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Neoblast Specialization in Regeneration of the Planarian *Schmidtea mediterranea*

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SUMMARY

Planarians can regenerate any missing body part in a process requiring dividing cells called neoblasts. Historically, neoblasts have largely been considered a homogeneous stem cell population. Most studies, however, analyzed neoblasts at the population rather than the single-cell level, leaving the degree of heterogeneity in this population unresolved. We combined RNA sequencing of neoblasts from wounded planarians with expression screening and identified 33 transcription factors transcribed in specific differentiated cells and in small fractions of neoblasts during regeneration. Many neoblast subsets expressing distinct tissue-associated transcription factors were present, suggesting candidate specification into many lineages. Consistent with this possibility, *klf*, *pax3/7*, and *FoxA* were required for the differentiation of *cintillo*-expressing sensory neurons, *dopamine-β-hydroxylase*-expressing neurons, and the pharynx, respectively. Together, these results suggest that specification of cell fate for most-to-all regenerative lineages occurs within neoblasts, with regenerative cells of blastemas being generated from a highly heterogeneous collection of lineage-specified neoblasts.

INTRODUCTION

Planarians are flatworms capable of regenerating any missing tissue after injury. Regeneration in the planarian *Schmidtea mediterranea* requires a population of small mesenchymal cells called neoblasts, which are the only dividing cells of the adult animal. Irradiation eliminates neoblasts, blocking regeneration and tissue turnover (Reddien et al., 2005). Following injury, neoblasts rapidly divide throughout the animal, with mitotic numbers peaking at 6 hr after wounding. If the wound requires the replacement of missing tissue, a second peak of neoblast proliferation occurs at 48 hr (Wenemoser and Reddien, 2010). At this time, neoblasts accumulate at the wound site and their progeny form an unpigmented bud of regenerated tissue called the blastema.

Recently, two neoblast models for planarian regeneration have been proposed: the naive and specialized models (Reddien, 2013). The naive model posits that all neoblasts are stem cells with the same potential and are therefore a largely homogeneous population with fate specification occurring only in nondividing neoblast progeny. By contrast, the specialized model predicts that neoblasts involved in producing missing cells have largely restricted fates and are therefore a heterogeneous population containing many different lineage-committed dividing cells.

Neoblasts have frequently been considered as a uniform population of pluripotent stem cells. Indeed, some neoblasts, termed cNeoblasts, are pluripotent stem cells that can rescue tissue homeostasis and regeneration in lethally irradiated animals by single-cell transplantation (Wagner

et al., 2011). The abundance of cNeoblasts in the neoblast population, however, is unknown. *smedwi-1* encodes a PIWI-family protein that is expressed in all dividing adult planarian cells (Reddien et al., 2005) and is a canonical neoblast marker. All *smedwi-1*⁺ cells rapidly disappear within 1 day following irradiation (Eisenhoffer et al., 2008). Some *smedwi-1*⁺ cells have been found to express tissue-specific transcription factors required for specification of a few distinct tissues, such as the eye (Lapan and Reddien, 2011, 2012), the nephridia (Scimone et al., 2011), the anterior pole (Scimone et al., 2014), and some neurons (Cowles et al., 2013; Currie and Pearson, 2013; Wenemoser et al., 2012). Expression of these transcription factors is induced in a small number of *smedwi-1*⁺ cells following wounding, with only rare neoblasts expressing these transcription factors in intact animals (Cowles et al., 2013; Lapan and Reddien, 2012). These data provide support for the specialized neoblast model for at least several lineages.

Determining whether the specification of most, or all, cell lineages occurs within neoblasts is essential for understanding the cellular basis for planarian regeneration. Specifically, at what cellular step in regeneration is the identity of new cells specified? On the basis of prior results demonstrating the specialization of *smedwi-1*⁺ cells for case study tissues, such as the eye, we sought to test the breadth of the specialized neoblast model. Because it is possible that the *smedwi-1*⁺ cell population contains both dividing cells (neoblasts) and immediate nondividing neoblast progeny cells, we purified S and G2/M phase neoblasts (X1 neoblasts) using fluorescence-activated cell sorting (FACS) and used RNA sequencing (RNA-seq) to identify



transcription factors upregulated in X1 neoblasts following wounding. We combined this approach with broad gene expression screening to identify transcription factors expressed in many different tissues and in neoblasts of wounded planarians. Several conserved transcription factors expressed in neoblasts following wounding were required for regeneration of specific cell types, as predicted by the specialized neoblast model. Our results identify a large collection of transcription factors expressed in small subsets of neoblasts at wounds. Together with previous data, these results indicate that the neoblasts that produce regenerative lineages are a highly heterogeneous population consisting of pluripotent stem cells and lineage-restricted progenitors.

RESULTS

X1 Neoblasts from Wounded Planarians Express Tissue-Associated Transcription Factors

Planarians are flatworms with complex internal anatomy (Figure 1A). They possess a branched intestine that connects to a centrally located pharynx (the only opening of the animal); a nervous system with two cephalic ganglia, two ventral nerve cords, sensory neurons including those of the eyes, and many peripheral neurons; an excretory system (a network of ciliated ducts); and body wall muscle fibers. Because a small planarian tissue fragment can regenerate an entire animal, regeneration requires mechanisms for the specification of the many distinct cell types of adult planarians.

Transcription factors can orchestrate the differential expression of regulatory and structural genes needed for cell differentiation in development. In planarians, several transcription factors are expressed in neoblasts for the specification of neoblast fate. In order to establish whether expression of transcription factors in neoblasts following wounding is a hallmark of the specification of most regenerative cell lineages, we first utilized FACS to purify neoblasts that were in the S or G2/M phases of the cell cycle (the X1 fraction: DNA content of more than 2C) (Hayashi et al., 2006) of the prepharyngeal region 48 hr following either head (posterior-facing wound) or trunk (anterior-facing wound) amputations. We subjected these neoblasts to RNA-seq analysis (Figure 1B). This experimental design allowed identification of genes activated during regeneration and controlled for natural variability in gene expression across the anterior-posterior axis by using the same (prepharyngeal) neoblast population. Expression in X1 neoblasts would confirm these transcription factors are active in cells that undergo one or more cell divisions. A few transcription factors were significantly (P_{adj} [adjusted p value] ≤ 0.05) upregulated in X1 neoblasts following wounding

(Figure 1C). We also examined (and tested below) transcription factors that were upregulated with a fold-change of $\log_2 \geq 0.7$ and P_{unadj} (unadjusted p value) ≤ 0.05 (Figure 1C; Table S1 available online). By these criteria, neoblasts from wounded planarians expressed the previously described genes *sp6-9*, which is expressed in optic cup cells and peripheral neurons, and required for eye regeneration (Lapan and Reddien, 2011); *meis* and *otxA*, which are expressed in and required for the regeneration of photoreceptor neurons (Lapan and Reddien, 2012); *eya*, which is expressed in and required for regeneration of eyes and protonephridia (Lapan and Reddien, 2011, 2012; Scimone et al., 2011); and *ap-2*, which is expressed in multiple neuron types and required for *TrpA*⁺ neuron regeneration (Wenemoser et al., 2012) (Figure 1C). In addition, we identified several transcription factor homologs that were expressed in X1 neoblasts 48 hr following wounding (Figures 1C, S1, and S2A; Table S1). We examined the expression of some of these transcription factor homologs (7 out of 13 genes were tested). In wild-type and irradiated uninjured animals, most (six out of seven genes; *zfp-2* did not show expression above background) were primarily expressed in differentiated cell types rather than in neoblasts (Figure S1C). To assess the expression in neoblasts, we isolated X1 cells of prepharyngeal regions at 48 hr following both anterior and posterior amputation and performed cell fluorescence in situ hybridizations (FISH). All of the tested transcription factors identified from this RNA-seq analysis (unknown and previously reported, 15 out of 23 genes were tested) were expressed in small subsets of X1 cells from wounded animals (Figures 1D, 2A, 3, and S1D).

