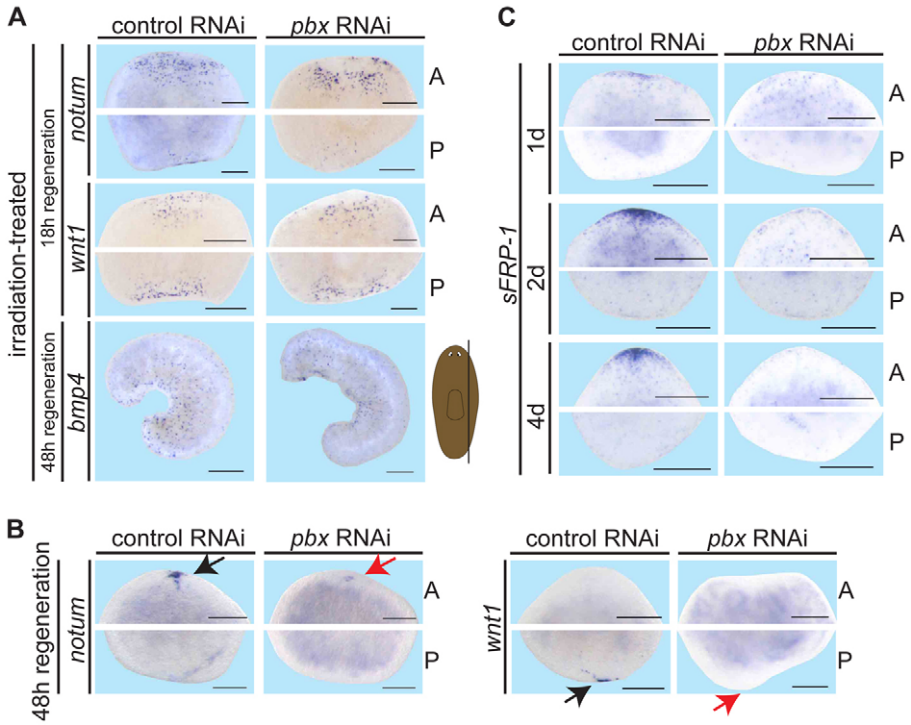

Fig. 4. Wound-induced patterning gene expression is not affected in *pbx(RNAi)* planarians. (**A**) Anterior (A) and posterior (P) blastemas of irradiated *pbx(RNAi)* trunk pieces exhibited normal irradiation-insensitive, wound-induced expression of *notum* (control, *n*=5/7; *pbx* RNAi, *n*=6/6; transversely amputated), *wnt1* (control, *n*=7/7; *pbx* RNAi, *n*=8/8; transversely amputated) and *bmp4* (control, *n*=8/8; *pbx* RNAi, *n*=13/13; parasagitally amputated). Cartoon depicts amputation site; thin fragments were used. (**B**) *notum* expression (control, *n*=6/6; *pbx* RNAi, *n*=2/6 no expression, *n*=3/6 reduced expression) in the anterior blastema and *wnt1* expression (control, *n*=6/6; *pbx(RNAi)*, *n*=0/7 trunk pieces) in the posterior blastema of transversely cut animals 48 hours post-cutting. Black and red arrows indicate normal and reduced/absent expression, respectively. (**C**) *sFRP-1* expression in transversely cut trunk pieces during regeneration; 1, 2 and 4 days (d) shown (*pbx* RNAi, *n*=14 trunk pieces for each condition). Scale bars: 200 μm.

pbx is not required for wound-induced patterning gene expression

The patterning gene *wnt1* is induced rapidly at virtually all wounds (Petersen and Reddien, 2009b), and is required for regeneration polarity (Adell et al., 2009; Petersen and Reddien, 2009b). *wnt1* is expressed at wounds of irradiated animals, indicating that wound-induced *wnt1* expression occurs even in the absence of new cell production (Petersen and Reddien, 2009b). *notum* is also induced at many wound types, at anterior-facing wound edges (Petersen and Reddien, 2011). Both *wnt1* and *notum* wound-induced expression were detected in irradiated *pbx(RNAi)* animals (Fig. 4A). Similarly, *bmp4* is expressed rapidly (within 12 hours) following parasagittal amputation in lateral fragments, even in irradiated animals (Reddien et al., 2007). This irradiation-insensitive *bmp4* expression observed 48 hours after amputation was normal in *pbx(RNAi)* animals (Fig. 4A). Therefore, early wound-induced patterning gene expression involved in axis polarization in regeneration did not require *pbx*.

By 48 hours after transverse amputation, *wnt1* expression is normally clustered at regenerating tail tips and *notum* expression is clustered at regenerating head tips. By contrast, this polarized *wnt1* and *notum* expression was greatly reduced in *pbx(RNAi)* animals (Fig. 4B). To determine more precisely when pole formation abnormalities appear in *pbx(RNAi)* animals, we analyzed *sFRP-1* expression, which is specific to anterior-facing blastemas and largely (but not completely) irradiation-sensitive (Petersen and Reddien, 2009b; Gurley et al., 2010). A low level of *sFRP-1* expression appears in the first 3-9 hours of regeneration, but significant numbers of *sFRP-1*$^{+}$ cells are not visible until 24 hours after amputation (Gurley et al., 2010), later than the robust wound-induced expression phases of *wnt1* and *notum*. *sFRP-1* expression was reduced, but initially present at 24 hour and 48 hour anterior-facing wounds in *pbx(RNAi)* animals, and was absent by 4 days of regeneration (Fig. 4C). Together, these data indicate that *pbx* is not required for the first step in regeneration of tissue pattern (wound-induced expression of AP and DV polarizing genes) but is required after this wound-induced gene expression phase for further development of poles.

The requirement of *pbx* for eye regeneration is separable from its requirement in pole regeneration

Several lines of evidence indicate that failed eye regeneration in *pbx(RNAi)* animals is not simply explained by anterior pole absence in these animals. First, whereas pole gene expression defects are observed in both *pbx* and *prep* RNAi animals, *pbx* RNAi caused a more severe eye regeneration defect than did *prep* RNAi. For instance, *prep(RNAi)* head blastemas are cyclopic (Felix and Aboobaker, 2010); 18/19 *prep* trunk fragments had one eye whereas 0/20 *pbx(RNAi)* animals regenerated eyes (Fig. 5A). Second, we investigated whether *pbx(RNAi)* animals could regenerate surgically removed eyes, while leaving the anterior pole intact. *notum* and *sFRP-1* had similar expression patterns in control, *prep(RNAi)* and *pbx(RNAi)* animals at this time point after RNAi, indicating that expression of patterning genes was intact (Fig. 5C-E). Both control and *prep(RNAi)* animals regenerated removed eyes within 8 days, whereas *pbx(RNAi)* animals had no detectable eye regeneration (Fig. 5B). Finally, we also investigated the requirement for *pbx* in eye progenitor formation 48 hours after amputation. Eye progenitors initially appear and are dispersed at the wound site/early blastema at this time (Lapan and Reddien, 2011). The number of these initial eye progenitors was severely reduced in *pbx(RNAi)* animals (supplementary material Fig. S7). These results suggest that *pbx* has a requirement in eye regeneration that is not caused by the absence of poles during regeneration.

pbx is broadly expressed, including in neoblasts

pbx displays broad, diffuse expression throughout the entire planarian body and pharynx, including prominent expression in the central nervous system (CNS) (Fig. 6A). Four days after irradiation,

