RESEARCH ARTICLE

Genome-wide analysis of the bHLH gene family in planarians identifies factors required for adult neurogenesis and neuronal regeneration

Martis W. Cowles1, David D. R. Brown2,3, Sean V. Nisperos1, Brianna N. Stanley1, Bret J. Pearson2,3,4 and Ricardo M. Zayas1,*

ABSTRACT
In contrast to most well-studied model organisms, planarians have a remarkable ability to completely regenerate a functional nervous system from a pluripotent stem cell population. Thus, planarians provide a powerful model to identify genes required for adult neurogenesis in vivo. We analyzed the basic helix-loop-helix (bHLH) family of transcription factors, many of which are crucial for nervous system development and have been implicated in human diseases. However, their potential roles in adult neurogenesis or central nervous system (CNS) function are not well understood. We identified 44 planarian bHLH homologs, determined their patterns of expression in the animal and assessed their functions using RNAi. We found nine bHLHs expressed in stem cells and neurons that are required for CNS regeneration. Our analyses revealed that homologs of coe, hes (hesl-3) and sim label progenitors in intact planarians, and following amputation we observed an enrichment of coe* and sim* progenitors near the wound site. RNAi knockdown of coe, hesl-3 or sim led to defects in CNS regeneration, including failure of the cephalic ganglia to properly pattern and a loss of expression of distinct neuronal subtype markers. Together, these data indicate that coe, hesl-3 and sim label neural progenitor cells, which serve to generate new neurons in uninjured or regenerating animals. Our study demonstrates that this model will be useful to investigate how stem cells interpret and respond to genetic and environmental cues in the CNS and to examine the role of bHLH transcription factors in adult tissue regeneration.

KEY WORDS: Basic helix-loop-helix, Coe, Single-minded, Hes, Neurogenesis, Lophotrochozoan, Planarians, Regeneration, Schmidtea mediterranea, Stem cells, Neoblasts

INTRODUCTION
The discovery that neurogenesis persists in the central nervous system (CNS) of adult animals (Gage, 2002) changed a long-held doctrine that neurons were only produced in the embryo (Ramón y Cajal, 1928; Kempermann, 2011). Although it is now well accepted that adult neurogenesis is a widespread phenomenon across diverse metazoans (Lindsey and Tropepe, 2006; Kempermann, 2012), the ability of most organisms to produce new neurons does not compensate for cells lost after injury or disease. Therefore, to examine how neural precursors could be directed to repair CNS neurons in vivo, comparative approaches using animal models of regeneration will help us to gain insights into the basic mechanisms needed to reestablish nervous system function after injury or the onset of neurodegenerative disease (Kempermann, 2011).

Freshwater planarians have emerged as an excellent model to examine the molecular mechanisms underlying stem cell biology and tissue replacement (Elliott and Sánchez Alvarado, 2013; King and Newmark, 2012). Following amputation, planarians are capable of restoring lost body parts from a population of adult pluripotent stem cells called neoblasts (Baguñà, 2012; Elliott and Sánchez Alvarado, 2013). Planarian stem cells share conserved pluripotency determinants with mammalian stem cells (Labbé et al., 2012; Önal et al., 2012; Solana et al., 2012) and serve to replace cells lost during normal physiological cell turnover or after amputation. In contrast to most model organisms currently studied, planarians have the remarkable ability to regenerate their CNS after injury. Thus, these animals provide an excellent opportunity to analyze mechanisms underlying stem cell regeneration and CNS regeneration in vivo. The planarian CNS consists of bi-lobed cephalic ganglia (brain) that are connected to two longitudinal ventral nerve cords projecting posteriorly along the length of the animal. Distinct neuronal cell types have been described by histochemistry, including unipolar and bipolar neurons as well as neurosecretory cells (Bullock and Horridge, 1965; Lentz, 1968). The generation of genomic resources has led to identification of hundreds of neural markers that have been used to show that the planarian CNS is molecularly complex (Gentile et al., 2011), but little is known about how these animals regenerate their nervous system. On the basis of elegant single cell transplantation studies in lethally irradiated planarians (Wagner et al., 2011), it has been estimated that less than 5% of the planarian stem cells are truly pluripotent (Rink, 2013). Therefore, we and others hypothesize that a fraction of the heterogeneous stem cell pool may be comprised of lineage-committed or specialized progenitor cells (Reddien, 2013). To fully understand the mechanisms underlying how neuronal diversity is maintained or reestablished in planarians it will be essential to define any neural precursor populations that may exist.