Known Tissue-Associated Transcription Factors Are Expressed in X1 Neoblasts following Wounding

We reasoned that known tissue-associated transcription factors might be expressed in neoblasts from wounded planarians, but expression of such genes might not be significantly detectable by RNA-seq differential gene expression analysis as a consequence of expression occurring in only rare neoblasts. We therefore analyzed the X1 neoblast expression of transcription factors expressed in distinct tissues. First, we determined the expression frequency of transcription factors previously shown to be associated with distinct tissues and expressed in *smedwi-1*⁺ cells using FISH in sorted X1 neoblasts. As expected, a small fraction of X1 cells expressed the nephridia transcription factors *POU2/3*, *sall*, and *osr* (1.6%) (Scimone et al., 2011); the eye-specific transcription factor *ovo* (0.5%) (Lapan and Reddien, 2012); and the anterior pole transcription factors *FoxD* and *prep* and the marker *notum* (0.4%) (Scimone et al., 2014) (Figure 2A).

Second, we examined the expression of transcription factors known or predicted to be active in the pharynx, gut,

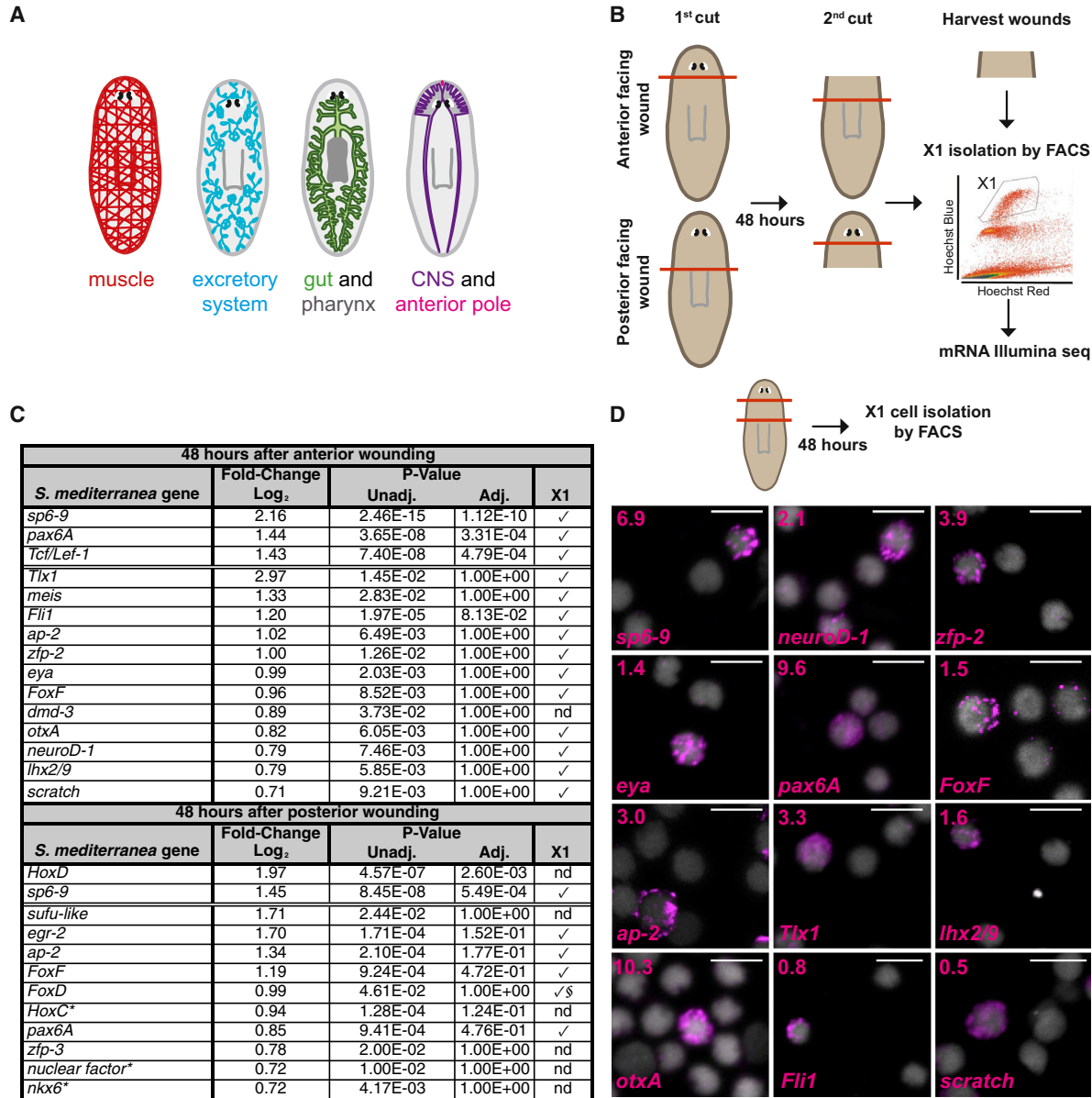


Figure 1. mRNA Sequencing Analysis of Sorted X1 Neoblasts from Regenerating Planarians

(A) Cartoon shows the planarian gastrovascular system with pharynx, the nervous system with photoreceptors, the excretory system, muscle, and the anterior pole.

(B) Schematic of the experimental approach. Prepharyngeal tissues were harvested 48 hr after amputation. Cell suspensions were labeled with Hoechst 33342 and X1 cells isolated by FACS. RNA was then purified from sorted X1 cells and mRNA Illumina sequencing performed.

(C) The table lists all the transcription factors that showed upregulated expression in X1 cells from wounded animals with $P_{adj} \leq 0.05$ (above double line) and $P_{unadj} \leq 0.05$ with a \log_2 fold change ≥ 0.7 (below double line). Check marks indicate expression was validated in sorted X1 cells from wounded planarians; nd, not determined. §From published work (Scimone et al., 2014); *best BLASTx hit to human transcripts.

(D) Schematic: X1 cells were isolated from prepharyngeal regions by FACS 48 hr after amputations, and cell FISH was performed using RNA probes from genes found in (C). DAPI labeled DNA (gray). Percentages of X1 cells expressing each transcription factor are shown in the upper left corners. Total number of X1 cells counted: *sp6-9*⁺ 14/202; *neuroD-1*⁺ 22/1034; *zfp-2*⁺ 7/180; *eya*⁺ 9/635; *pax6A*⁺ 107/1113; *FoxF*⁺ 3/205; *ap-2*⁺ 34/1137; *Tlx1*⁺ 5/152; *lhx2/9*⁺ 20/1222; *otxA*⁺ 16/155; *Fli1*⁺ 11/1463; *scratch*⁺ 4/766. Images shown are maximal-intensity projections. Scale bars, 10 μ m.

