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

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**SUMMARY**

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

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

**INTRODUCTION**

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

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

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

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

MATERIALS AND METHODS

Planarian culture and irradiation

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

Cloning Smed-pbx/extradenticle

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

RNAi

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

*In situ* hybridization

Whole mount *in situ* hybridization and *in situ* hybridization on paraffin sections were performed as described previously (Umesono et al., 1997; Cardona et al., 2005; González-Estévez et al., 2009). The following probes were used: Smed-laminin-α, Smed-sFRP, Smed-FZ4, Smed-GP4S, Smed-NB.21.11.e, Smed-AGAT1, Smed-septin, Smed-forcin1, Smed-Wnt1, Smed-Wnt11-2, Smed-slit, Smed-H2B and Smed-notum (Cebrià and Newmark, 2005; Cebrià et al., 2007; Cebrià and Newmark, 2007; Eisenhoffer et al., 2008; Gurley et al., 2008; Iglesias et al., 2011; Petersen and Reddien, 2011; Solana et al., 2012). RNA *in situ* probes were generated as described previously (González-Estévez et al., 2009). Bright-field images were taken on a Zeiss Discovery V8 using an Axiocam MRC (Carl Zeiss). Bright-field images of adjacent sections were false colored and overlaid to demonstrate colocalization of pbx and Smed-H2B in neoblasts. For quantification of Smed-NB.21.11.e- and Smed-AGAT1-expressing cells by fluorescent *in situ* hybridization, maximum projections were created from ~20 μm optical sections from the anterior-dorsal domain. ImageJ was used to quantify the number of cells. Confocal laser scanning microscopy was performed using a Leica SP2 confocal microscope (CLSM, Leica Lasertechnik).

Immunohistochemistry

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

Pharynx amputation

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

Analysis of proliferation

Mitotic figures were visualized by anti-H3P immunostaining and counted. Adobe Photoshop CS4 was used to determine the area of each sample analyzed and the number of mitoses/mm² was calculated.

RESULTS

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

A single ortholog of the *extradenticle/pbx* family was identified and named Smed-pbx (pbx for short). pbx expression in multiple RNAseq experiments was reduced by irradiation or loss of neoblasts induced by genetic means, and enriched in stem cells and stem cell progeny in FACS sorted populations (Blythe et al., 2010; Labbé et al., 2012; Onal et al., 2012; Solana et al., 2012). *In situ* hybridization demonstrated that *pbx* is expressed broadly in intact planarians in a...
pattern that resembled that exhibited by neoblasts (Fig. 1A; supplementary material Fig. S1A). We found pbx expression was depleted from the parenchyma 24 hours after γ-irradiation to remove neoblasts, revealing the remaining expression of pbx in the cephalic ganglia (CG) (Fig. 1A). pbx was expressed throughout the parenchyma of regenerating pieces amputated anterior and posterior to the pharynx (Fig. 1B), and within and adjacent to the regenerating blastemas (Fig. 1B). As regeneration proceeded, pbx expression was broadly observed throughout anterior and posterior blastemas, indicative of expression in neoblast progeny forming this structure (supplementary material Fig. S1B).

To reveal expression in post-mitotic differentiated cells, regenerating pieces were irradiated 24 hours after γ-irradiation to remove neoblasts, revealing the remaining expression of pbx in the cephalic ganglia (CG) (Fig. 1A). pbx was expressed throughout the parenchyma of regenerating pieces amputated anterior and posterior to the pharynx (Fig. 1B), and within and adjacent to the regenerating blastemas (Fig. 1B). As regeneration proceeded, pbx expression was broadly observed throughout anterior and posterior blastemas, indicative of expression in neoblast progeny forming this structure (supplementary material Fig. S1B).