Transcription factors from the basic helix-loop-helix (bHLH) gene family play vital regulatory roles throughout the different stages of neurogenesis in embryos, including neural fate commitment, subtype specification, migration and axon guidance (Bertrand et al., 2002; Guillemot, 2007). Genes of the Drosophila achaete-scute complex represent the prototypical proneural genes that are important for development of the peripheral and central nervous systems (Jan and Jan, 1994). Proneural genes have been identified...
in sponges (Richards et al., 2008) and their roles are conserved from cnidarians to vertebrates (Lindsey and Tropepe, 2006; Galliot et al., 2009). However, the precise function of bHLH genes in embryos or adult neural stem cells remains poorly understood (Kintner, 2002). Here we have performed a genome-wide analysis of bHLH family genes to identify factors essential for CNS tissue renewal in adult planarians. Our screen identified nine genes that are expressed in both the stem cells and neurons and are required for normal CNS regeneration, including homologs of collier/olfactory-1/early B-cell factor (coe), hairy/enhancer of split (hes-like-3) and single-minded (sim). To characterize and follow the fate of coe+, hesl-3+ and sim+ stem cells, we performed bromodeoxyuridine (BrDU) pulse-chase experiments and found that coe and sim are expressed in proliferating cells adjacent to the CNS, which can be traced to the brain or ventral nerve cords over the course of 2 days. During regeneration, we observed an enrichment of sim+ progenitors near the wound site. Furthermore, RNAi knockdown of coe, hesl-3 or sim led to defects in CNS regeneration, including failure of the cephalic ganglia to reorganize or pattern, and a loss of expression of genes unique to distinct neuronal subtypes. Together, these data suggest that coe, hesl-3 and sim are expressed in neural progenitor cells and that these bHLH genes are required to generate new neurons in uninjured and regenerating animals. Our study demonstrates that this model will be useful to investigate how stem cells interpret and respond to genetic and environmental cues in the CNS and to examine the role of bHLH transcription factors in adult tissue regeneration.

RESULTS
Identification of bHLH family genes in planarians
We identified 44 sequences in the planarian Schmidtea mediterranea predicted to encode a bHLH motif and named them according to their homology, as described in the Materials and methods (supplementary material Fig. S1, Table S1; for brevity we have omitted the Smed prefix from the gene names). Recent transcriptional profiles generated from sorted cell populations indicate that most bHLH homologs are expressed in the planarian stem cells and their postmitotic progeny (Labbé et al., 2012; Önal et al., 2012; Resch et al., 2012). To investigate cell- and tissue-specific patterns of bHLH gene expression, we performed whole mount in situ hybridization (WISH). We confirmed the presence of transcripts in stem cell or their progeny by testing for the loss of gene expression throughout the parenchyma (mesenchyme) 6 days following exposure to γ-irradiation, a treatment that depletes all stem cells and postmitotic progeny (Reddien et al., 2005b; Eisenhoffer et al., 2008). Consistent with the transcriptome data, we found that 35/43 bHLH genes tested are expressed in stem cells and their progeny (supplementary material Table S1). As expected, we also observed bHLH expression in differentiated tissues, including the CNS (21 genes), epidermis (three genes), pharynx (14 genes) or intestine (nine genes) (supplementary material Fig. S2, Table S1). One gene, neuroD-2, was not detected by WISH.

A subset of bHLH genes is expressed in neurons
Of the 12 CNS- and stem-cell-expressed bHLH genes, we selected atoh, coe, fer3l-1, hesl-3 and sim for detailed expression analyses because their transcripts were detected in discrete cell populations (supplementary material Fig. S2). To confirm the pattern of mRNA expression in the CNS and visualize the distribution of these cell populations in reference to the brain, we performed double-fluorescent in situ hybridization experiments (dFISH) using the pan-neural marker pc2 (Collins et al., 2010). atoh, coe, fer3l-1, hesl-3 and sim were expressed in discrete neural populations throughout the brain and in regenerating tissues (Fig. 1; supplementary material Fig. S3). In addition, fer3l-1 (supplementary material Fig. S3D), hesl-3 (Fig. 1D) and sim (Fig. 1G) were detected in cells distributed throughout the mesenchyme. We then investigated the expression of coe, hesl-3 and sim in specific neuronal subtypes by performing dFISH with markers of cholinergic, GABAergic, octopaminergic, dopaminergic and serotonergic neurons (Umesono et al., 2011). coe, hesl-3 and sim were each co-expressed in cholinergic neurons (Fig. 1J-L). We also detected transcripts for coe in GABAergic, octopaminergic, dopaminergic and serotonergic neurons and sim in octopaminergic and dopaminergic neurons (Fig. 1J,L). These results demonstrate that a subset of bHLH genes is expressed in molecularly distinct differentiated neurons.

coe, hesl-3 and sim label cycling cells in close proximity to the CNS
Following amputation, stem cells proliferate beneath the wound site (post-blastema) and give rise to the regeneration blastema, the structure where postmitotic cells differentiate to form the missing tissues. atoh, coe, fer3l-1, hesl-3 and sim were expressed in the newly regenerated tissues, but it was also noted that these mRNAs were present in cells located in the post-blastema (Fig. 1; supplementary material Fig. S3). Therefore, we examined whether atoh, coe, fer3l-1, hesl-3 and sim could be detected in mitotic cells. We found that, with the exception of atoh, their transcripts could be visualized in a subset of anti-phosphohistone-H3+ cells (Fig. 1C,F,I; supplementary material Fig. S3C,F), which we also observed in uninjured planarians (data not shown).