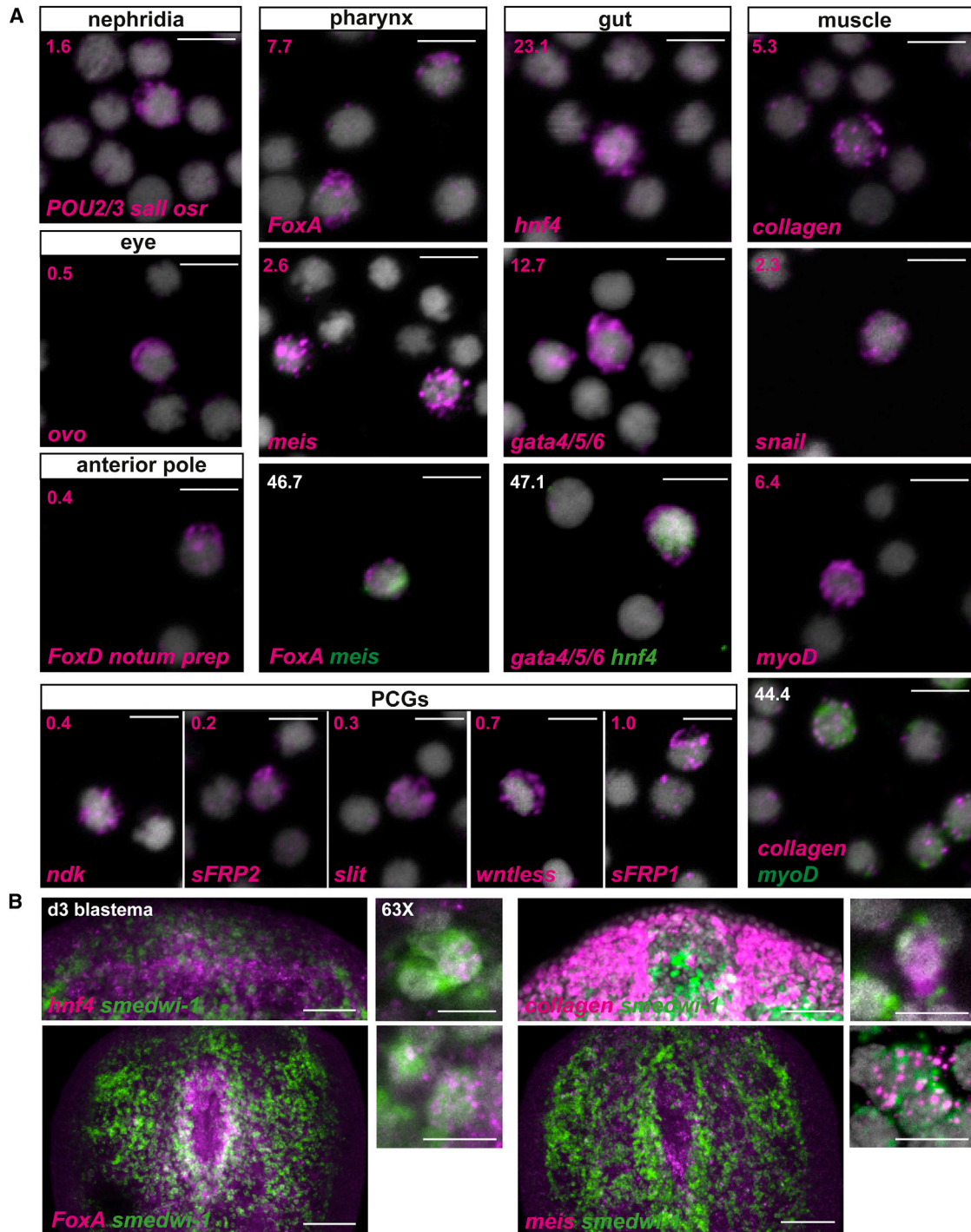


Figure 2. A Candidate Gene Approach Identified Tissue-Associated Transcription Factors Expressed in X1 Neoblasts from Regenerating Planarians

(A) FISH using previously known and new tissue-associated transcription factors with sorted X1 cells from prepharyngeal regions of amputated planarians. Percentages of X1 cells expressing the transcription factors are shown in the upper left corner. Total number of X1 cells counted: for protonephridia, a mixture of RNA probes *POU2/3*, *odd-skipped related (osr)*, and *sal-like (sall)* $n = 8/504$; for the eye, *ovo* $n = 1/194$; for the anterior pole, a mixture of the RNA probes *FoxD*, *prep*, and *notum* $n = 1/250$; for pharynx, *FoxA* $n = 54/704$ and *meis* $n = 20/777$, with *FoxA* and *meis* coexpression observed in $n = 7/15$ of *meis*⁺ X1 cells; for the gut, *hnf4* $n = 337/1459$ and *gata4/5/6*

(legend continued on next page)



and muscle (Figures 2A and S1E). *FoxA* is a forkhead transcription factor expressed in the endoderm lineage across metazoans. It is expressed in cells that intercalate, polarize, and form tight junctions in the digestive tracts of the mouse, the sea urchin, and the nematode *C. elegans* (deLeon, 2011). A planarian *FoxA* homolog is expressed in the pharynx of the related species *Dugesia japonica* (Koinuma et al., 2000). We found that during regeneration, *FoxA* was expressed in the pharynx primordium in the planarian *S. mediterranea* (*Smed-FoxA*; Figures S2A, S2B, 2B and 6B). *FoxA* expression occurred in *smedwi-1*⁺ cells and in isolated X1 neoblasts following wounding (7.7% of X1 cells) (Figure 2). The homeobox gene *meis* was previously shown to be expressed in eyes and required for the regeneration of photoreceptor neurons (Lapan and Reddien, 2012) and was also expressed at the pharynx primordium during regeneration (Figures 2B and S2B). *meis* was expressed in isolated X1 neoblasts (2.6%) following wounding, and some *meis*⁺ X1 neoblasts also coexpressed *FoxA* (46.7% of *meis*⁺ cells, which is higher than expected if expression of these genes in neoblasts was independent; $p = 0.0001$, Fisher's exact test), suggesting that both genes might be involved in pharynx specification (Figure 2A). Expression of *meis* was also observed in *smedwi-1*⁺ cells during pharynx regeneration in tail fragments 3 days following wounding (Figure 2B). *FoxA* and *meis* were expressed in the pharynx of intact animals (Figure S2C). Finally, a homolog of *twist* was expressed in the pharynx in intact animals as well as during regeneration (Cowles et al., 2013; Figures S2B and S2C), and it was expressed in *smedwi-1*⁺ cells during regeneration (Figure S2B). Moreover, *twist* was expressed in isolated X1 neoblasts following wounding (2.1%; Figure S2B).

Endodermal transcription factors that control differentiation of the gut and its derivatives during development in multiple species include the hepatocyte nuclear factor (HNF) and GATA families (Zaret, 1999). An HNF homolog, *hnf4*, and a GATA4/5/6 homolog, *gata4/5/6*, are both expressed in the gut of *S. mediterranea* (Wagner et al., 2011). These genes were expressed together in the same cells during gut regeneration (day 3 anterior blastemas; Figure S2D) and were expressed in isolated X1 cells from wounded planarians (*hnf4*: 23.1% of X1 and *gata4/5/6*: 12.6%; Figure 2A).

Moreover, *hnf4* and *gata4/5/6* were coexpressed together in the same X1 cells (47.1% of *hnf4*⁺ cells; $p = 0.0038$, Fisher's exact test; Figure 2A). In addition, *hnf4* was expressed in *smedwi-1*⁺ cells during gut regeneration (Figure 2B).

Muscle cells develop in most embryos from the mesoderm tissue layer and frequently involve expression of *myoD*, a well-known myogenic basic-helix-loop-helix transcription factor (Weintraub et al., 1991). Muscle cells are marked by *collagen* expression in *S. mediterranea* (Witchley et al., 2013). *collagen* and *myoD* (Cowles et al., 2013) were expressed in isolated X1 neoblasts following wounding (*myoD*: 6.4% and *collagen*: 5.3%), and 44.4% of *myoD*⁺ cells coexpressed *collagen* (greater than expected by chance: $p < 0.0001$, Fisher's exact test; Figure 2A). *myoD* was also coexpressed with *collagen* in intact animals (95.1% of *myoD*⁺ cells; Figure S2E). In addition, *collagen* was coexpressed with *smedwi-1* in cells of day 3 blastemas (Figure 2B). The Snail gene family encodes zinc finger proteins that can act as transcriptional regulators and control entry into myogenic differentiation (Soleimani et al., 2012). A planarian *snail* homolog (Figure S1A) was expressed together with *collagen* in intact animals (83.8% of *snail*⁺ cells; Figure S2E). *snail* was also expressed in X1 neoblasts (2.3%; Figure 2A) and was coexpressed with *myoD* in X1 cells from wounded animals (35.7%; Figure S2E) and expressed in *smedwi-1*⁺ cells during regeneration (Figure S2E), suggesting a possible role for this gene in muscle specification.

A different category of genes, the positional control genes (PCGs), described or predicted to have roles in planarian patterning (Reddien, 2011), are highly expressed in planarian muscle cells (Witchley et al., 2013), and are required for the patterning of regenerated tissue. We observed that in addition to this muscle expression, several PCGs were expressed at low frequency in the X1 compartment during regeneration (Figure 2A); these PCGs included *slit*, a midline-expressed repulsive axon guidance cue; *nou-darake* (*ndk*), which is required for proper restriction of the brain to the planarian head; *sFRP1* and *sFRP2*, which are expressed as anterior gradients; and the wound-induced and posteriorly expressed gene *wntless* (Reddien, 2011). Although these PCGs are not transcription factors, these results further demonstrate the gene expression heterogeneity that exists in neoblasts during regeneration.

$n = 665/5249$, with coexpression observed in $n = 56/119$ of *hnf4*⁺ X1 cells; for the muscle, *collagen* $n = 72/1357$ and the transcription factors *myoD* $n = 47/732$ and *snail* $n = 45/1945$, with *myoD* and *collagen* coexpression observed in $n = 20/45$ *myoD*⁺ X1 cells. Positional control genes were also expressed in wounded X1 cells: *nou-darake* (*ndk*, $n = 23/6432$), *secreted related-frizzled 2* (*sFRP-2*, $n = 9/4402$), *slit* ($n = 8/2763$), *wntless* ($n = 24/3367$), and *secreted related-frizzled 1* (*sFRP-1*; $n = 7/732$). Because some genes were expressed in multiple cell types, for some tissues, progenitor numbers will be an overestimate. DAPI labeled DNA (gray). Scale bars, 10 μm .