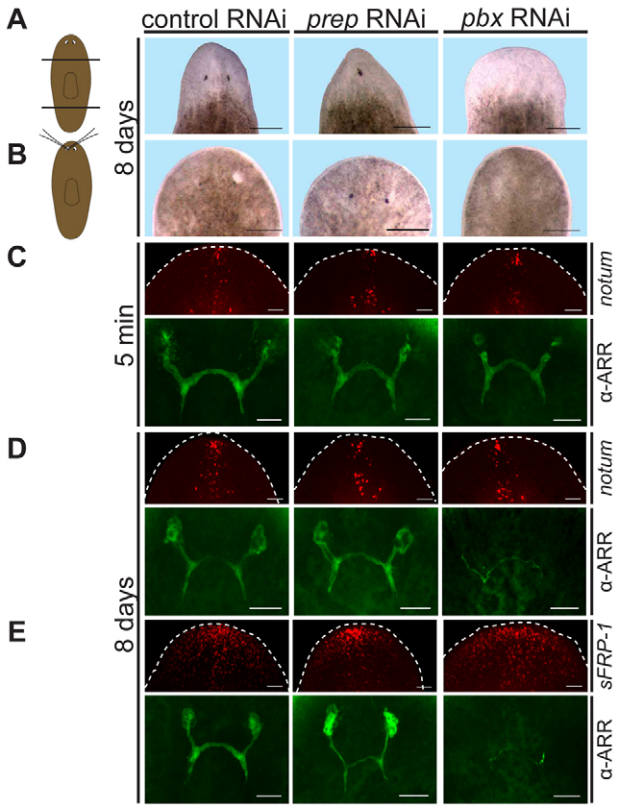

Fig. 5. The requirement of *pbx* for eye regeneration is separable from its requirement for pole regeneration. (**A**,**B**) Cartoons depict head or eye removal. (A) *pbx* (*n*=0/20 no eyes) and *prep* (*n*=18/19 one eye; *n*=1/19 no eyes) RNAi planarians exhibited different eye regeneration phenotypes after transverse amputation. (B) Both control (*n*=25/25) and *prep* RNAi (*n*=24/24) animals regenerated eyes but *pbx(RNAi)* animals (*n*=0/25) did not 8 days after eye removal with glass needles. (**C-E**) Double-fluorescent labeling of *notum* or *sFRP-1* FISH with α-ARRESTIN immunostaining were used to assess anterior pole gene expression and eye regeneration in each animal. (C) Photoreceptor cell bodies were mostly absent 5 minutes after eye removal, with axons (green fluorescence) remaining (control, *n*=9/9; *prep* RNAi, *n*=10/10; *pbx* RNAi, *n*=10/10). *notum* expression was normal. (D,E) Eye regeneration defects were observed in *pbx(RNAi)* animals but not in *prep(RNAi)* or control animals. The size of the cluster of photoreceptor cells increased in *prep(RNAi)* (*n*=24/24) and control (*n*=25/25) animals with time, but did not in *pbx(RNAi)* (*n*=25/25) animals with only sparse pre-existing axon fragments remaining. *notum* and *sFRP-1* expression were normal. Dashed line depicts animal boundary. Anterior is to the top. Scale bars: in A,B, 200 µm; in C-E, 50 µm.

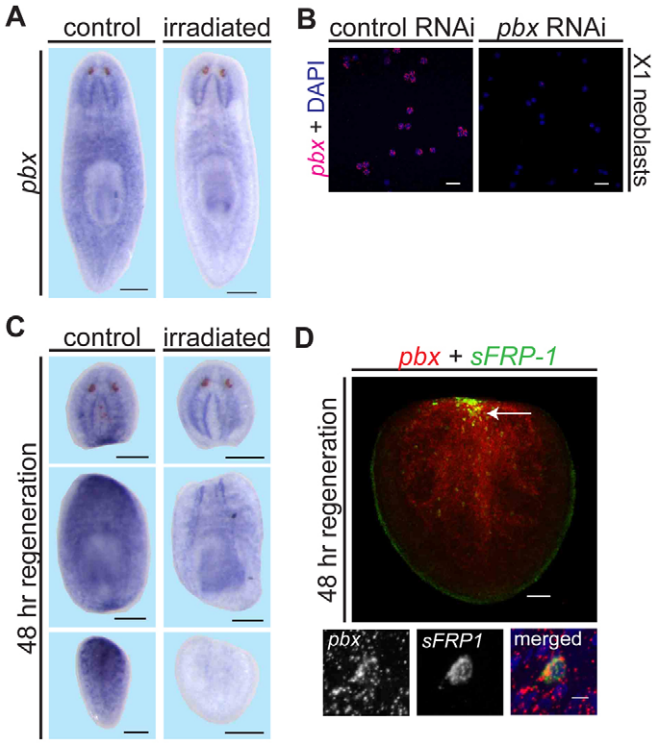

Fig. 6. *pbx* displays broad expression, including in neoblasts. (**A**) Whole-mount ISH showing *pbx* expression in non-irradiated (control) and irradiated planarians (*n*=6 for each). Scale bars: 200 µm. (**B**) *pbx* expression in neoblasts (referred to as X1 cells) sorted using FACS (Hayashi et al., 2006) was detected using FISH. Expression was absent following *pbx* RNAi. Scale bars: 20 µm. (**C**) *pbx* mRNA levels were strong and irradiation sensitive near wounds, 48 hours after amputation (*n*=6 for each). Scale bars: 200 µm. (**D**) Double FISH of *pbx* (red) and *sFRP-1* (green) with DAPI (blue) in a regenerating tail (*n*=10) fragment at 48 hours. Some cells displayed both *pbx* and *sFRP-1* signal, indicated by an arrow. Scale bars: upper panel, 50 µm; lower panels, 5 µm.

pbx^{+} signal was reduced, with the exception of expression in the CNS and pharynx (Fig. 6A). Because neoblasts are specifically eliminated by irradiation, this result suggests that some *pbx* expression might occur in neoblasts. Neoblasts can be isolated using fluorescence-activated cell sorting (FACS) based upon their >2N DNA content (Hayashi et al., 2006). We isolated neoblasts and found these cells to display *pbx* expression (Fig. 6B). *pbx* expression was strong at anterior and posterior wounds 24-48 hours after amputation, correlating with the time *pbx* is required for patterning gene expression (Fig. 6C; supplementary material Fig. S8). *pbx* expression near wounds during regeneration was also irradiation sensitive (Fig. 6C). Cells expressing both *pbx* and the patterning gene *sFRP-1* were detected at 48 hours after amputation. However, the diffuse nature of the *pbx* signal made it difficult to resolve expression at the cellular level, and *pbx* expression appeared to be present in most cells of the blastema (Fig. 6D). These results suggest that *pbx* is expressed in neoblasts that give rise to many newly forming blastema cells where it can regulate expression of pole markers and formation of eye progenitors.

pbx inhibition causes gradual loss of patterning gene expression and eyes during tissue turnover

Does *pbx* have a regeneration-specific role in pole formation? We investigated this question by inhibiting *pbx* during homeostatic tissue turnover. Unamputated *pbx(RNAi)* animals displayed uncoordinated movement (by 20 days of RNAi), whereas neither the control nor *prep(RNAi)* animals displayed a mobility defect (supplementary material Movies 1-3). By more than 6 weeks of RNAi, both *prep* and *pbx* RNAi animals displayed decreased anterior *sFRP-1* and *notum* expression, and increased anterior *wnt2* expression, suggesting that *pbx* and *prep* are both required to maintain normal patterning gene expression at the anterior pole (Fig. 7A). By contrast, only *pbx(RNAi)* animals exhibited reduced *wnt-1*$^{+}$ cell numbers, suggesting that *pbx* functions independently of *prep* in maintenance of posterior patterning gene expression (Fig. 7A). Additionally, photoreceptor neuron cluster size was