To distinguish between gene expression in stem cells/progeny or differentiated neurons, we examined the expression of atoh, coe, fer3l-1, hesl-3 and sim following 6 days of γ-irradiation treatment. Compared with control animals, atoh expression was reduced throughout the mesenchyme, but we were unable to detect changes in expression in the head or the pre-pharyngeal area even when we used FISH (supplementary material Fig. S3G-J). In contrast to atoh, we observed a reduction of coe+ cells near the brain and between the cephalic ganglia and ventral nerve cords (VNCs) (supplementary material Fig. S3K,L). fer3l-1 expression was broadly reduced in the mesenchyme, except for a few cells located on the dorsal surface of the cephalic ganglia and distributed throughout the mesenchyme (supplementary material Fig. S3L). hesl-3 and sim staining were also reduced in the mesenchyme and near the cephalic ganglia following γ-irradiation (supplementary material Fig. S3M). To validate further that coe, hesl-3 and sim were expressed in a subset of stem cells, we co-stained these genes with the stem cell marker smedwi-1 (Reddien et al., 2005b; Eisenhoffer et al., 2008) (Fig. 1M-O). Taken together, our analyses confirmed that bHLH genes were expressed in subsets of stem cells and postmitotic progeny. In addition, we noted that cell populations that expressed coe, hesl-3 and sim near the CNS were γ-irradiation-sensitive, further supporting potential roles of these genes in differentiation of neural precursor-like cells.

coe and sim are expressed in differentiating neurons
Stemming from our observations that coe, hesl-3 and sim were expressed in stem cells and in diverse neural subtypes, we reasoned that these genes label lineage-committed progenitors and differentiating neurons. To address this possibility, we sought to label stem cells expressing coe, hesl-3 or sim and map their relative positions in the animal over time. Although the most commonly used tools to trace cell lineages (e.g. genetic marks) (Kretzschmar
and Watt, 2012) are not yet available in planarians, it is possible to label S-phase neoblasts with the thymidine analog BrdU and then determine the location of label-retaining cells (Newmark and Sánchez Alvarado, 2000; Eisenhoffer et al., 2008). This approach has been used to study planarian eye (Lapan and Reddien, 2011) and intestinal (Forsthoefel et al., 2011) cell differentiation. Previous studies have estimated that the length of S/G2/M in planarians is between 12 and 16 hours (Newmark and Sánchez Alvarado, 2000). We predicted that at later time points BrdU+ cells in the head marked by any of these bHLH genes would represent differentiating stem cells. Accordingly, we noted that, as expected, coe+ and sim+ cells were smedwi-1+ in the anterior region of the brain (supplementary material Fig. S4). We pulsed animals with BrdU and inspected animals for BrdU+ and coe+ or sim+ progenitors near the CNS over time, suggesting that coe+ progenitors, BrdU+ cell populations could be traced to the CNS over time; by 24 hours, progenitors were observed near the posterior end of the brain, and by 48 hours, we observed BrdU+/sim+ cells at the most anterior tip of the cephalic ganglia (Fig. 2E,F). Cells progressing through S-phase that expressed hesl-3 were observed throughout the animal (Fig. 2G,H). In contrast to coe+ and sim+ progenitors, the distribution of BrdU+/hesl-3+ cells remained relatively consistent over the course of 48 hours (Fig. 2I-J). To determine whether coe, hesl-3 and sim label unique cell populations, we performed dFISH to either coe or hesl-3 with sim. We found that coe, hesl-3 and sim were not co-expressed in the head; however, although rare, we did observe coe+/sim+ cells in the pre- pharyngeal area (supplementary material Fig. S5A-E). Consistent with the expression of these genes in smedwi-1+ cells, these data suggest that coe, hesl-3 and sim are expressed in lineage-committed progenitors. Moreover, we observed coe+ and sim+ progenitors near the CNS over time, suggesting that these genes are expressed in differentiating neurons.

In addition to determining the location of BrdU+ cells marked by bHLHs, we quantified the number of double-positive cells over time (Fig. 2J-M). After 4 hours, BrdU+/coe+ cells remained relatively consistent over the course of 48 hours (Fig. 2H,I). To determine whether BrdU+/hesl-3+ cells remained consistent throughout the head and pre-pharyngeal area over the course of 48 hours (Fig. 2L). By contrast, the proportion of BrdU+/hesl-3+ cells remained consistent throughout the head and pre-pharyngeal area over the course of 48 hours (Fig. 2M). Intriguingly, we also noted that after 48 hours we still observed...
BrdU+ cells expressing coe, hesl-3 or sim in the pre-pharyngeal area (yellow arrowheads in Fig. 2C,F,I), the same location where most coe+, hesl-3+ and sim+ cycling cells were first detected following a 4-hour chase period (Fig. 2A,D,G), which suggests that new progenitors were generated or differentiating cells turned on expression of these genes. The increase in the proportion of BrdU+ cells that expressed coe or sim near the brain and VNCs, combined with the observation that these genes were expressed in diverse neuronal subtypes (Fig. 1J,L), demonstrate that at least some coe+ and sim+ progenitors differentiate into neurons. The fact that we did not observe changes in the proportion of hesl-3 suggests a potential role of this gene in progenitor cell maintenance (Ishibashi et al., 1995; Kageyama et al., 2008).

In addition, we investigated whether these coe+ and sim+ progenitor populations contribute to the generation of the regeneration blastema following amputation. By 2 and 3 days of regeneration, BrdU+/coe+ and BrdU+/sim+ cells were detected in the post-blastema of animals regenerating new heads, with most BrdU+/coe+ progenitors located directly adjacent to the VNCs and many BrdU+/sim+ progenitors located between the VNCs (Fig. 3A-D). In S. mediterranea, head regeneration is completed within 7 days following amputation, and we found that the distribution of BrdU+/coe+ or BrdU+/sim+ cells observed in uninjured animals was reestablished by this time point (Fig. 2A,D, Fig. 3G-J). Taken together, these data are consistent with the hypothesis that the blastema is generated from a heterogeneous population of lineage-committed cells (Reddien, 2013).
FGF signaling modulates expression of sim+ and coe+ neurons and progenitors