(B) Coexpression of the gut transcription factor *hnf4*, the muscle gene *collagen*, and the pharynx transcription factors *FoxA* and *meis* (magenta) with the neoblast marker *smedwi-1* (green) in day 3 regenerating anterior blastemas or tail fragments (for the pharynx genes). Higher magnification on the right shows cells coexpressing both genes (scale bars, 10 μm). DAPI labeled DNA (gray). All images shown are maximal-intensity projections. Anterior, up. Scale bars, 100 μm .

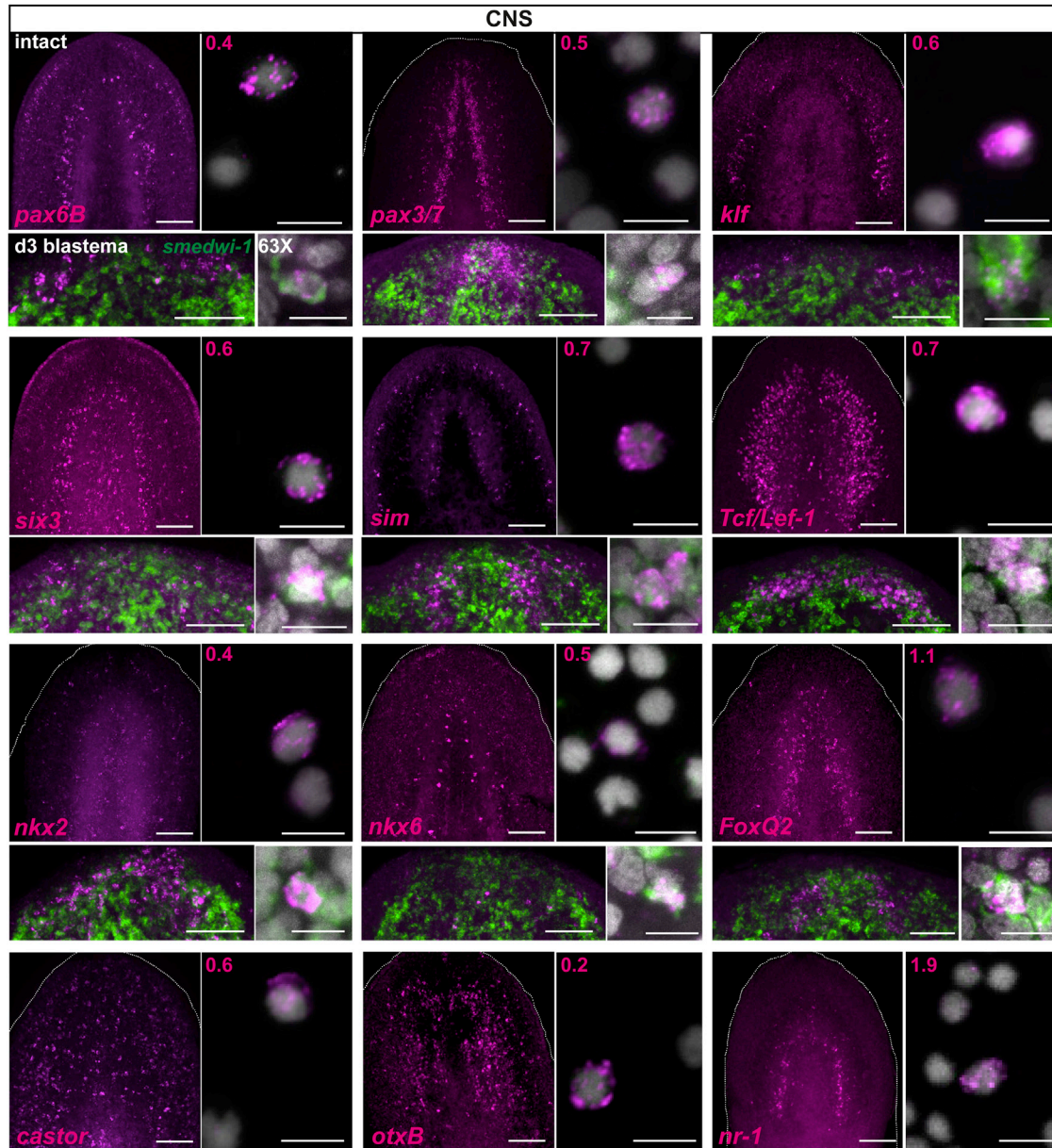


Figure 3. A Broad Panel of CNS-Associated Transcription Factors Was Expressed in X1 Neoblasts

For each gene tested (except last row), the left upper panel shows the expression of the transcription factor (magenta) in the intact head (scale bars, 100 μ m), the right upper panel shows expression in sorted X1 cells from prepharyngeal regions of amputated animals 48 hr after wounding (scale bars, 10 μ m), the left bottom panel shows the expression of the transcription factor and *smedwi-1* (green) in day 3 regenerating anterior blastemas (scale bars, 100 μ m), and the bottom right panel shows a higher magnification of a cell coexpressing the transcription factor (magenta) and *smedwi-1* (green) in the regenerating blastema (scale bars, 10 μ m). DAPI labeled DNA (gray). Percentages of X1 cells expressing the transcription factors are shown in the upper left corner. Numbers of X1 cells counted: *pax6B* n = 10/2302, *pax3/7* n = 12/2299, *klf* n = 15/2746, *six3* n = 11/1826, *single minded (sim)* n = 1/150, *Tcf/Lef-1* n = 3/432, *nkx2* n = 9/2435, *nkx6* n = 2/395, *FoxQ2* n = 2/180, *castor* n = 11/1954, *otxB* n = 4/2111, and *nr-1* n = 31/1637. Images shown are maximal-intensity projections. Anterior, up.



Numerous Transcription Factors Expressed in Cells of the Nervous System Are Expressed in Neoblasts following Wounding

Despite the ability of planarians to completely regenerate the nervous system following head amputation, few genes required for the regeneration of specific neuronal lineages have been described (Cowles et al., 2013; Currie and Pearson, 2013; März et al., 2013; Wenemoser et al., 2012). We reasoned that many transcription factors might exist that specify the diverse, numerous cell types in the brain, and the specialized neoblast model predicts many of these would be expressed in neoblasts during regeneration. The nervous system was therefore a good target for testing the specialized neoblast hypothesis.

The neoblast RNA-seq data described above identified upregulation of several transcription factors associated with the nervous system in the neoblast population following wounding (Figure 1C), such as *sp6-9*, *eya*, *ap-2*, *pax6A*, and *otxA* (Figure S1C). In addition, expression of other nervous system-associated genes such as *Tcf/Lef-1*, *neuroD-1* (Cowles et al., 2013), and *lhx2/9* was observed in neoblasts following wounding (Figure 1C). These genes were indeed expressed in the nervous system of *S. mediterranea* (Figures S1C, S3A, and 3), as well as in X1 neoblasts from wounded planarians and in *smedwi-1*⁺ cells near day 3 regenerating anterior blastemas (Figures 1D, 3, and S3B).

We next searched for conserved transcription factors with roles in nervous system development in other organisms. We found 40 putative transcription factors homologs that were expressed in the planarian brain, coexpressing or closely associated with the neuronal marker *cholinergic acetyltransferase* (*ChAT*) gene. From those 40 genes, 18 genes were detectably expressed following wounding either in *smedwi-1*⁺ cells in regenerating blastemas and/or in isolated X1 neoblasts of wounded animals (Figures 3, S3B, and S3C). Collectively, from the RNA-seq and candidate approaches, a total of 26 neuron-associated transcription factors displayed expression in neoblasts of regenerating planarians.