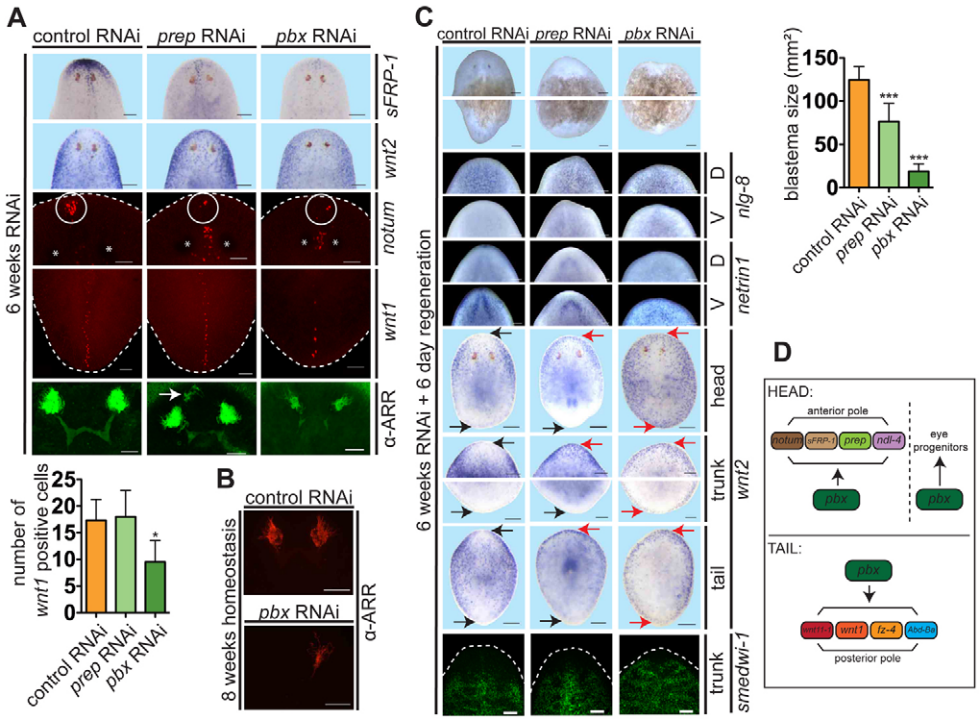

Fig. 7. *pbx* is required for maintenance of pole gene expression and eyes during homeostatic tissue turnover. (**A**) Six weeks of *prep* and *pbx* RNAi caused reduced *sFRP-1* expression (control, *n*=0/13; *prep* RNAi, *n*=13/13; *pbx* RNAi, *n*=10/10) and aberrant *wnt-2* (control, *n*=0/4; *prep* RNAi, *n*=3/3; *pbx* RNAi, *n*=3/3) and *notum* (control, *n*=0/6; *prep* RNAi, *n*=6/6; *pbx* RNAi, *n*=3/3; white circle) expression. *pbx* RNAi, but not *prep* RNAi, caused *wnt1*$^{+}$ cell reduction at the posterior pole (control, *n*=7; *prep* RNAi, *n*=7; *pbx* RNAi, *n*=6; shown are mean±s.e.m.; one-way ANOVA test followed by a Dunnet post-hoc test; *P≤0.05 between experimental condition and control). *pbx(RNAi)* animals, labeled with α-ARRESTIN antibody gradually lost photoreceptors and *prep(RNAi)* animals exhibited extra photoreceptors (white arrow) (control, *n*=6; *prep* RNAi, *n*=6; *pbx* RNAi, *n*=3). Asterisks in images indicate eyes. Scale bars: 50 μm. (**B**) Unamputated *pbx(RNAi)* animals failed to homeostatically maintain photoreceptor neurons after 8 weeks of RNAi (labeled with α-ARRESTIN antibody; *pbx* RNAi, *n*=8/10; control, *n*=0/10). Scale bars: 50 μm. (**C**) Control, *prep* or *pbx* RNAi animals at 6 days of regeneration. Blastema size quantification is shown at right (one-way ANOVA test followed by a Dunnet post-hoc test; ***P≤0.001 between experimental condition and control; *n*=10 for each condition). Dorsal expression of *nlg-8* and ventral expression of *netrin* in trunk anterior blastemas (*n*=14/14 for all conditions). Both *prep* and *pbx* RNAi animals exhibited aberrant *wnt-2* expression at the anterior tip of all regenerating pieces (control, *n*=0/16; *prep* RNAi, *n*=16/16; *pbx* RNAi, *n*=11/12). However, only *pbx(RNAi)* animals showed aberrant *wnt-2* expression at posterior blastemas (control, *n*=1/16; *prep* RNAi, *n*=1/16; *pbx* RNAi, *n*=11/12). Black arrows, normal expression; red arrows, aberrant expression. Neoblasts (*smedwi-1*$^{+}$) were similar in distribution among all RNAi treatments (control RNAi *n*=15/15; *prep* RNAi *n*=16/16; *pbx* RNAi, *n*=14/14 regenerating pieces; shown are mean±s.e.m.). Scale bars: 100 μm. (**D**) Data summary. *pbx* is required for expression of anterior and posterior pole genes in head and tail blastemas, respectively. *pbx* is also required for eye progenitor formation. Dashed lines indicate animal boundaries. All animals are oriented with anterior to the top.

reduced by *pbx* but not *prep* RNAi (Fig. 7A; supplementary material Fig. S9). By 8 weeks of *pbx* inhibition, most animals had completely lost one or both eyes (Fig. 7B). We conclude that the roles of *pbx* in regeneration and tissue turnover are similar for the regulation of gene expression at poles and for eye formation.

Because pole marker expression decreased in *pbx(RNAi)* animals during tissue turnover, we tested whether these animals could regenerate. This experiment differs from prior RNAi experiments because amputation occurred after pole marker expression had decreased. Animals amputated after 6 weeks of *pbx* RNAi produced very small blastemas with 100% penetrance (Fig. 7C). These blastemas displayed normal DV polarized *nlg-8* (dorsally) and *netrin-1* (ventrally) (Cebrià and Newmark, 2005) expression, and generated differentiated cells (Fig. 7C; supplementary material Fig. S10). *wnt2* was expressed at the anterior pole of *pbx(RNAi)* tail fragments, similar to results described above, but was also expressed around the entire animal periphery. Similar *wnt2* levels were present from the anterior to the posterior pole around the periphery of these *pbx(RNAi)* tail fragments (Fig. 7C). In *pbx(RNAi)* head fragments, *wnt2* expression failed to scale away from the posterior pole (Fig. 7C). Control and *prep(RNAi)* trunks and tails had some, but weaker, *wnt2* expression in the posterior periphery. These data indicate that significant blastema formation failed to occur in long-term *pbx* RNAi animals and that these animals display defects in the AP-polarized character of the primary body axis. Very small blastemas could in principle be caused by defects with neoblasts manifesting after long-term *pbx* RNAi. However, approximately normal distribution of and numbers of *smedwi-1*$^{+}$ neoblasts and *agat-1*$^{+}$ neoblast progeny were present in these RNAi animals. Whereas the reason for the severe blastema growth defect in these long-term *pbx(RNAi)* animals is unknown, these data suggest that regeneration failure is not caused by loss of the neoblast population or of the general capacity of neoblasts to differentiate (Fig. 7C; supplementary material Fig. S10).

DISCUSSION

The recent application of cellular and molecular methods to the study of planarian regeneration has allowed identification of genes controlling multiple aspects of regeneration and tissue turnover. Many conserved genes and signaling pathways with central roles

in metazoan development have crucial roles during planarian regeneration, such as involvement in stem cell regulation, organ regeneration and body patterning (Sánchez Alvarado, 2007; Forsthoefel and Newmark, 2009; Adell et al., 2010; Shibata et al., 2010; Aboobaker, 2011; Reddien, 2011). The *Smed-pbx* RNAi phenotype described here identifies *pbx* as a new player in multiple steps of planarian regeneration and homeostatic tissue turnover.

pbx and pole regeneration

Planarian regeneration involves molecular mechanisms that direct restoration of tissue identity (Reddien, 2011). Regeneration polarity (the choice to regenerate a head or tail at transverse amputation planes) is initiated by wound-induced *wnt1* expression (Petersen and Reddien, 2009b). Feedback inhibition of Wnt signaling by *notum* at anterior-facing wounds controls the regeneration polarity switch (Petersen and Reddien, 2011). Once the polarity decision is made at wounds, neoblast-dependent blastema formation is necessary for expression of additional patterning genes (e.g. other Wnt genes and Wnt inhibitory genes) at anterior and posterior poles (Gurley et al., 2010). Despite emerging data regarding signaling molecule expression for polarity initiation, little is understood about the molecules that control expression of pole-specific gene programs.

Our data indicate that *pbx* acts in regeneration at a step after initial expression of *wnt1* and *notum* to control pole-specific patterning gene expression (Fig. 7D). The anterior pole defect in *pbx(RNAi)* animals is similar to that in *prep(RNAi)* animals (Felix and Aboobaker, 2010). *pbx* is required for *prep* expression in regeneration, suggesting that *pbx* functions a step prior to *prep* expression in promoting anterior pole gene expression. The posterior pole defect is similar to that of *Djislet(RNAi)* animals (Hayashi et al., 2011). However, *pbx* is unique in being required for regeneration of both poles. *pbx* is also required for expression during regeneration of *bmp4* medially on the dorsal side and *admp* medially on the ventral side, indicating that the patterning role for *pbx* is not restricted to anterior and posterior extremities.

Several observations suggest that the requirement for *pbx* in regulating gene expression at poles reflects a specific role for *pbx* in pole development rather than being explained as a non-specific consequence of a defect in blastema growth. First, smaller blastemas than those present in *pbx(RNAi)* animals can express pole markers. Second, the effects of *pbx* RNAi on pole expression are robust; little to no expression is observed for some genes, such as *sFRP-1*, in *pbx(RNAi)* blastemas that are only slightly small. Third, *pbx(RNAi)* animals show defects in pole gene expression by 48 hours after amputation and substantial blastema growth and differentiation occurs subsequently, indicating that growth is not arrested before the pole should have formed. Finally, *pbx* is required for homeostatic maintenance of pole gene expression rather than this defect being observed only in blastemas.

Tissue polarization still occurred in *pbx(RNAi)* animals, despite decreased pole marker expression. For example, although blastemas were abnormal following *pbx* RNAi, cephalic ganglia still formed at anterior-facing wounds and DV polarized gene expression (*nlg-8* and *netrin-1*) remained restricted in the normal manner. However, markers that are normally strongest in the pre-pharyngeal region or the posterior base of the head (*wnt2* and *ndl-3*) extended to the anterior head tip of *pbx(RNAi)* animals. These observations indicate that reduced pole marker expression in *pbx* RNAi did not ablate regeneration polarity, but can be associated with axial patterning abnormalities.

Normally posterior features of heads were also present at the anterior end of *pbx(RNAi)* head blastemas. These features include expression domains for *wnt2* and *ndl-3*, a decreased gap between neoblasts (*smedwi-1*$^{+}$) and the head tip, and medial/anterior compression of brain (*chat*$^{+}$) and *cintillo*$^{+}$ domains. One possible interpretation for these defects is that *pbx* specifically promotes anterior pole formation, with pole absence resulting in failed restriction of the posterior head blastema domain. A similar, but alternative explanation is that *pbx* RNAi causes a Hox-like phenotype involving replacement of anterior regions with more posterior ones. However, *pbx* RNAi also resulted in failed posterior pole regeneration. If *pbx* RNAi caused expansion of posterior tail blastema regions at the expense of normally anterior tail blastema regions, tails with expanded, rather than absent, poles would be predicted. Therefore, posterior pole absence in *pbx(RNAi)* animals suggests that *pbx* has a specific role in pole formation and not simply a role in preventing posterior regions from expanding anteriorly. The pole-absence phenotype described in this article and the molecular mechanisms involved in pole formation will be an important area of continued investigation for understanding regeneration.