Gene silencing of non-darake (ndk), an FGF receptor-like gene, disrupts anterior patterning and leads to ectopic expression of brain-specific neurons outside of the head domain (Cebría et al., 2002). We hypothesized that ndk silencing would cause an increase in the number of coe+ and sim+ progenitor cells. As we expected, ectopic expression of brain-specific markers (npp-4 and gpas) extended from the posterior end of the brain to the anterior boundary of the pharynx following 14 days of ndk RNAi treatment (Fig. 4A,B). We then examined sim and coe mRNA expression in the pre-pharyngeal area of control and ndk(RNAi) animals after 14 days of RNAi; we also exposed animals from each RNAi group to a lethal dose of γ-irradiation to distinguish stem cell or progeny expression from differentiated neurons (Fig. 4C-E). In control animals, we consistently observed coe+ and sim+ cells in the pre-pharyngeal region and found that most irradiation-sensitive cells were located between the VNCs. Following ndk RNAi, we found there were nearly twice the number of coe+ and ~40% more sim+ cells between the VNCs (Fig. 4F-H). Irradiated ndk(RNAi) animals confirmed that the majority of the additional cells between the VNCs are stem cells or early progeny. We conclude that coe+ and sim+ progenitor generation is regulated by signals downstream of FGF signaling.

Analysis of bHLH gene function in CNS regeneration

We took advantage of the experimental ease to inhibit gene function in planarians by RNAi to investigate the role of all 44 bHLH genes in planarian tissue regeneration. To screen for defects in CNS architecture and stem cell regulation, animals were stained with the pan-neural marker anti-SYNAPSIN and the mitotic cell marker anti-phosphohistone-H3 following dsRNA treatment (supplementary material Fig. S6A). We observed a wide range of regeneration phenotypes following RNAi knockdown of 11 bHLH genes, including lesions (mitfl-1), defects in CNS morphology (arn, arh, atoh8-1, coe, da, max, mxi-1 and sim) and patterning (hesl-3 and myoD) (Table 1; supplementary material Fig. S6B). We did not observe obvious defects in cell division following knockdown of any bHLH gene (data not shown). mitfl-1 was primarily detected in differentiated intestinal cells (supplementary material Fig. S2), and gene knockdown caused severe regeneration abnormalities and dorsal lesions that resulted in death, a phenotype reminiscent of proneural factors are co-silenced with da (Reddien et al., 2005a). Proneural bHLHs form heterodimers with ubiquitously expressed E proteins (such as daughterless, da) to bind DNA and function to commit progenitors to the neural fate during development (Bertrand et al., 2002). We did not observe regeneration defects following RNAi of candidate proneural gene homologs, such as acheate-scute or neuroD, which we validated by real-time quantitative PCR and found that our treatment strongly silenced each gene that we tested (supplementary material Fig. S6C). It has been shown that developmental defects are exacerbated in Drosophila when proneural factors are co-silenced with da (Goulding et al., 2000; Huang et al., 2000). Therefore, we performed double-RNAi experiments of da and ascl-1, ascl-2, atoh, neuroD-1 or neuroD-2. We also co-silenced predicted bHLH paralogs to test if genes may be functionally redundant (ascl-1,ascl-2 and hesl-1;hesl-2 RNAi). Due to the possibility that these proteins perdure, we also conducted long-term knockdown experiments (6 weeks of RNAi treatment; ascl-1, ascl-2, hesl-1, hesl-2, sim). Neither combinatorial nor long-term RNAi experiments revealed any additional regeneration defects. However, extended knockdown experiments increased the penetrance of sim(RNAi) animals from ~50% to 100% (n=10/10; data not shown).
Furthermore, we capitalized on the robust ndk RNAi phenotype to test the hypothesis that bHLHs required for neurogenesis would suppress ectopic nervous system expansion. We performed combinatorial RNAi experiments using ndk, which has been successfully used to investigate the role of other genes in planarian body patterning and CNS regeneration (Felix and Aboobaker, 2010; Iglesias et al., 2011; Blassberg et al., 2013), and screened 15 genes (ascl-1, ascl-2, atoh, atoh8-1, coe, da, hesl-1, -2, -3, hh, id4, neuroD-1, neuroD-2, sim and usf) by inspecting bHLH:ndk(RNAi) animals for changes in gpas and npp-4 expression. Induction of gpas expression posterior to the cephalic ganglia was not suppressed by inhibiting any of the bHLH genes together with ndk. However, ascl-1;ndk(RNAi) animals exhibited a 60% decrease in ectopic npp-4+ cells, whereas ndk;hesl-3(RNAi) and ndk;neuroD-1(RNAi) animals exhibited a 40% decrease of ectopic npp-4+ cells when compared with gfp;ndk(RNAi) animals (supplementary material Fig. S7A-G). These data suggest that ascl-1 and neuroD-1 may function in neural specification, but do not cause gross morphological CNS regeneration defects following gene knockdown.

coe, hesl-3 and sim are required for neuronal regeneration or maintenance

Our RNAi screen revealed that coe, hesl-3 or sim led to clear defects in brain regeneration (Fig. 5A-D). coe(RNAi) regenerates displayed photoreceptors with abnormal morphology and smaller cephalic ganglia that failed to form anterior commissures (n=94/114; Fig. 5B). In hesl-3(RNAi) regenerates, the CNS was abnormally patterned, with animals regenerating a single or an ectopic eyespot and brains with abnormal morphology (n=20/35; Fig. 5C). sim(RNAi) animals regenerated photoreceptors with reduced pigmentation (n=17/65) and displayed reduced density of the brain neuropil (n=30/65; Fig. 5D). Gene knockdown of arnt or sim resulted in similar regeneration defects and these genes have been shown to interact with each other (Probst et al., 1997). Hence, we also tested the effect of co-silencing sim and arnt; however, sim;arnt(RNAi) did not increase the severity of the phenotype above single RNAi treatments (data not shown). The coe, hesl-3 and sim knockdown phenotypes, together with the expression of these genes in progenitors and neurons (Figs 1-3), led us to further investigate their potential roles in neuronal regeneration and homeostasis.