Paired-box homeodomain-encoding genes are transcription factors with essential roles in organogenesis, including brain development and patterning (Robson et al., 2006). Planarians have two genes encoding Pax6 homologs, *pax6A* and *pax6B* (Figure S4A; Pineda et al., 2002). *pax6A* was abundantly expressed throughout the brain (Figures S1C and S3A), was significantly upregulated in X1 cells (Figure 1C), and was expressed in isolated X1 cells from wounded planarians (9.6%; Figure 1D). *pax6A* was also expressed in *smedwi-1*⁺ cells during regeneration (Wenemoser et al., 2012; Figure S3B). *pax6B* expression was most abundant in the lateral cephalic ganglia of the planarian brain in intact animals (Figures 3 and S3A), was present in rare X1

cells from wounded animals (0.4%), and was coexpressed with *smedwi-1* during head regeneration (Figure 3). In addition, a planarian gene encoding a homolog of Pax3/7-family transcription factors (Figure S4A) was expressed in the ventral midline of the brain and within the ventral nerve cords (Figures 3 and S3A). *pax3/7* was expressed in isolated X1 cells (0.5%) from wounded planarians and coexpressed with *smedwi-1*⁺ cells during anterior regeneration (Figure 3).

Drosophila cephalic gap genes have important roles in brain development, as do their orthologs in vertebrates (Kammermeier and Reichert, 2001). Planarians have two homologs of the *Drosophila orthodenticle* gene, *otxA* and *otxB*. Both genes were abundantly expressed in the planarian brain (Figures 3 and S3A; Umesonu et al., 1999; Lapan and Reddien, 2011). *otxA* upregulation in X1 neoblasts was indicated by RNA-seq (Figure 1C), was detected in isolated X1 cells from wounded animals (10.3%; Figure 1D), and was coexpressed with *smedwi-1* during anterior regeneration (Figure S3B); *otxB* was also expressed in X1 cells from wounded animals (0.2%; Figure 3).

The *nkx2.2* gene functions in establishing progenitor domains of the ventral spinal cord and hindbrain of vertebrates during embryogenesis (Briscoe et al., 1999). A planarian homolog, *nkx2* (Figure S1A), was expressed in the medial planarian brain; cells expressing *nkx2* mostly localized to the space between the midline neurons and the lateral region of the cephalic ganglia (Figures 3 and S3A). *nkx6.1* is another homeobox gene with important roles in patterning of the ventral vertebrate CNS (Sander et al., 2000). A planarian homolog, *nkx6* (Figure S1A), was expressed in scattered cells throughout the medial brain region (Figures 3 and S3A). *nkx2* and *nkx6* were expressed in *smedwi-1*⁺ cells during regeneration and in isolated X1 cells from wounded animals (*nkx2*, 0.4% and *nkx6*, 0.5%; Figure 3).

castor encodes a zinc-finger transcription factor expressed in a subset of *Drosophila* embryonic neuroglioblasts and controls neural differentiation (Mellerick et al., 1992). A planarian *castor* homolog (Figure S1B) was expressed ventrally in many scattered cells, with some of these cells coexpressing the neuronal marker *ChAT* (Figure S3A). Following wounding, *castor* was expressed in 0.6% of X1 neoblasts (Figure 3).

Nuclear receptors are ligand-activated transcription factors that have several roles during development. We found a *nuclear receptor* (*nr-1*) gene in *S. mediterranea* to be expressed in neurons in an inner arc of the brain (Figures 3 and S3A). *nr-1* was also expressed in 1.9% of isolated X1 neoblasts from wounded animals (Figure 3).

Several genes found in a transcriptome study of the planarian eye (Lapan and Reddien, 2012) were also expressed in different domains of the brain. For example,



the *forkhead*-family gene *FoxQ2*, as well as *klf*, are expressed in the eye and required for photoreceptor neuron regeneration (Lapan and Reddien, 2012). In addition, *FoxQ2* was expressed in the ventral midline of the brain and in ventral nerve cords, and *klf* was expressed in peripheral sensory neurons and in the outer brain branches (Figures 3 and S3A). Both of these genes were also expressed in isolated X1 cells from wounded planarians (1.1% and 0.6%, respectively) and were expressed in *smcdwi-1*⁺ cells during anterior regeneration (Figure 3).

The HLH-Pas domain transcription factor *single minded* (*sim*) is expressed in the planarian brain and is required for the regeneration of several neuron types (Cowles et al., 2013). In agreement with this report, we found expression of *sim* in isolated X1 neoblasts from wounded planarians (0.7%) and in *smcdwi-1*⁺ cells in day 3 regenerating anterior blastemas (Figure 3).

An ortholog of the *sine oculis/Six* family transcription factor *six3* (Pineda and Saló, 2002; Wenemoser et al., 2012) was expressed in the lateral cephalic ganglia of the planarian brain and in peripheral neurons (Figures 3 and S3A) and in X1 neoblasts following wounding (0.6%) and was coexpressed with *smcdwi-1* in day 3 regenerating blastemas (Figure 3). Moreover, genes encoding two SOX proteins (*soxB-2* and *soxB*; Figure S1A; Lapan and Reddien, 2012), as well as the transcription factor-encoding genes *glass*, *lhx3/4*, *prox-2*, and *elf-1* (Figures S1A and S1B), were coexpressed with *smcdwi-1*⁺ cells during anterior regeneration (Figure S3B), and some of these were expressed in isolated X1 cells from wounded planarians (Figure S3C).

In conclusion, the large number of transcription factors expressed in distinct brain regions and in neoblasts from wounded planarians supports a model of neuronal fate specification occurring for most or all lineages within the neoblast compartment during regeneration.

Tissue-Associated Transcription Factors Are Expressed in Nonoverlapping Subsets of Neoblasts from Wounded Planarians

We hypothesized that if the neoblast expression of tissue-associated transcription factors is a result of the specification of distinct lineages, as opposed to reflecting stochastic gene expression or other roles for these genes, only specific combinations of genes would be expressed together in the same neoblast cells. Indeed, for the eye and protonephridia, combinations of transcription factors expressed in differentiated cells are coexpressed in a small number of neoblasts (Lapan and Reddien, 2011, 2012; Scimone et al., 2011). We found multiple other instances of transcription factors (and/or specific markers) expressed together in the same differentiated tissue in intact animals that were coexpressed in the same isolated X1 neoblasts following wounding (Figure 2). Specifically, *hmf4* and

gata4/5/6 (gut-associated), *collagen*, *myoD*, and *snail* (muscle-associated), and *FoxA* and *meis* (pharynx-associated) were expressed together in neoblasts (Figure 2).

To test for the possibility that coexpression of transcription factors could be coincidental, we performed double FISH with isolated X1 cells from wounded planarians using combinations of RNA probes for genes expressed in distinct differentiated cell types. In all such tested cases, we failed to detect any neoblast that displayed coexpression of transcription factors associated with different tissues (taking these results together, these data suggest substantial restriction against coexpression; one-tailed paired t test, $p = 0.0064$; Figure 4). Specifically, *pax6A* (neurons) was never detectably coexpressed in the same X1 cell with *FoxA* (pharynx) or with *POU2/3*, *sall*, and *osr* (nephridia) or with the anterior pole genes *FoxD*, *notum*, and *prep* (Figure 4). Similarly, *FoxA* was never detectably coexpressed in the same X1 cell together with the nephridia genes, the muscle gene *collagen*, or the eye gene *ovo* (Figure 4). Moreover, the gut genes *hmf4* and *gata4/5/6* were never detectably coexpressed with the neuronal and eye gene *sp6-9*, the neuronal genes *neuroD-1* and *pax6A*, or the nephridia genes in X1 cells (Figure 4). Together, these results indicate that multiple distinct neoblast populations express specific combinations of transcription factors during regeneration that are associated with differentiated tissues.