Multiple roles for *pbx* in regeneration

pbx is required for normal animal locomotion and eye regeneration. Although the cellular basis for the locomotion defect remains to be identified, we determined that *pbx* is required for formation of eye progenitor cells. Planarian eyes are an attractive system for studying eye biology and regeneration, because regenerative eye precursor cells have recently been identified (Lapan and Reddien, 2011). These precursors originate in the neoblasts, migrate into the head blastema, and coalesce to form the regenerating eye. *pbx* was required for *otxA*$^{+}$ *eya*$^{+}$ eye progenitor formation during regeneration, and ultimately for formation of photoreceptor neurons and pigmented optic cups, indicating that *pbx* is a new regulator of planarian eye formation (Fig. 7D). Involvement of *pbx* genes in eye development has been reported in mouse, zebrafish and *Drosophila*, although the role of *pbx* in these cases can be different and its mechanism of action remains to be further elucidated. Conditional *Pbx1* knockout in mouse corneal epithelial cells causes corneal clouding, probably because of failure in proper corneal tissue turnover (Murphy et al., 2010). In zebrafish, *pbx2/4* null embryos have a disorganized photoreceptor layer and retinal ganglion cells that fail to innervate the optic tectum (French et al., 2007). By contrast, *exd* suppresses eye formation in *Drosophila* (Pai et al., 1998). The requirement of *pbx* in eye development in planarians, a member of the Lophotrochozoan superphylum, raises the possibility of a broad use of *pbx* genes in animal eye biology. Further comparison of *pbx* roles in animal eye formation will be important for testing this possibility.

The multiple aspects of the *pbx(RNAi)* phenotype appear to be explained by separable roles for *pbx* in regeneration. For example, *pbx(RNAi)* animals display eye replacement defects following specific eye removal in animals with pole gene expression still present. Therefore, pole absence does not appear to explain the requirement for *pbx* in eye regeneration. Similarly, the eye and locomotion defects observed in *pbx(RNAi)* animals were not observed in *prep(RNAi)* animals, suggesting that these defects are not explained by the pole and brain abnormalities seen under both RNAi conditions. Furthermore, *pbx* is required for the earliest stage of eye progenitor formation, when these progenitors are broad at the wound site (48 hours after amputation). Finally, during tissue turnover in unamputated animals, *pbx* and *prep* RNAi animals lose anterior pole gene expression, but only *pbx(RNAi)* animals lose eyes. Mutants in Pbx genes in other organisms, including in mouse,

zebrafish, *Drosophila* and *C. elegans*, also exhibit a pleiotropic phenotype, consistent with the multiple patterning and cell type specification roles for *pbx* in planarian regeneration (Kurant et al., 1998; Pöpperl et al., 2000). Expression data suggest that *pbx* is expressed in neoblasts and their progeny in blastemas to regulate patterning and eye regeneration (Fig. 7D). A detailed molecular investigation of the role of *pbx* in eye regeneration will be an important future direction.

PBX proteins can interact with other transcription factors, such as Hox, non-Hox homeodomain-containing proteins, or basic helix-loop-helix (bHLH) proteins, for controlling distinct developmental processes (Moens and Selleri, 2006). Therefore, it is possible that SMED-PBX functions with different partner proteins in the control of gene expression important for pole regeneration, eye regeneration and locomotion. Some of the genes for which expression requires *pbx* in planarians, including Wnt and Hox genes, are targets of *pbx* gene action in vertebrate limb development (Capellini et al., 2011). Further analysis of the targets and partner proteins of SMED-PBX during planarian regeneration will be important for understanding its conserved and divergent roles in patterning and cell type specification.

In conclusion, we report that *Smed-pbx* is an important component of multiple steps of planarian regeneration and homeostatic tissue turnover. Formation of new poles of body axes is an essential step in regeneration, and no gene was previously known to be required for this process at both AP poles. *pbx* is therefore an attractive target for molecular dissection of the mechanisms by which re-establishment of tissue pattern occurs in regeneration.

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Competing interests statement
The authors declare no competing financial interests.

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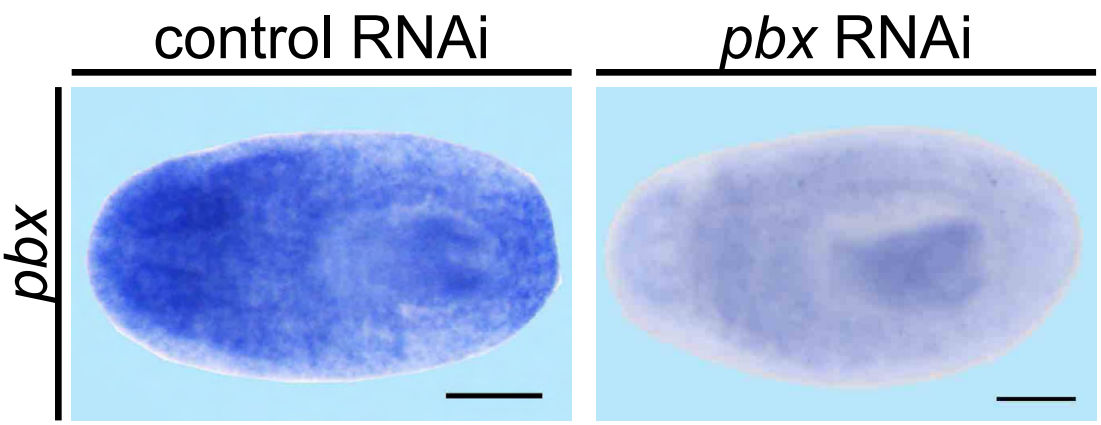

Fig. S1. RNAi of *pbx* by feeding results in reduction of *pbx* mRNA. Worms were fed with control or *pbx* RNAi food as described in Materials and methods. Two days after the last feeding worms were transversely amputated. Day 6 regenerating worms were fixed for whole-mount ISH. Control RNAi animals (6/6) exhibited broad *pbx* expression whereas *pbx(RNAi)* animals (6/6) had only light background staining.

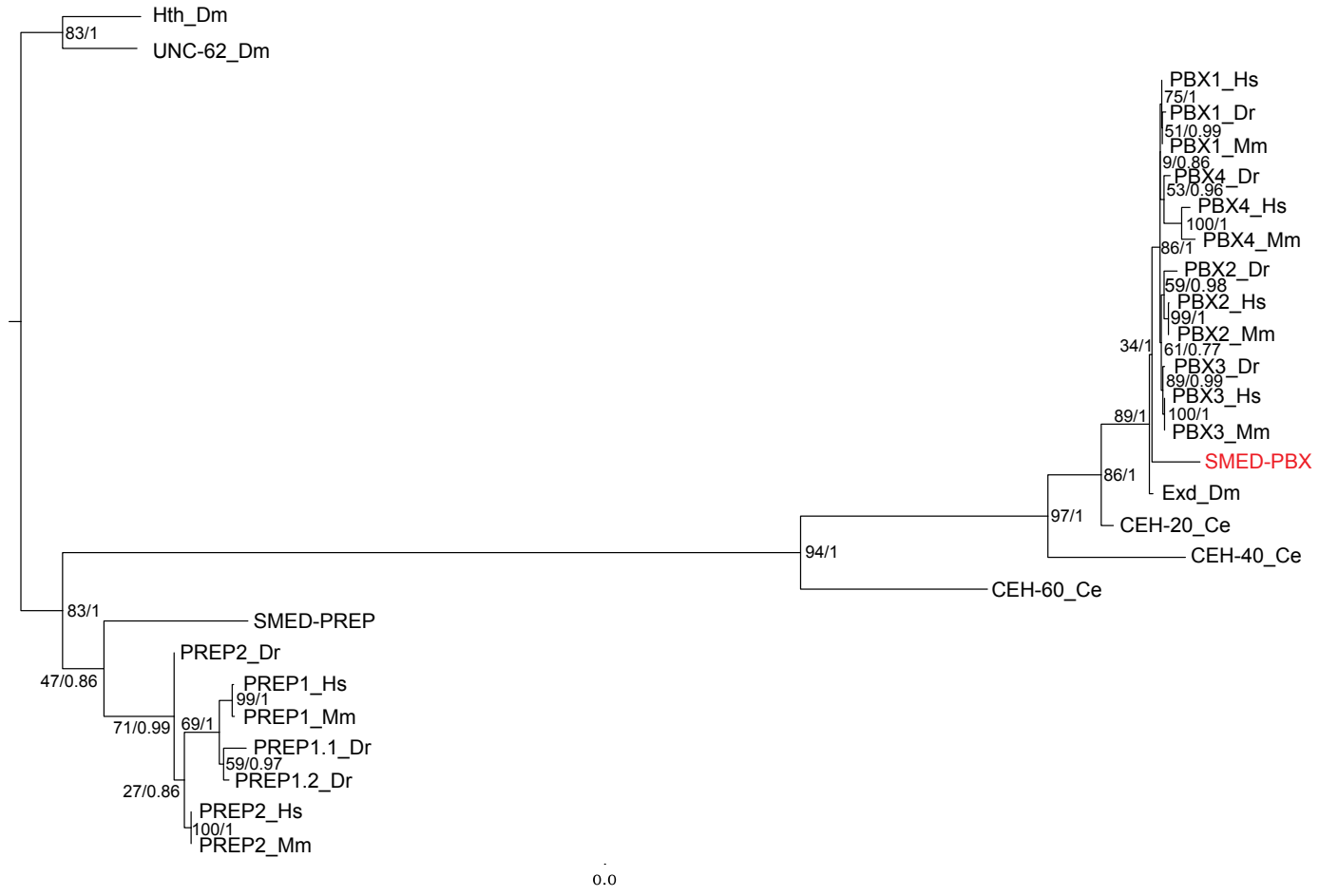

Fig. S2. Phylogenetic analysis of SMED-PBX. Seventeen Pbx, eight Prep and two Meis proteins from diverse organisms were aligned using ClustalW with default settings and trimmed with Gblocks. Maximum likelihood analyses were run using PhyML with 100 bootstrap replicates, the WAG model of amino acid substitution, four substitution rate categories and the proportion of invariable sites estimated from the dataset. The result provides strong support for SMED-PBX (highlighted in red) to be a member of the PBX subfamily of the TALE protein family. All ML bootstrap values are shown. Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Smed, *Schmidtea mediterranea*.