Next, we examined the specific roles of coe, hesl-3 and sim in nervous system differentiation by evaluating the effect of gene knockdown on the expression of neuronal subtype-specific genes (Fig. 5E-H). We selected the neural marker ChAT, which is broadly expressed in the CNS and was co-detected with coe+, hesl-3+ and sim+ cells (Fig. 1J-L), and cpp-1, npp-4 and npy-2, which are strongly expressed in neuropeptidergic neurons in the brain (Collins et al., 2010). Using ChAT staining we measured the brain area of 7-day regenerates and found that coe and sim RNAi animals regenerated smaller brains (Fig. 5I). In addition, we observed a significant reduction of cpp-1+ cells in coe(RNAi) animals (Fig. 5F,J) and of npp-4+ and npy-2+ cells in coe(RNAi) and sim(RNAi) animals (Fig. 5F,H,K,L). Furthermore, we observed ChAT+, npy-2+ and npp-4+ cells in aberrant locations following coe RNAi (Fig. 5F). Although the brain area difference in hesl-3(RNAi) regenerates was not statistically significant, we did detect fewer cpp-1+, npp-4+ and npy-2+ neurons (Fig. 5G,I-L). Due to the fact that we observed CNS patterning-like defects in coe(RNAi) and hesl-3(RNAi) animals, we tested whether these abnormalities were caused by defects in the stem cells (smedwi-1), progeny (NB.32.1g, early progeny; agat-1, late progeny) or midline signals (slit expression) (Cébrèi et al., 2007), but we did not find obvious changes in the expression of these markers after coe or hesl-3 RNAi (supplementary material Fig. S8A-C). These data demonstrate that coe, hesl-3 and sim are required for expression of neuronal-specific genes and may be necessary for the replacement of neurons following injury. In combination with our expression analyses, these data suggest that coe, hesl-3 and sim are expressed in a subset of stem cells committed to neural fates and their function is crucial for neural progenitor maintenance or differentiation.
Planarians continuously replace cells during normal tissue homeostasis. Therefore, we also assessed the roles of *coe*, *hesl-3* and *sim* in nervous system maintenance by performing extended RNAi treatments (6 weeks) on intact planarians. Knockdown of *hesl-3* and *sim* resulted in no external phenotype or alterations in CNS architecture (data not shown). By contrast, long-term *coe* RNAi resulted in a strong behavioral phenotype in which animals exhibited impaired negative phototaxis and a flattened and stretched body shape with ruffling along the body margin (Fig. 5M; supplementary material Movies 1, 2). Analysis of ChAT+ and pc2+ neurons in *coe(RNAi)* animals showed that the CNS appeared largely intact except for the absence of ChAT+ and pc2+ neurons located at the anterior brain commissure (Fig. 5N). This phenotype was reminiscent of the defect observed in *coe* knockdown regenerates, in which the brain fails to reconnect (Fig. 5B). Because *coec(RNAi)* regenerates showed a dramatic reduction of *cpp-1*+ brain neurons, we examined whether this cell population was also affected in uninjured *coe(RNAi)* animals. Strikingly, we observed an 80% reduction in the number of *cpp-1*+ cells in *coe(RNAi)* planarians (Fig. 5O,P). Furthermore, when we performed dFISH to *coe* and *cpp-1*, we found that a majority of *cpp-1*+ cells also expressed *coe* (81±1.3%; Fig. 5Q). Taken together, our data indicate that *coe* is required for normal function and maintenance of neural tissues and strongly suggest that *cpp-1* may be downstream of *coe*.

**DISCUSSION**

Although it has been demonstrated that planarians possess pluripotent stem cells (Baguña et al., 1989; Wagner et al., 2011; Guedelhoefer and Sánchez Alvarado, 2012), several studies support the hypothesis that the stem cell population is heterogeneous (Elliott and Sánchez Alvarado, 2013; Reddien, 2013; Rink, 2013). Analyses of the planarian photoreceptor, excretory and serotoninergic cells have shown that tissue-specific transcription factors are detected in the stem cells in intact (Lapan and Reddien, 2012) and regenerating tissues (Lapan and Reddien, 2011; Scimone et al., 2011; Currie and Pearson, 2013;); these studies have identified the first sets of precursor cells in planarians outside of the germ cells (Newmark et al., 2008) and have generated a working model in which planarians possess diverse lineage-committed progenitors that contribute to the maintenance and regeneration of tissues (Reddien, 2013; Rink, 2013). In contrast to the well-defined excretory system and photoreceptors, the nervous system represents a formidable challenge. At the molecular level, there are potentially dozens of neuronal subtypes (Cebría, 2007; Collins et al., 2010; Gentile et al., 2011; Umesono et al., 2011), and it is largely unknown whether the generation of neural diversity is solely dependent on the pluripotent stem cells or lineage-restricted progenitors. In our study, we investigated this question by analyzing the bHLH gene family. By combining *in situ* hybridization analyses and RNAi studies, we identified nine bHLH genes expressed in specific neural and stem cell subpopulations that were required for regeneration (Fig. 6A), which strongly suggested that these phenotypes could be due to abnormal neural differentiation and/or function.

