**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 Smad-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 regenerating pathways and transcription factors are essential for regulation of planar 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; Almudo-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). Smad-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, Dijislet, 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 *lzl (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
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 Podbieliewicz, 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 lkr (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 Wntb9 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 Gammaccell-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 feeding (Fig. 1A,B; Fig. 2B,C,E; Fig. 4A,B) of 3-5 mg/ml 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 fluorescence 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, thus treated as described (Newmark and Sánchez Alvarado, 2000). α-Myosin heavy chain, TMUS-13 antibody (Romero et al., 1991), rabbit α-trypothan hydroxylase (α-TH) antibody against peptide HPSDPFYTPEDCC, 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 proenephrine system (cubulin) (Scimone et al., 2011), muscle fibers (myosin heavy chain) (Cebrià and Vispo, 1997), subepidermal marginal adhesive gland cells (mag-l1) (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).
**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 Nodarake-family FGF-receptor-like protein; the related planarian *nodarake* gene is expressed anteriorly and is required for anterior cephalic ganglia
pbx(RNAi) animals. These data indicate that the wild-type anterior-most gene expression domain is absent in 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.
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 wnt11-3 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 uniformly stable in tail fragments and recedes towards the posterior pole during regeneration (Petersen and Reddien, 2009a; Gurley et al., 2010). pbx(RNAi) animals failed to re-scale 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 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, ndl-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.
**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). 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 (Feliz 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,
**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 aberrant expression at the anterior tip of all regenerating pieces (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 expressing the smedwi-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 notch 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 notch 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 Dijislet(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+ eye+ progenitor regeneration 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 properties 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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