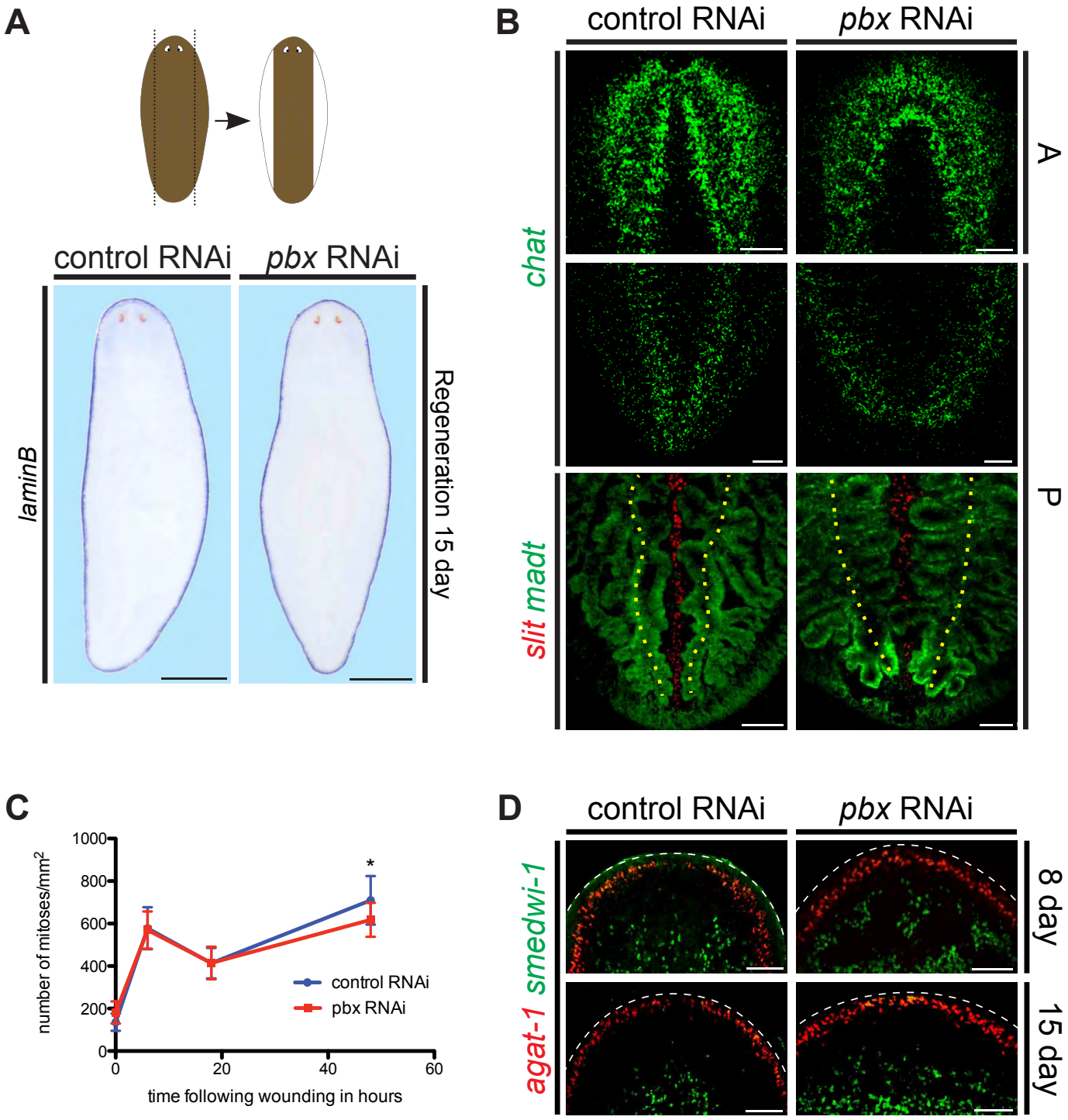

Fig. S3. Additional analysis of the *pbx(RNAi)* phenotype, including neoblasts and late regeneration time points. (**A**) *pbx(RNAi)* animals exhibited normal lateral boundary regeneration 15 days post-amputation (control, *n*=13/14; *pbx* RNAi, *n*=19/21 have uninterrupted *laminB* domains). Cartoon depicts lateral amputation. Scale bars: 0.5 mm. (**B**) *pbx(RNAi)* animals exhibited fused cephalic ganglion lobes (control, *n*=0/6; *pbx* RNAi, *n*=6/6) and truncated posterior nerve cords (control, *n*=0/6; *pbx* RNAi, *n*=6/6) that appeared joined at the posterior end (control, *n*=0/5; *pbx* RNAi, *n*=3/6) at 15 days post-amputation. *pbx(RNAi)* animals displayed widened *slit* expression domains (control, *n*=0/6; *pbx* RNAi, *n*=4/5) and longer medial intestinal branches adjacent to the ventral *slit* expression domain (control, *n*=0/6; *pbx* RNAi, *n*=2/5) at 15 days post-amputation. Yellow dotted lines indicate primary posterior intestinal branches. Scale bars: 200 μm for *chat*; 100 μm for *slit+MADT*. (**C**) Assay of wound response by mitoses per unit area shows no significant difference in mitotic cell density between control and *pbx(RNAi)* animals at the 6 hour global response peak and lower mitotic density in *pbx(RNAi)* animals at the 48 hour missing tissue response peak (**P*<0.05, *t*-test). Error bars represent s.d. (**D**) *pbx(RNAi)* animals exhibited reduced separation between *smedwi-1*$^{+}$ and *agat-1*$^{+}$ domains at 8 days post-amputation (control, *n*=1/6; *pbx* RNAi, *n*=6/6) and 15 days post-amputation (control, *n*=0/6; *pbx* RNAi, *n*=5/5). White dashed lines indicate head rim. Scale bars: 100 μm.

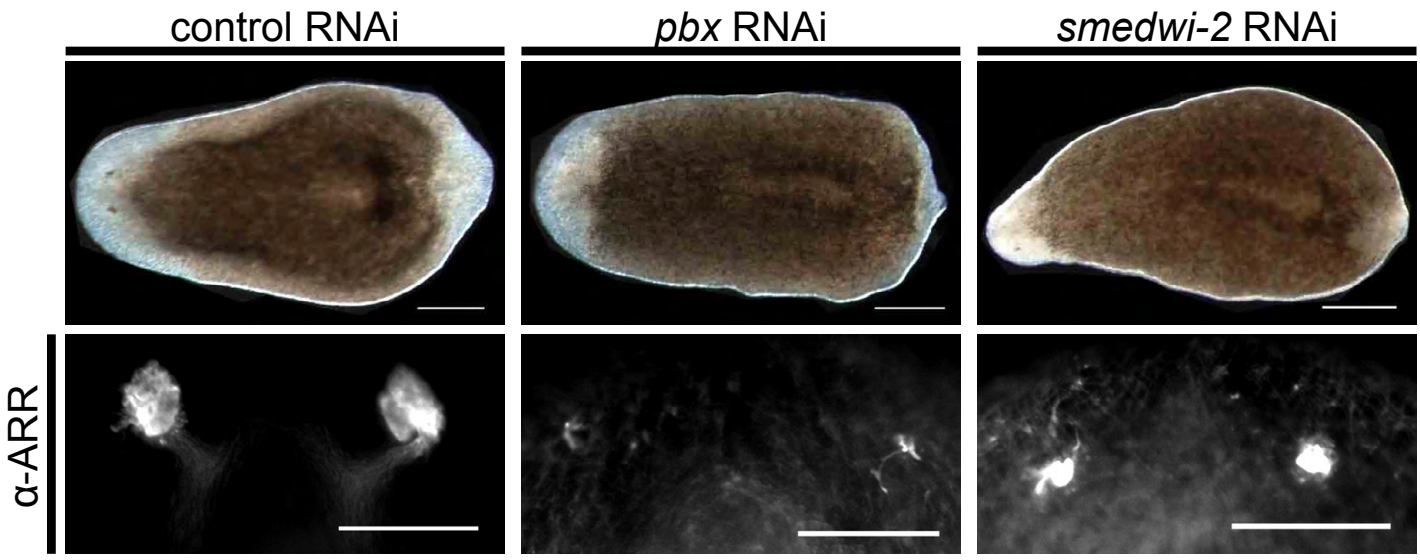

Fig. S4. The influence of blastema size on eye regeneration. Worms were injected with dsRNA against control, *pbx* or *smedwi-2* at 600 μg/ml to achieve weak RNAi. Both control (*n*=10) and *pbx(RNAi)* (*n*=10) animals regenerated blastemas with similar size but *smedwi-2(RNAi)* (*n*=8) animals produced smaller blastemas. However, *pbx(RNAi)* animals exhibited less eye regeneration than did *smedwi-2(RNAi)* animals, as shown with anti-ARRESTIN labeling of photoreceptor neurons. Scale bars: 100 μm.