**Identification of neuronal progenitor cells in planarians**

Owing to the mRNA expression in stem cells and neurons, we focused our analyses on *coe*, *hesl-3* and *sim*, which are known to serve major roles in neurogenesis in both vertebrate and invertebrate organisms (Dubois and Vincent, 2001; Kewley et al., 2004; Kageyama et al., 2008). As we expected, using BrdU, we observed *coe*, *hesl-3* and *sim* expression in cycling stem cells located in the mesenchyme of intact animals. Over the course of 48 hours, we observed an increase in the proportion of BrdU+ cells that expressed *coe* and *sim* and detected many of these cells in the cephalic ganglia. We hypothesize that the observed increase in the proportion of BrdU+/*coe* and BrdU+/*sim* cells over time is from both progenitors that maintain expression of *coe* or *sim* as they divide and begin to differentiate and additional cells that turn on expression of these genes during differentiation. Additionally, the increase in the proportion of double-labeled cells could also be accounted for by a contribution of newly generated progenitor cells. Together with the observation that the number of *coe*+ and *sim*+ cells increases following induction of ectopic neurogenesis and the requirement of *coe* and *sim* during CNS regeneration (RNAi studies discussed below), our data suggest that a subset of *coe*- and *sim*-expressing cells represent multipotent neural progenitors (Fig. 6B). We propose that these *coe*+ and *sim*+ progenitors migrate and terminally differentiate in the CNS. By contrast, we did not observe an increase.

**Table 1. Summary of phenotypes observed following RNAi of bHLH homologs in *S. mediterranea***

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Phenotype</th>
<th>Developmental role</th>
</tr>
</thead>
<tbody>
<tr>
<td>aryl hydrocarbon receptor</td>
<td>arh</td>
<td>Reduced brain neuropil density (14/20)</td>
<td>B-cell and nervous system differentiation</td>
</tr>
<tr>
<td>aryl hydrocarbon receptor nuclear</td>
<td>amt</td>
<td>Delayed regeneration and reduced brain neuropil density (12/40)</td>
<td>Differentiation of multiple cell types</td>
</tr>
<tr>
<td>translocator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>atonal homolog 8-1</td>
<td>atoh8-1</td>
<td>Delayed regeneration and smaller cg (19/35)</td>
<td>Nervous system differentiation</td>
</tr>
<tr>
<td>collariofactory-1/early B-cell</td>
<td>coe</td>
<td>Abnormal pr morphology, flattened morphology, and failure of cg to reconnect (94/114)</td>
<td>B-cell, muscle and nervous system differentiation</td>
</tr>
<tr>
<td>factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>daughterless</td>
<td>da</td>
<td>Ruffled body margin edges and reduced cg neuropil (70/70)</td>
<td>Neurogenesis, oogenesis and sex determination</td>
</tr>
<tr>
<td>hairy and enhancer of split like-3</td>
<td>hesl-3</td>
<td>Abnormal pr morphology; single or third pr (20/35)</td>
<td>Negative regulation of Notch signaling</td>
</tr>
<tr>
<td>max-interactor-1</td>
<td>mxi-1</td>
<td>Abnormal pr morphology (13/40), expanded and disorganized cg (20/40)</td>
<td>Negative regulation of cell proliferation</td>
</tr>
<tr>
<td>microphthalmia-associated</td>
<td>mifl-1</td>
<td>Lyseis (8/40), smaller and disorganized cg (12/40)</td>
<td>Osteoclast differentiation</td>
</tr>
<tr>
<td>transcription factor like-1</td>
<td>max</td>
<td>Lighter pr and smaller cg (15/20)</td>
<td>Negative regulation of gene expression</td>
</tr>
<tr>
<td>myc associated factor X</td>
<td>max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myogenic differentiation</td>
<td>myoD</td>
<td>Failure to regenerate (8/30), abnormal pr morphology; cyclops or bowtie-shaped pr pair (14/30)</td>
<td>Mesoderm specification</td>
</tr>
<tr>
<td>single-minded</td>
<td>sim</td>
<td>Reduced pr pigmentation (17/65) and cg neuropil (30/65)</td>
<td>Axon guidance, nervous system differentiation</td>
</tr>
</tbody>
</table>

The number of animals showing the phenotype(s) among the total number examined is indicated in parentheses. cg, cephalic ganglia; pr, photoreceptor.
in the proportion of BrdU+/hesl-3+ cells near the brain; it is possible that hesl-3 expression is downregulated during cell-fate specification and that this gene may be regulating progenitor maintenance or the timing of neural stem cell differentiation, scenarios that are consistent with known roles of HES genes (Hatakeyama et al., 2004; Kageyama et al., 2008). Although some coe+ and sim+ cells were observed in the posterior end of the animal, neural progenitors were most prevalent in the area anterior to the pharynx and posterior to the base of the brain, the location where eye progenitors (ovo+/smedwi-1+ cells) were also detected (Lapan and Reddien, 2012). Interestingly, our observation that the proportion of BrdU+/coe+ and BrdU+/sim+ cells located in the pre-pharyngeal area increased over time suggests that this area may represent a ‘neurogenic zone’ in planarians. Our data support a model in which