Tissue-Associated Transcription Factors Are Required for Regeneration of Distinct Cell Types

The large collection of transcription factors identified here provides a resource for detailed investigation of different candidate lineage-specification events in planarian regeneration. The specialized neoblast model predicts that most of the transcription factors described above will be essential for the specification of regenerative lineages. Identifying all of such candidate lineages is beyond the scope of this work; however, we sought to test this prediction by the analysis of case studies. To assess the role of some of the identified neoblast-expressed transcription factors in the regeneration of specific cell types, we performed RNAi and analyzed regeneration outcomes. We restricted our analysis to three transcription factors expressed in cell types for which we had other markers as the result of extensive in situ screening: the neuronal expressed genes *klf* and *pax3/7* and the pharynx gene *FoxA*.

klf was shown to be required for the proper formation of photoreceptor neurons during eye regeneration (Lapan and Reddien, 2012). However, *klf* had a second expression domain in the outer branches of the planarian head, suggesting a possible role in the differentiation of sensory neurons (Figure 5A). *cintillo* encodes a protein similar to the degenerin/epithelial superfamily of sodium channels and is expressed in the anterior dorsal margin of the

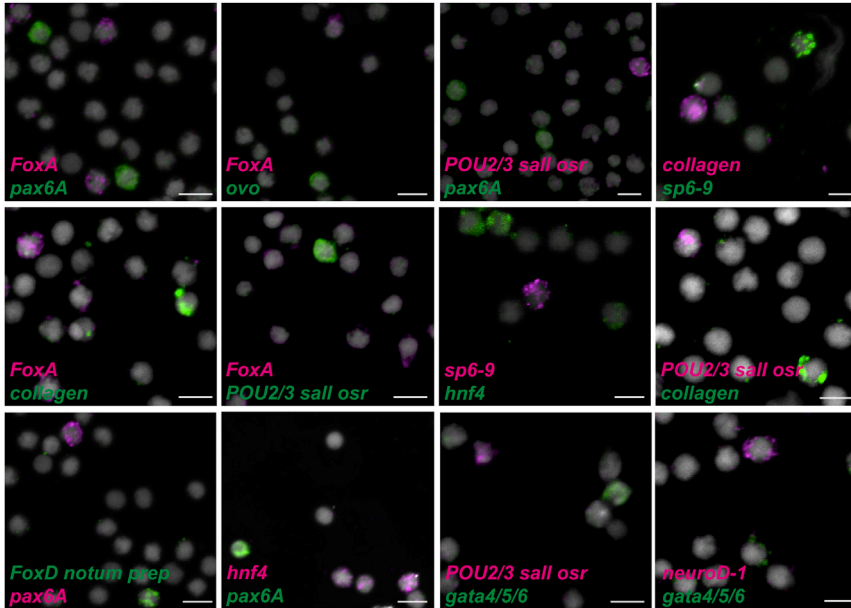


Figure 4. Nonoverlapping Expression of Different Tissue-Associated Genes in X1 Neoblasts

Cell FISH from sorted X1 cells from pre-pharyngeal regions 48 hr following amputations using several combinations of genes expressed in different tissues. Pharynx: *FoxA*; CNS: *pax6A*, *neuroD-1*, and *sp6-9*; eye: *ovo* and *sp6-9*; protonephridia: *POU2/3*, *sall*, *osr*; muscle: *collagen*; anterior pole: *FoxD*, *notum*, *prep*, and gut: *hnf4* and *gata4/5/6*. No overlapping expression within the same X1 cells was detected (paired t test one-tailed, $p = 0.0064$) when compared to expected frequencies. Total number of X1 cells counted: *FoxA/pax6A* $n = 693$, *FoxA/ovo* $n = 198$, *POU2/3/sall/osr/pax6A* $n = 489$, *FoxD/prep/notum/pax6A* $n = 255$, *collagen/sp6-9* $n = 198$, *collagen/FoxA* $n = 443$, *FoxA/POU2/3/sall/osr* $n = 295$, *hnf4/sp6-9* $n = 448$, *collagen/POU2/3/sall/osr* $n = 427$, *hnf4/pax6A* $n = 216$, *POU2/3/sall/osr/gata4/5/6* $n = 2422$, *neuroD-1/gata4/5/6* $n = 201$. DAPI labeled DNA (gray). Images shown are maximal-intensity projections. Scale bars, 10 μm .

planarian head (Oviedo et al., 2003). We found that neurons expressing *klf* in the outer head rim also expressed *cintillo* both in intact and regenerating animal heads (Figures 5A and S4B). *pax3/7* was expressed in the ventral midline of the planarian brain (Figure 5A). We identified the planarian homolog of the enzyme dopamine β -hydroxylase (*DBH*), which is required for the conversion of dopamine into the neurotransmitter norepinephrine. *DBH* was also expressed in scattered cells on the ventral midline of the planarian brain, and some of these cells coexpressed *pax3/7* in intact and regenerating planarians (Figures 5A and S4B).

klf, *pax-3/7*, and control RNAi animals regenerated anterior blastemas with largely normal brain formation and patterning (Figures 5B and S4C). Specifically, normal expression of *Smed-tryptophan hydroxylase* (*tph*) (Nishimura et al., 2007; Lapan and Reddien, 2012), *sim*, and a gene expressed in eyes and cephalic branches with homology to a sodium-dependent chloride/bicarbonate exchanger (Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$) was observed (Figures 5B and S4C). *klf* and *pax3/7* transcripts were greatly reduced in *klf* and *pax3/7* RNAi animals, respectively (Figure S5D). Despite displaying normal regeneration of many neurons, *klf*(RNAi) animals had a near-complete absence of *cintillo*-expressing cells ($n = 14/14$; Figure 5B) and also lacked photoreceptor neurons (Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ -expressing cells) (Lapan and Reddien, 2012), suggesting a specific role for *klf* in the regeneration of this subset of peripheral sensory neurons. Similarly, *pax3/7*(RNAi) animals showed

significantly reduced numbers of *DBH*-expressing cells ($n = 12/13$; Figure 5B), consistent with a role for this gene in the specification of this subset of neurons. In both cases, whether neuron classes are absent or simply not expressing the appropriate genes is unknown.

FoxA was expressed in the pharynx primordium and in neoblasts during pharynx regeneration (Figures 2 and S2B). We inhibited *FoxA* with RNAi and amputated animals into head, trunk, and tail fragments. Seven days later, all regenerating trunk pieces (which started regeneration with pharynges) had lesions at the pharynx ($n = 18/18$ animals; Figure 6A). Most tail fragments failed to regenerate a pharynx ($n = 15/20$; Figure 6A). Because the pharynx is a muscular organ, the *mhc-1* muscle marker was used to determine the presence and structure of the pharynx. We found that *FoxA* (RNAi) animals showed limited pharynx regeneration ($n = 9/9$), indicating a requirement for *FoxA* in pharynx specification (Figure 6B). *FoxA* mRNA levels in *FoxA*(RNAi) animals were significantly reduced compared to control RNAi animals (Figure S5A). Regenerating tail fragments of *FoxA*(RNAi) animals 4 days after amputation completely lacked expression of both *FoxA* and *mhc-1* ($n = 9/9$; Figure 6B). By contrast, control RNAi animals showed *FoxA* expression together with *mhc-1* in the pharynx primordium (Figure 6B). *FoxA* expression was not exclusively restricted to the pharynx in intact animals (Figure S2C). Similarly, the gut transcription factor *hnf4* was expressed in few scattered cells in and around the pharynx

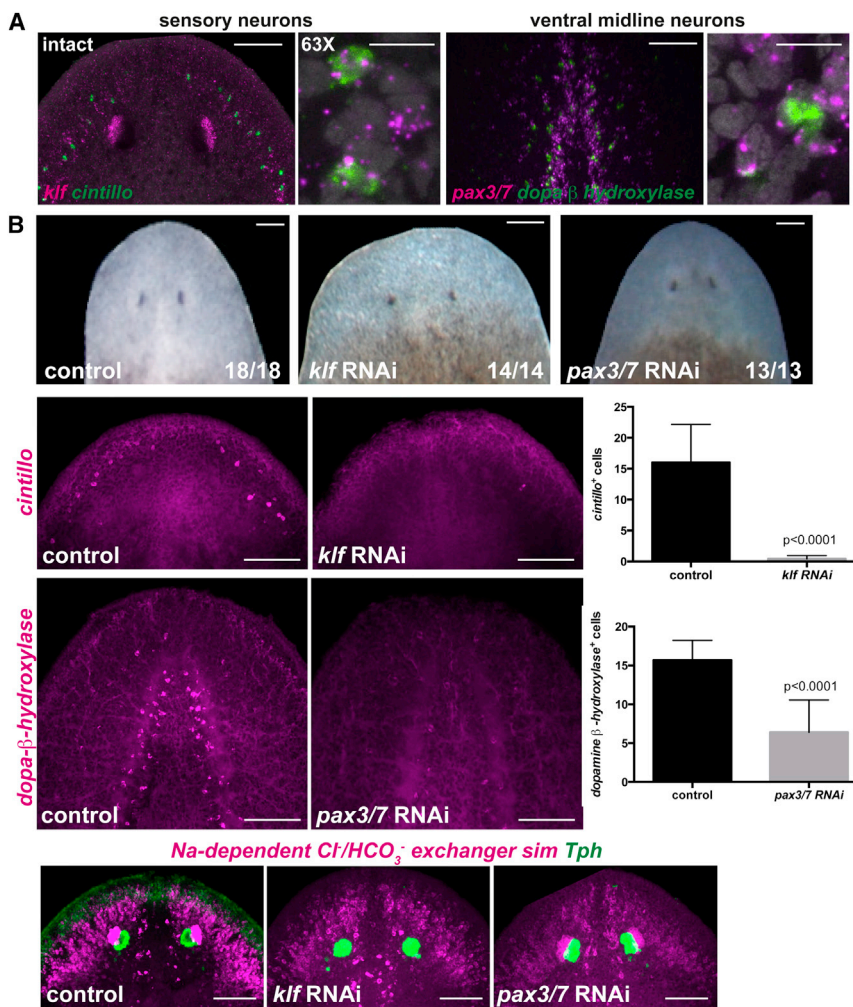


Figure 5. Regeneration Defects of Neural Cell Types following Tissue-Associated Transcription Factor RNAi