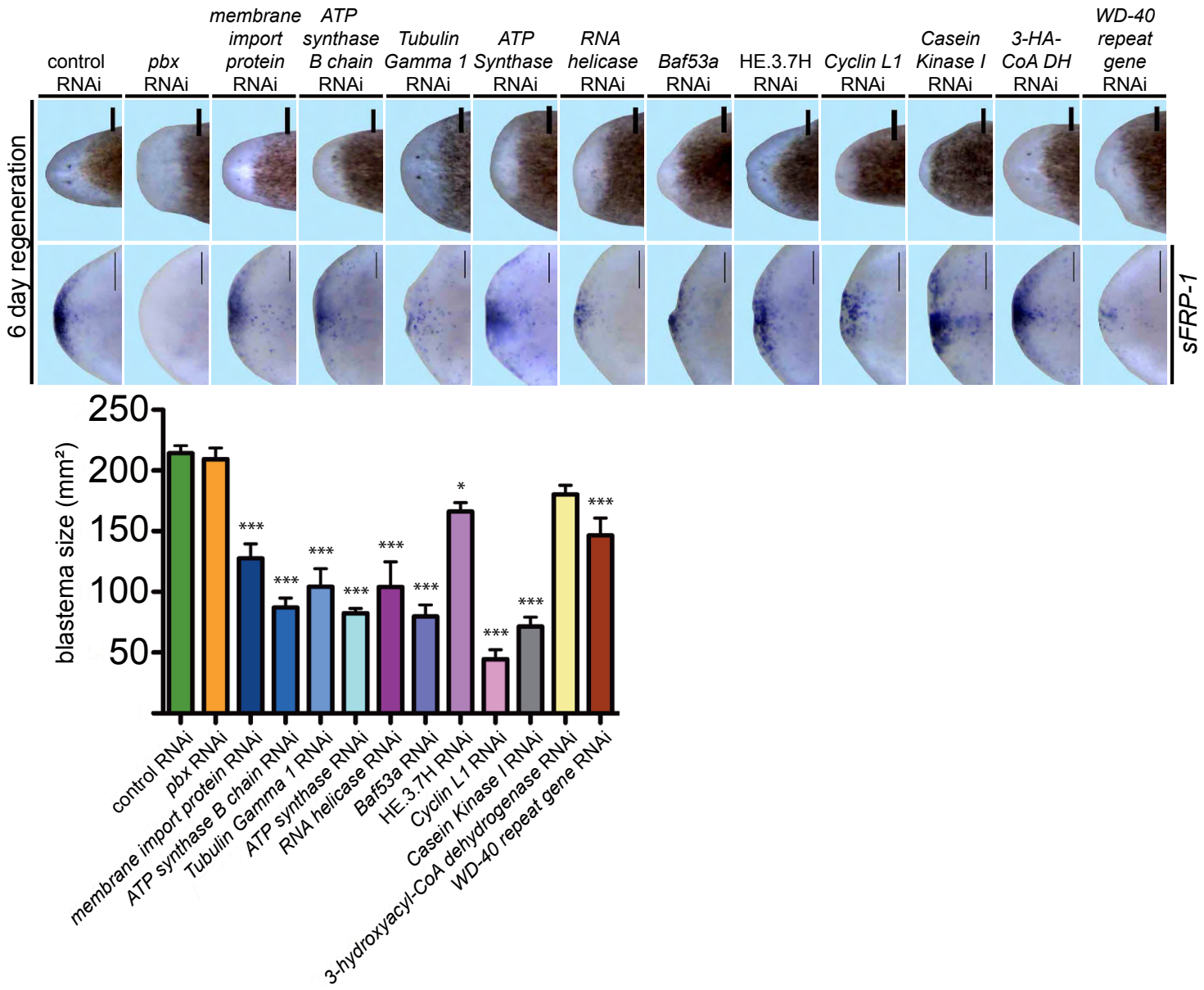

Fig. S5. Examination of *sFRP-1* expression in head blastemas following RNAi of 11 randomly selected genes that affect blastema formation. RNAi of 11 genes reported to be required for blastema formation by Reddien et al. (Reddien et al., 2005a) resulted in animals with head blastemas smaller than those of *pbx(RNAi)* animals, but more *sFRP-1* expression in each case. Animals were fed RNAi food three times, transversely amputated and fixed 6 days after amputation for *in situ* hybridization. Quantification of blastema size is shown in the bottom panel (one-way ANOVA test followed by a Dunnet post-hoc test; ***$P \leq 0.001$ between the experimental condition and the control; *$P \leq 0.05$ between the experimental condition and the control. cDNA clones used for each gene are as follow: *membrane import protein* (HE.4.1B), *ATP synthase B chain* (HE.4.2D), *Tubulin Gamma 1* (NBE.8.2D), *ATP Synthase* (NBE.8.9G), *RNA helicase* (HE.2.9G), *Baf53a* (HE.3.10F), *cyclin L1* (NBE.2.9B), *casein kinase I* (HE.3.9F), *3-hydroxyacyl-CoA dehydrogenase* (NBE.2.3C) and *WD-40 repeat* (NBE.2.9G).