---

**Fig. 5. coe, hesl-3 and sim are required for CNS regeneration.** (A–D) Images of live or immunostained RNAi-treated animals after 10 days of regeneration. The yellow arrowhead in B marks a commissure defect, and white arrowheads in C and D mark the abnormal brain morphology and a dramatic reduction of neuropil density observed in hesl-3 and sim RNAi planarians, respectively. (E–H) Seven days following amputation, RNAi animals were processed for FISH to ChAT, cpp-1, npp-4 or npy-2 (n=20). Arrowheads in F denote aberrant location of npp-4+ and npy-2+ neurons (the latter were counterstained with DAPI to visualize the brain). (I–L) Quantification of neurons shown in E–H; the total number of cells counted are indicated within each bar. (M–O) After 24 days of RNAi treatment uninjured control and coe(RNAi) animals were imaged live (M) or processed for FISH to pc2 or ChAT (N; n=10) or WISH to cpp-1 (O; n=15). (P) Quantification of cpp-1+ cells in O. (Q) FISH to cpp-1 and coe, and quantification of cpp-1+ cells that also expressed coe. Scale bars: 100 μm.
pluripotent stem cells (cNeoblasts) maintain lineage-committed progenitors, which generate most if not all of the cells required to meet normal physiological demands in uninjured planarians (Fig. 6C).

**bHLH genes with roles in planarian CNS regeneration**

Planarians possess members from all of the families of proneural factors, including homologs of acheate-scute, atonal, neuroD and da, all of which are primarily expressed in the stem cells. With the exception of atoh8-1 RNAi, which caused animals to regenerate smaller brains, we found that gene knockdown of most proneural homologs failed to cause overt regeneration defects, even after long-term or combinatorial RNAi. Nonetheless, we did find that co-silencing of ascl-1 or neuroD-1 together with ndk suppressed ectopic formation of npp-4' neurons. We surmise that knockdown of some bHLHs may cause subtle defects in neural specification, which are difficult to detect with the use of pan-neural markers. Future functional studies using discrete nervous system markers may reveal additional roles of bHLH genes in CNS differentiation.

On the bases of gene expression patterns and RNAi phenotypes, we further explored the function of coe, hesl-3 and sim. coe genes are conserved in metazoans and are known to play roles in neuronal specification, migration, axon guidance, dendritogenesis, neuronal subtype specification (Wightman et al., 1997; Dubois et al., 1998; Prasad et al., 1998; Garel et al., 2000; Pozzoli et al., 2001; Garcia-Dominguez et al., 2003; Hattori et al., 2007; Jinushi-Nakao et al., 2007; Crotzatier and Vincent, 2008; Demilly et al., 2011; Kratsios et al., 2012), and cellular reprogramming (Richard et al., 2011). In planarians, coe knockdown led to a failure of animals to connect the cephalic ganglia. Analysis of this defect using neural subtype markers showed that neurons were found in aberrant locations. In addition, long-term silencing caused animals to exhibit abnormal locomotion and decreases of cholinergic and pc2' neurons at the anterior commissure and brain cpp-1' neurons. In *C. elegans*, coe (unc-3) mutants exhibit behavioral abnormalities (Wightman et al., 1997), a defect that is caused by a loss of cholinergic motoneuron properties (Kratsios et al., 2012). Our data show that coe is playing a conserved role in neuronal differentiation during both CNS regeneration and maintenance. coe homologs in humans (EBF transcription factors) have been associated with cancers of the nervous system (Liao, 2009), yet the genetic targets of coe homologs have not been fully characterized. Thus, further investigation of coe function in planarians may reveal mechanisms regulating neural progenitor populations.

**hes genes** are a primary target of Notch signaling and defects in hes genes cause premature neural differentiation and depletion of the neural progenitor pool in mice (Ishibashi et al., 1995; Kageyama et al., 2008). In planarians, hesl-3 knockout led animals to regenerate mispatterned brains and a reduction of cpp-1', npp-4' and npp-2' brain neurons. These data suggest hesl-3 plays a role in neural fate regulation during CNS repair. At present, the role of Notch signaling in planarians has not been extensively characterized. Thus, analysis of hesl genes in stem cell regulation should be a focus of future investigations.

Finally, in flies and crustaceans, **sim** functions as a master regulator of midline cells by regulating the specification of midline progenitors (Nambu et al., 1991; Vargas-Vila et al., 2010), whereas in vertebrates, sim controls the differentiation (Michaud et al., 1998; Eaton and Glasgow, 2006) and migration (via plexinC1) (Xu and Fan, 2007) of certain neuroendocrine lineages. sim does not appear to function as a master regulator of the midline in planarians. However, sim RNAi caused animals to regenerate smaller brains.
with fewer *npp-4* and *npy-2* neurons, suggesting a potential role in specification and/or guidance of cells from the neuroendocrine lineage. To further explore this possibility, future experiments should test the effects of *sim* knockdown on the fate of all neuropeptide-expressing neurons (Collins et al., 2010) or the expression of guidance molecules, such as plexin homologs.

**Conclusions**

Our work has revealed that planarians possess lineage-committed progenitors that contribute to the maintenance and regeneration of the CNS. We also identified nine bHLH genes that regulate adult neurogenesis and are required for nervous system repair. This study sets the stage to use planarians as a model to elucidate roles of bHLH genes in adult pluripotent stem cell differentiation. Furthermore, by extending our analysis of bHLH factors genome-wide, this study will serve as a resource for future investigation into bHLH evolution and function.