(A) Two regions of the brain are shown: the left panel depicts sensory neurons coexpressing the transcription factor *klf* and the marker *cintillo*, and the right panel shows ventral midline neurons coexpressing the transcription factor *pax3/7* and the enzyme *dopamine-β-hydroxylase* (scale bars, 100 μm). Higher-magnification panels show coexpression of the genes analyzed in intact head regions (scale bars, 10 μm). DAPI labeled DNA (gray).

(B) Upper row: normal head regeneration (day seven) in control, *klf*, or *pax3/7* RNAi animals. Animals were fed four times followed by head amputation. Second row: *cintillo*⁺ cells are not detected in *klf*(RNAi) animals; graph on the right shows total number of *cintillo*⁺ cells (mean ± SD) in regenerating heads (n > 14 animals per RNAi condition; Student's t test, p < 0.0001). Third row: *dopamine-β-hydroxylase*-expressing cells are strongly reduced in regenerating *pax3/7*(RNAi) animals; graph on the right shows total number of *dopamine-β-hydroxylase* cells (mean ± SD) in regenerating heads (n > 13 animals per RNAi condition; Student-t test, p < 0.0001). Fourth row: dorsal view of control labelings using the RNA probes *sim*, the outer branches and photoreceptor neuron gene *Na⁺-dependent Cl⁻/HCO₃⁻*, and the *tryptophan hydroxylase (Tph)* gene, show normal expression of these genes in the

regenerating heads of the different RNAi animals. Images are maximal-intensity projections and representative of results seen in more than five animals (except otherwise labeled) per panel. Anterior, up. Scale bars, 100 μm.

(Figure S5B) in intact animals. Cells around the pharynx coexpressed *FoxA* and *hnf4* in intact animals (Figure S5B). Moreover, some isolated X1 cells from wounded animals coexpressed *FoxA* and *hnf4* (27.1% of *FoxA*⁺ cells; Figure S5C). Anterior blastemas in *FoxA*(RNAi) animals showed defects in the regeneration of gut branches (Figure S5C), reflecting either a direct role in anterior gut formation or a secondary effect of failed pharynx regeneration. Animals from cnidarians to humans have *FoxA* transcription factors that are commonly associated with development of digestive tracts (Mango, 2009). The phenotype observed in *FoxA*(RNAi) in planarians is reminiscent of the *C. elegans pha-4* mutants that lack a pharynx and *Drosophila forkhead* mutants that lack foregut and hindgut, consistent with an evolutionary ancient role for *FoxA* transcription factors in foregut/pharynx specification and differentiation (Mango, 2009).

DISCUSSION

Planarian neoblasts have long attracted interest as adult dividing cells that are required for regeneration and cell turnover. A largely unaddressed question has been whether neoblasts are a homogeneous population or are constituted of multiple different cell types, such as stem cells and lineage-committed progenitors. Two models for regeneration have been considered (Reddien, 2013): the naive neoblast model unites all neoblasts as pluripotent stem cells, with fate specification occurring in nondividing neoblast progeny. The specialized neoblast model posits that fate of regenerating cells is specified in neoblasts themselves.

A variety of recent reports have demonstrated instances of *smedwi-1*⁺ cell specialization. Transcription factors required for the regeneration of the eye (Lapan and Reddien, 2011, 2012), nephridia (Scimone et al., 2011), the

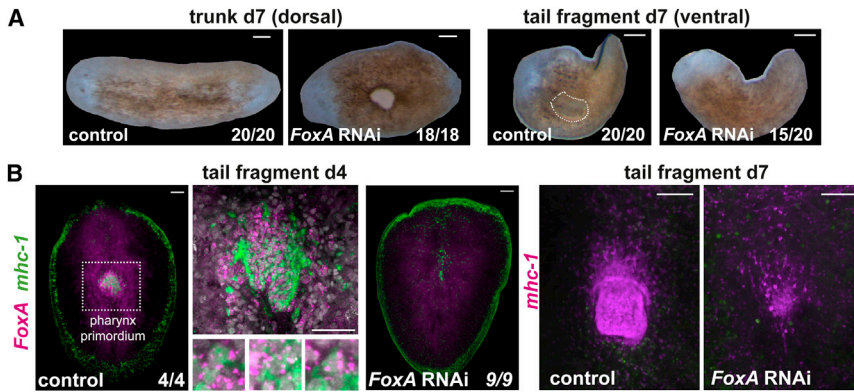


Figure 6. FoxA RNAi Disrupts Pharynx Regeneration

(A) *FoxA(RNAi)* animals show dorsal lesions near the old pharynx (in trunk pieces, $n = 18/18$) and do not regenerate a new pharynx (in tail fragments, $n = 15/20$) 7 days following amputation. Light images show no pharynx opening on the ventral side in regenerating tail fragments of *FoxA(RNAi)* animals.

(B) FISH using the RNA probes *mhc-1* and *FoxA* shows a defective regenerating pharynx in *FoxA(RNAi)* tail fragments at 4 and 7 days following amputation. Coexpression of the muscle marker *mhc-1* (green) and

the pharynx transcription factor *FoxA* (magenta) is observed in cells of the pharynx primordium in control RNAi animals at 4 days following tail amputation. Right panels: *mhc-1* expression (magenta) in control and *FoxA* RNAi day 7 regenerating tail fragments. DAPI labeled DNA (gray). Images are maximal intensity projections and representative of results seen in more than nine animals per panel. Anterior, up. Scale bars for all panels, 100 μm .

anterior pole (Scimone et al., 2014), and several neuron classes (Cowles et al., 2013; Currie and Pearson, 2013; Wenemoser et al., 2012) are expressed in *smcdwi-1*⁺ cells during regeneration. These data provide support for the specialized neoblast hypothesis. If this hypothesis is correct, it predicts that numerous additional transcription factors - perhaps for the specification of every missing cell type - would be expressed in distinct subsets of neoblasts during regeneration.

We tested this prediction here by seeking transcription factors expressed in small numbers of X1 neoblasts and/or *smcdwi-1*⁺ cells following wounding. We coupled an RNA-seq approach with expression screening approaches using transcription factors expressed in specific differentiated planarian tissues. In total, 41 transcription factors (8 known from prior reports and 33 reported here) were expressed in X1 neoblasts and/or *smcdwi-1*⁺ cells (Figure S1E). A total of 36 out of these 41 transcription factors were detectably expressed in sorted X1 neoblasts from wounded planarians, indicating expression in cells that will complete at least one round of division. These identified transcription factors displayed expression in specific differentiated cells and in small numbers of neoblasts at wound sites, consistent with the specialized neoblast model. Many specific lineages could be investigated to understand the formation of specific cell types during regeneration, and the transcription factors described here provide a resource for such inquiry. In vivo lineage-tracing methods are presently limited in planarians. Therefore, in principle, a transcription factor induced in neoblasts at wounds might have some role in neoblast physiology other than lineage specification. However, two lines of evidence suggest that many or all of these transcription factors will be involved in specifying progenitors for the regeneration

of specific differentiated cell types. First, RNAi of a number of identified transcription factors, reported here and in published work, ablate the regeneration of their specific tissues. Here, we demonstrated a requirement for *FoxA* for the pharynx; *pax3/7* for *DBH*⁺ ventral midline neurons, and *klf* for *cintillo*⁺ sensory neurons. In prior work, *sp6-9*, *eya*, and *ovo* were required for the eye (Lapan and Reddien, 2011, 2012); *six1/2-2*, *POU2/3*, and *sall* were required for the nephridia (Scimone et al., 2011); *FoxD* was required for the anterior pole (Scimone et al., 2014); and *ap-2*, *lhx1/5-1*, *pitx*, *coe*, *hes1*, and *sim* were required for different subsets of neurons (Cowles et al., 2013; Currie and Pearson, 2013; Wenemoser et al., 2012). Second, we found that transcription factors that were expressed in different differentiated tissues were expressed in distinct subsets of neoblasts. By contrast, in multiple cases, transcription factors expressed together in the same differentiated tissues were expressed together in the same neoblasts. Some described transcription factors might prove to have roles in the specification of more than one lineage. For instance, *klf* is required for the specification of both photoreceptor neurons as well as for the sensory *cintillo*⁺ neurons (this study and Lapan and Reddien, 2012). Similarly, *eya* has been shown to be required for the specification of eye progenitors (Lapan and Reddien, 2011) as well as nephridia cells (Scimone et al., 2011).