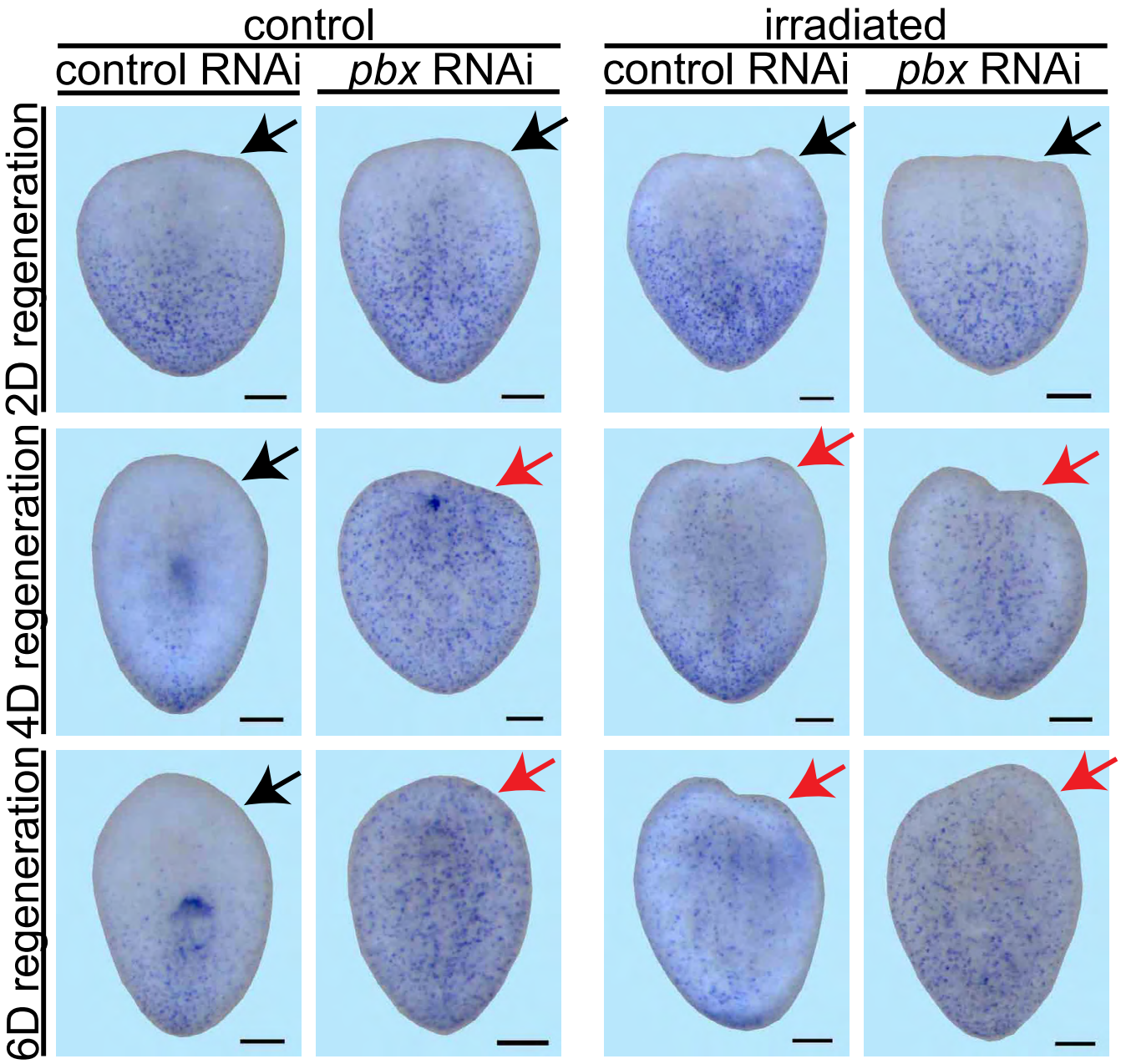

Fig. S6. *pbx* is required for irradiation-sensitive rescaling of the *wntP-2/wnt11-5* expression domain in regenerating tail pieces. Animals were fed with RNAi food four times, transversely amputated and fixed at the indicated regeneration time points for *in situ* hybridization with a *wntP-2* RNA probe. Irradiated animals were exposed to 6000 rads of radiation 4 days prior to amputation. Control animals exhibited retraction of the *wntP-2* expression domain to the tail tip at 4 days following amputation [as described by Peterson et al. (Petersen et al., 2009b)]. *wntP-2* expression subsequently expanded anteriorly to approximately where the new pharynx was forming at 6 days following amputation. Irradiated animals and *pbx(RNAi)* animals displayed initial clearing of *wntP-2* expression from the wound site (2 days after amputation) followed by expansion back towards the wound (4 days after amputation) ($n \geq 7$ for each condition). This behavior of *wntP-2* expression in irradiated tail fragments is as described by Gurley et al. (Gurley et al., 2011). Black arrows, normal expression pattern; red arrows, aberrant expression pattern. Scale bars: 100 μm.

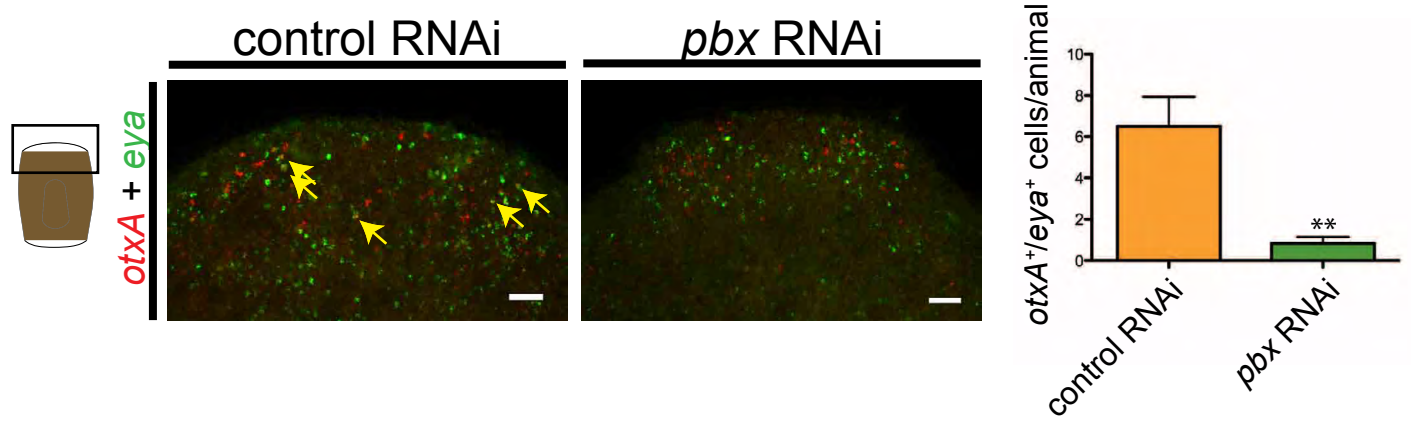

Fig. S7. Eye progenitor formation in *pbx(RNAi)* animals. *pbx(RNAi)* animals are required for *otxA*$^+$ *eya*$^+$ eye progenitor formation at 48 hours after amputation. Worms were fed with RNAi food four times, transversely amputated and fixed after 48 hours of amputation. FISH was performed to examine presence of *otxA*$^+$ *eya*$^+$ double-positive cells in or near the head blastema. Quantification of the number of double-positive cells is shown at right (n=6 for both conditions, **P=0.0031, t-test). Error bars represent s.e.m.

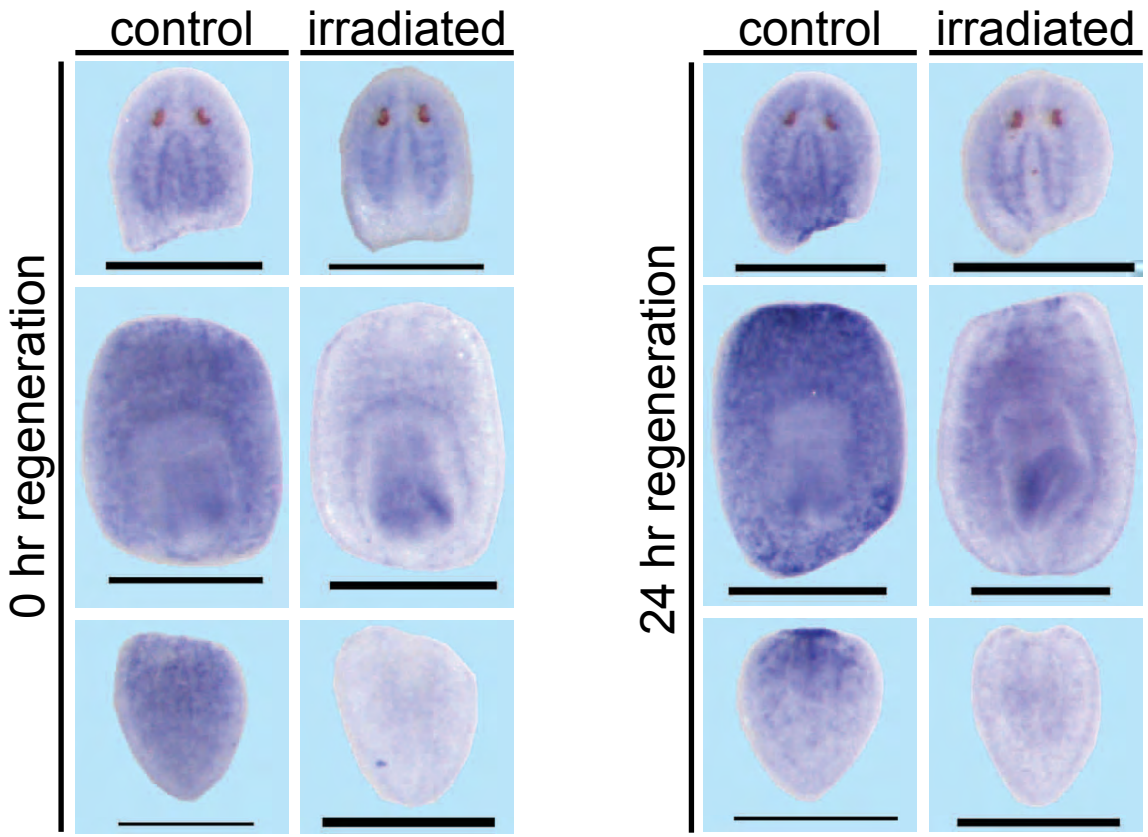

Fig. S8. *pbx* expression during early regeneration time points. Worms were transversely amputated and *pbx* expression accumulated at wound sites at 24 hours (n=6) after cutting and compared with freshly amputated animals (n=6). Worms were irradiated 4 days before amputation. Scale bars: 200 μm.

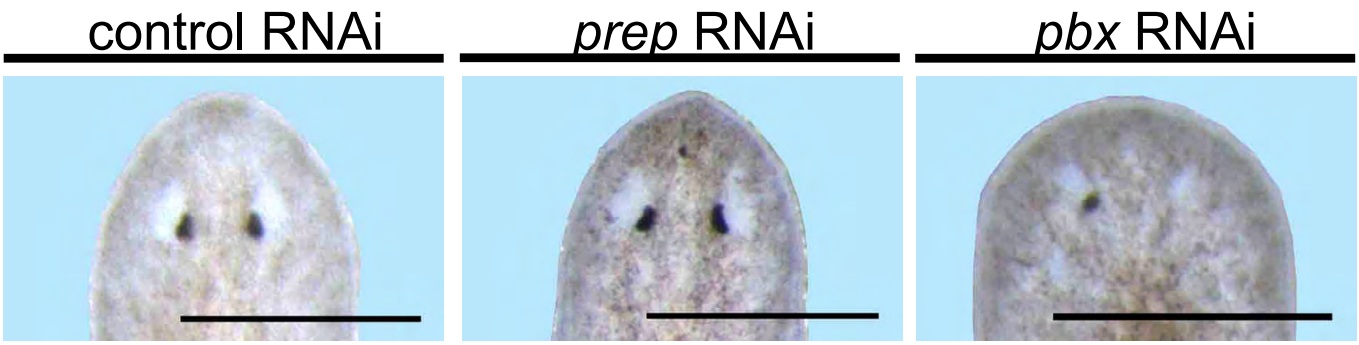

Fig. S9. *pbx(RNAi)* causes eye maintenance defects in unamputated animals. Light microscope images of the eyes of unamputated animals following 6 weeks of RNAi. Scale bars: 200 μm.