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

pbx is required for regeneration along the AP axis RNA interference (RNAi) was used to knock down pbx expression (see supplementary material Fig. S2A). We observed clear defects in the ability of pbx(RNAi) animals to regenerate correctly. Animals formed regeneration blastemas but head pieces failed to regenerate a tail or a pharynx, tail pieces failed to regenerate a head or a pharynx, and trunk pieces failed to regenerate a head or a tail (Fig. 2A). Using a pan-neural marker against synapsin, we observed that anterior blastemas had highly reduced CG labeling, and head and trunk pieces failed to correctly regenerate ventral nerve cords (VNCs) posteriorly after 14 days post amputation (dpa) (Fig. 2B). VNCs failed to extend into the posterior blastema in head pieces and were prematurely rounded and fused behind the pre-existing pharynx in trunk pieces (Fig. 2B). To confirm failure in pharynx regeneration, we used the pharynx-specific marker Smed-laminin.
**Fig. 3. pbx is required for CG patterning.** (A) By 3 dpa, CG primordia expressed the neural marker GPAS in the anterior blastema of regenerating trunk pieces in control (10/10) and pbx(RNAi) (10/10). At 4 dpa, these structures remained separate in control regenerates (8/8), whereas they started to coalesce in pbx(RNAi) (8/8) (red arrowhead). By 14 dpa, the CG was completely regenerated in controls (10/10), whereas in pbx(RNAi) regenerates the fused CG did not enlarge significantly (10/10). (B) Following longitudinal amputation the structure of the CG was correctly regenerated by 14 dpa in control lateral regenerates (12/12). Following pbx(RNAi), CG tissue was regenerated to the same extent as in controls; however, the structure was fused at the midline (17/17 fused, red arrowhead). (C) The CG structure was comparable in intact control (12/12) and pbx(RNAi) (14/14) planarians, as was the presence of cintillo-expressing sensory cells (8/8 and 8/8, respectively). In control planarians, sFRP was expressed at the anterior margin (13/13), whereas it was lost following pbx(RNAi) within 3 weeks of homeostasis (9/13). (D,E) In control gfp(RNAi) background, ndk(RNAi) resulted in expansion of the regenerated CG in 14 dpa tail and trunk pieces (17/17 control animals normal, 18/18 tails and 20/20 trunks in ndk(RNAi) expanded, red arrow). CG regeneration was not observed following pbx/gfp(RNAi) in tails (22/22) and was greatly reduced in trunks (20/20). Tail and trunk pieces regenerated mis-patterned CG at the anterior following combined pbx/ndk(RNAi) (20/20 animals). Scale bars: 100 μm.

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

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

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

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

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

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

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

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

The defect in midline patterning was also observed when assessing the regeneration of the dorsal cilia. On the dorsal surface of pbx(RNAi) animals, the stripe of prominent cilia along the midline is absent (supplementary material Fig. S4B). Cilia are regenerated normally on the ventral surface of pbx(RNAi) animals and on the dorsal surface away from the midline (supplementary
material Fig. S4B), suggesting that defects observed in the regeneration of cilia on the dorsal surface result from a failure to re-establish the pattern of the dorsal midline stripe rather than from a general defect in ciliogenesis.

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

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

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

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

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

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

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

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

Another planarian tissue that displays polarity along the AP axis is the gut, which in Triclad species has one major anterior branch and two major posterior branches. During regeneration, the gut is coordinately remodeled in existing tissue and regenerated in new tissue to reconstitute this characteristic pattern (Forsthoefel et al.,...
Fig. 5. pbx coordinates AP patterning of the regenerating gut. (A) The anterior gut branch was regenerated and the existing posterior gut branches re-patterned by 14 dpa in control tail pieces (14/14), revealed by gut-specific Smed-porcupine expression. The anterior gut branch failed to regenerate and the existing posterior gut did not re-model following pbx(RNAi) (1/11). The posterior gut remodeled in prep(RNAi) tail pieces; however, the anterior gut branch did not (8/8). Schematic representations of gut morphology summarize the phenotypes observed. (B) By 14 dpa, the posterior gut branch was restored in control lateral regenerates (10/10). The gut extended into the lateral blastema following pbx(RNAi); however, patterning of the posterior branch was not regenerated (10/10). Regeneration of the posterior gut was comparable with controls following prep(RNAi) (10/10). Schematic representations of gut morphology summarize the phenotypes observed. (C) Gut morphology is maintained during 3 weeks of homeostasis following pbx(RNAi) (17/17), resembling controls (16/16). Scale bars: 200 μm.

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

pbx activity promotes brain regeneration independently of anterior polarity specification

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

Taken together, these data suggest that the role of pbx in establishing anterior polarity could be separated from its role in promoting CG tissue differentiation. Furthermore, we interpret these data to suggest that pbx activity normally promotes CG
Fig. 7. pbx is required for expression of AP polarity determinants. (A) wnt1 was expressed in cells adjacent to the posterior wound in control regenerating head pieces (12/12) and following pbx(RNAi) (11/11) at 1 dpa. The sustained posterior wnt1 expression observed at 4 dpa in control animals (16/18) was absent following pbx(RNAi) (16/17). wnt11-2 expression was not observed at 1 dpa in control or pbx(RNAi) animals (8/8 and 7/7), became expressed within the posterior blastema of controls by 4 dpa (17/17) and remained absent from pbx(RNAi) animals (17/17). (B) notum was expressed in cells adjacent to the anterior wound within 18 hours of regeneration in control tail pieces (6/6) and became localized to the anterior tip of the blastema by 50 hours (6/6), whereas its expression was not observed at either time point following pbx RNAi (16/16 and 16/16). (C) wnt1 expression was observed adjacent to the anterior wound in control (6/7) and pbx(RNAi) (6/7) regenerating tail pieces at 1 dpa. Anterior wnt1 expression did not persist to 4 dpa in control (6/6) or pbx RNAi (8/8) regenerating tail pieces. Scale bars: 100 μm.

DISCUSSION

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

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

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

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

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

Conserved roles for TALEs in axial regeneration?

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

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Competing interests statement

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Supplementary material

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