The concept of neoblast specialization at wounds is important in understanding planarian regeneration and opens many avenues for future inquiry. For example, how is the neoblast response tailored to the identity of missing tissues? Do specialized neoblasts amplify their population, or do specialized neoblasts rapidly cease division and differentiate? There is an ongoing need for differentiated cells in planarians, and low levels of specialized neoblasts appear

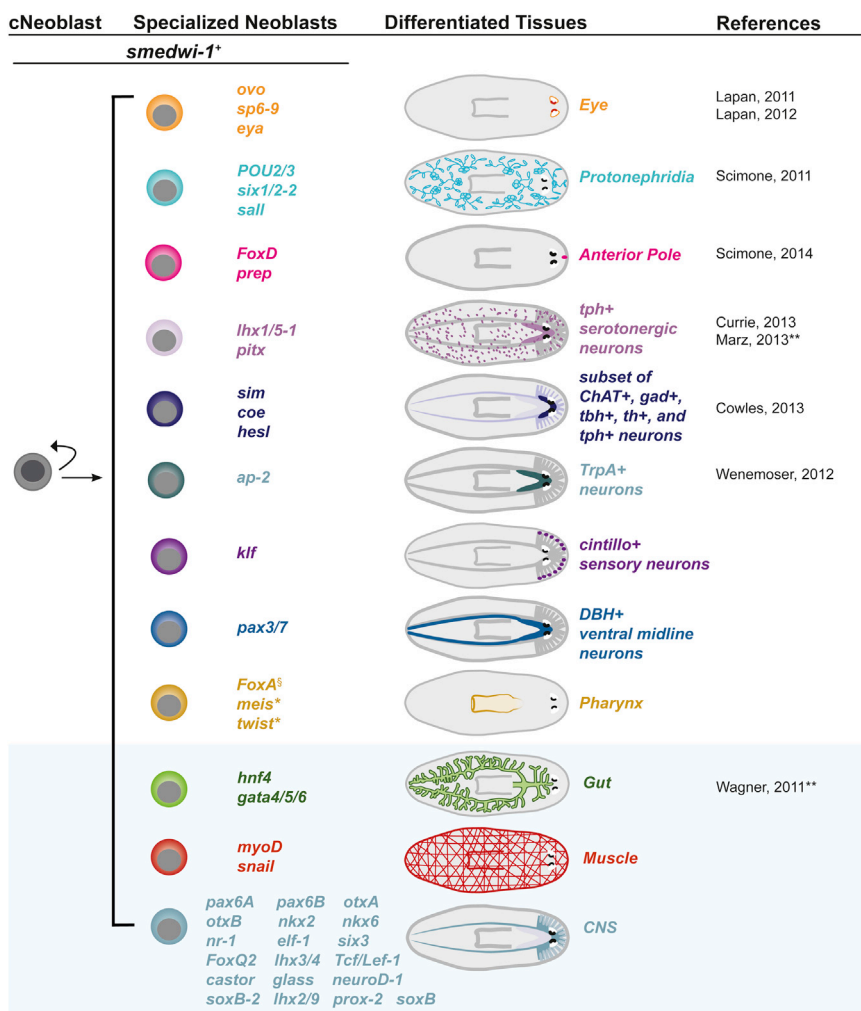


Figure 7. Proposed Model: Specialization of Neoblasts into Different Lineages following Wounding

cNeoblasts will give rise, directly or indirectly, to specialized neoblasts for most-to-all tissues. Both the cNeoblasts and the specialized neoblasts express the *smcdwi-1* marker. As specialized neoblasts further differentiate, they will lose expression of *smcdwi-1*. A summary of all known transcription factors expressed in neoblasts and functionally associated with distinct lineages by RNAi analysis are shown in the upper part of the model (white background). The transcription factors expressed in neoblasts and in specific tissues, but that have not been functionally shown to be required for their specification, are shown in the lower part of the model (blue background).[§] During the production phase of this manuscript, the role of *FoxA* in pharynx regeneration was also described in (Adler et al., 2014). *Functional requirement for pharynx specification was not tested. **Coexpression with the SMEDWI-1 protein was shown. The transcription factors *dlx*, *six1/2*, *soxB*, and *otxA*, as well as *hunchback* and *eya*, have been shown to be required for the regeneration of the eye (Lapan and Reddien, 2011, 2012) and the nephridia (Scimone et al., 2011), but no coexpression with the neoblast gene *smcdwi-1* or expression in X1 neoblasts has been tested.

to exist in intact animals (Cowles et al., 2013; Lapan and Reddien, 2012). For example, the *ovo*⁺ eye progenitors are abundant near anterior-facing wounds at 48 hr following amputation, but a small number of them can also be observed near the head of intact animals (Lapan and Reddien, 2012). Similarly, we observed some X1 neoblasts expressing *FoxA*, *klf*, and *pax3/7* from macerated prepharyngeal regions of uninjured animals, with an increased number present at 48 hr following wounding (Figure S6).

Regeneration involves instructions that specify the identity of missing tissue to be replaced. To understand the connection between regenerative instructions and the production of appropriate cell types, it is essential to address the cellular step at which specification of the fate of regenerative cells occurs. We propose that cNeoblasts (directly, or via their descendants) begin expressing numerous transcription factors of specific lineages in distinct neoblast cells (Figure 7). In this model, almost all of the lineages formed during development could be reconstituted during regeneration, with progenitors that generate and comprise

planarian blastemas being a heterogeneous patchwork of lineage-specified cells.

EXPERIMENTAL PROCEDURES

Animals, Radiation Treatment, and RNAi

Asexual *S. mediterranea* (CIW4) animals starved 7–14 days prior experiments were used. Animals were exposed to 6,000 rads of radiation using a dual Gammacell-40 137 cesium source and fixed 3 days after irradiation. Double-stranded RNA-expressing bacteria cultures were mixed with 70% liver solution in a 1:300 ratio. RNAi animals have been fed four times (days 0, 4, 7, and 11) and amputated at day 12. Seven days following amputation, animals were fixed and in situ hybridizations performed. Details of histological methods can be found in Supplemental Experimental Procedures.

mRNA Purification and Illumina Sequencing

Total RNA was purified using TRIzol reagent, and mRNA sequenced using TruSeq RNA Sample Preparation Kit v2 (Illumina) on a HiSeq.



Details of RNA-seq analysis can be found in [Supplemental Experimental Procedures](#).

Statistical Analysis

Coexpression frequencies were tested using a two-tailed Fisher's exact test. Nonoverlapping expression in neoblast subsets was tested using a one-tailed paired t test. Comparisons between RNAi treatments were analyzed using unpaired t tests. Details can be found in [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

Illumina sequencing data were deposited to the Gene Expression Omnibus with the accession number GSE57226. Sequences of genes identified in this study were deposited to GenBank with the accession numbers KJ934799–KJ934818.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.06.001>.

AUTHOR CONTRIBUTIONS

M.L.S., K.M.K., S.W.L., and P.W.R. were responsible for the overall study design and analysis. M.L.S. and K.M.K. performed RNAi experiments; K.M.K. and M.L.S. performed X1 FISH; K.M.K. performed X1 RNA sequencing; M.L.S. and S.W.L. performed in vivo neoblast FISH; M.L.S. and S.W.L. performed the neural transcription factor screen; and K.M.K., M.L.S., and S.W.L. performed other transcription factor identification. M.L.S., K.M.K., S.W.L., and P.W.R. wrote the manuscript.

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