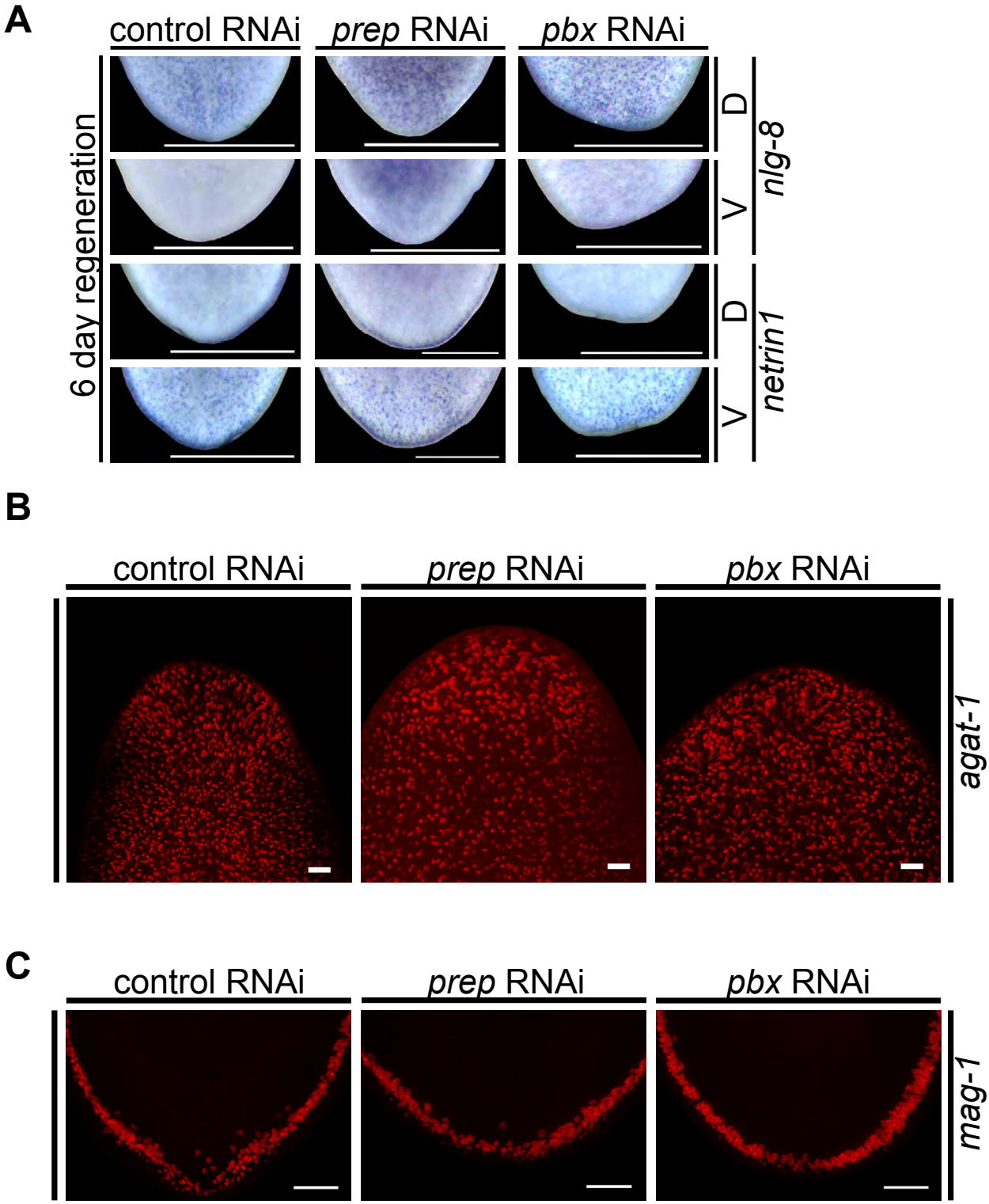

Fig. S10. Additional analysis of the *pbx(RNAi)* phenotype regenerating animals after long-term RNAi: expression of *nlg-8* and *netrin1* and neoblast differentiation. Animals underwent 6 weeks of RNAi, followed by amputation, and were analyzed 6 days after amputation. (**A**) Differential gene expression along the DV axis remained normal as shown by dorsal expression of *nlg-8* in regenerating posterior blastemas (control, *n*=16/16; *prep* RNAi, *n*=16/16; *pbx* RNAi, *n*=15/15) and ventral expression of *netrin1* (control, *n*=15/15; *prep* RNAi, *n*=16/16; *pbx* RNAi, *n*=15/15). (**B**) *agat-1*$^{+}$ neoblast progeny were also normal (control RNAi, *n*=15/15; *prep* RNAi, *n*=16/16, *pbx* RNAi; *n*=14/14 regenerating fragments). (**C**) *mag-1*$^{+}$ cells were regenerated (control RNAi, *n*=15/15; *prep* RNAi, *n*=16/16; *pbx* RNAi, *n*=14/14 regenerating fragments). Scale bars: in A, 500 μm; in B,C, 100 μm.

Table S1. List of primers used in this study. T7 promoter sequences and AttB1/2 adaptor sequences are shown in orange and red, respectively.

For riboprobes		
Smed-sFRP-1	TTGAATTCATGGAAATGACCAA	forward
Smed-sFRP-1	CATGTAATACGACTCACTATAGGGAATCAATGAAATGTTTTGTTGTGA	reverse
Smed-ndl-4	TGCAAATTGGTTCCACGTTA	forward
Smed-ndl-4	CATGTAATACGACTCACTATAGGGGAAGGCGACGACGAATTTT	reverse
Smed-prep	GCAACAAGCTGATCCTGGTT	forward
Smed-prep	CATGTAATACGACTCACTATAGGGGGAATGGTTGAAAACCGAAT	reverse
Smed-wnt2	ATCTTTCTTCACCTGGTTCTGG	forward
Smed-wnt2	CATGTAATACGACTCACTATAGGGTGATGAAGCGAGAGAAAATGAA	reverse
Smed-ndl-3	TGGAAATAATCTGTCCGCTTG	forward
Smed-ndl-3	CATGTAATACGACTCACTATAGGGGGATGAAGGATAATACGGATGG	reverse
Smed-wntP-2	TTAAATGTTCTAAGCCAAAACAACA	forward
Smed-wntP-2	CATGTAATACGACTCACTATAGGGAAAACTTTTATGATCAATCTGAATGC	reverse
Smed-abd-Ba	TTTTAAAGCATTGGATTTTCAC	forward
Smed-abd-Ba	CATGTAATACGACTCACTATAGGGAAGCTTGTTGATTAGGATTTCT	reverse
Smed-wnt11-1	GGCCGTTGTTCATGCTTTTA	forward
Smed-wnt11-1	CATGTAATACGACTCACTATAGGGCATGAGCCAGTAAATGAAATGGT	reverse
Smed-fz-4	CATGTAATACGACTCACTATAGGGGGAATAGCCCAACTCACCAA	reverse
Smed-fz-4	TGCCGAATTTAGTTGGAAGC	forward
Smed-wnt1	CCTCAAAATCGAATTTTACACTCA	forward
Smed-wnt1	CATGTAATACGACTCACTATAGGGTGGGACAAAAATAAAATTCCACA	reverse
Smed-notum	AAAATTTCTGAGGATCGAAAAA	forward
Smed-notum	CATGTAATACGACTCACTATAGGGTGAAGCTAGATTTATGTGAAAAACCA	reverse
Smed-pbx	CACCAGCCTCCGAGTAGTTG	forward
Smed-pbx	CATGTAATACGACTCACTATAGGGTGTCAATGCTATCAAGGAATCAA	reverse
cubilin	Scimone et al., 2011	
chat	Wagner et al., 2011	
mag-1	H1.3B	
laminB	Reddien et al., 2007	
otxA	Lapan and Reddien, 2011	
eya	Lapan and Reddien, 2011	
Smed-bmp4	Reddien et al., 2007	
Smed-admp	Gaviño and Reddien, 2011	

For RNAi		
Smed-pbx	CATGTAATACGACTCACTATAGGGCACCAGCCTCCGAGTAGTTG	forward
Smed-pbx	TGTCAATGCTATCAAGGAATCAA	reverse
Smed-pbx	CACCAGCCTCCGAGTAGTTG	forward
Smed-pbx	CATGTAATACGACTCACTATAGGGTGTCAATGCTATCAAGGAATCAA	reverse
Smed-prep	AAGCTGGAGCTCCACCGCGGtgaacaagcaactgcctcac	forward
Smed-prep	GGGCGAATTGGGTACCGGGcctgttgctcttcccatgat	reverse

Movie 1. Control RNAi animal at homeostasis day 45+ exhibiting normal locomotion.

Movie 2. *pbx(RNAi)* animal at homeostasis day 45+ exhibiting locomotive defect.

Movie 3. ***prep(RNAi)*** **animals at homeostasis day 45+ exhibiting normal locomotion.**