**MATERIALS AND METHODS**

**Animals**

*Asexual Schmidtea mediterranea* (CIW4) were maintained as previously described (Cebrià and Newmark, 2005). Animals 2-5 mm in length that were starved for 1 week were used for all experiments.

**bHLH identification, phylogenetic analysis and cloning**

To identify planarian bHLH genes, TBLASTN searches were performed against the *S. mediterranea* genome (Robb et al., 2008) and several transcriptomes (Zayas et al., 2005; Adamidi et al., 2011; Labbé et al., 2012; Önal et al., 2012) using bHLH protein sequences from human, mouse and fly. Putative planarian bHLH homologs were validated by performing reciprocal BLASTX against the nr database (NCBI). The bHLH superfamily consists of six monophyletic groups (Groups A-F), which are also characterized by the presence or absence of various additional protein domains (Simionato et al., 2007). Due to the large number of putative paralogs in Groups A and B, the predicted protein sequences were aligned using T-Coffee (Notredame et al., 2000) and subjected to Bayesian analyses as described previously (Currie and Pearson, 2013; Zhu and Pearson, 2013); Group C-F genes were categorized based on clear top BLASTP hits against the Swiss-Prot database (UniProt) and the presence of class-specific protein domains (supplementary material Table S1). bHLH sequences were obtained from a cDNA collection (Zayas et al., 2005) or cloned from cDNA into domains (supplementary material Table S1). bHLH sequences were deposited as described previously (Currie and Pearson, 2013; Zhu and Pearson, 2013); paralogs in Groups A and B, the predicted protein sequences were aligned using T-Coffee (Notredame et al., 2000) and subjected to Bayesian analyses as described previously (Currie and Pearson, 2013; Zhu and Pearson, 2013); Group C-F genes were categorized based on clear top BLASTP hits against the Swiss-Prot database (UniProt) and the presence of class-specific protein domains (supplementary material Table S1). bHLH sequences were obtained from a cDNA collection (Zayas et al., 2005) or cloned from cDNA into domains (supplementary material Table S1). bHLH sequences were deposited in GenBank. The primers used and GenBank accession numbers are listed in supplementary material Table S2.

**In situ hybridization**

Riboprobes were synthesized and animals were processed for *in situ* hybridization as previously described (Pearson et al., 2009). For γ-irradiation treatments, animals were exposed to 100 Gy in a JL Shepherd Mark I Cesium-137 irradiator and fixed 6 days after treatment. To visualize bHLH transcripts by multiple fluorescent *in situ* hybridization (FISH), we used horseradish peroxidase substrates as described previously (Pearson et al., 2009) or the alkaline phosphatase (AP) substrate Fast Blue (Lauter et al., 2011; Currie and Pearson, 2013). For Fast Blue staining, animals were developed in 0.25 mg/ml Fast Blue BB (Sigma F3378) and NAMP (Sigma 855) in AP staining buffer (0.1 M Tris-HCl pH 8.2, containing 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20) (Hauptmann, 2001; Lauter et al., 2011).

**BrdU staining**

Experiments were conducted by soaking animals in BrdU for 1 hour as previously described (Cowles et al., 2012), chasring for 4, 24 or 48 hours before fixation and processing for FISH, and then processing for BrdU labeling starting with the HCl treatment.

**RNA interference**

For regeneration studies, we administered six feedings of bacterially expressed dsRNA over 3 weeks as previously described (Gurley et al., 2008). *gfp* was used as a control for all experiments. 24 hours following the final RNAi treatment, animals were amputated anterior to the pharynx, observed for 10 days and then processed for *in situ* hybridization or immunostaining. For long-term experiments, animals were fed 12 times over 6 weeks before amputation; uninjured animals were fixed 1 week after the final feeding. Relative gene expression after RNAi was determined by real-time quantitative PCR as described previously (Hubert et al., 2013); primers are listed in supplementary material Table S2.

**Immunohistochemistry**

Immunostaining with anti-SYNAPSIN (1:400, 3C10, Developmental Studies Hybridoma Bank) and anti-phosphohistone-H3 (S10) (1:1000, D2C8, Cell Signaling) were performed as previously described (Cowles et al., 2012). Images were acquired using a Leica DFC450 camera mounted on a Leica M205 stereomicroscope. Animals labeled with fluorescent probes were imaged with an Axioimc MRm camera mounted on a Zeiss SteREO Lumar V12 or Axio Observer.Z1 equipped with an ApoTome, or a Hamamatsu ImagEM C9100-13 camera mounted on an Olympus IX81 microscope equipped with a Yokogawa CSU X1 spinning-disk confocal scan head.

**Cell counting**

Ten 1-μm optical sections were captured from selected regions and merged, and cells were hand-counted using ImageJ 1.43u software. The proportions of cells co-expressing specific neurotransmitters or *smedwi-1* and *coes*, *hsl-3* or *sim* were calculated from >100 cells counted from three to five animals. The proportion of *BrdU*+ cells co-expressing specific genes was calculated from >300 *BrdU*+ cells counted from three to five animals. For analysis of *ndk* RNAi animals, *coes* and *sim*+ cells were counted and normalized per mm3; ectopic *npp-4*+ cells were counted from the posterior end of the brain to the posterior boundary of the pharynx and normalized to the length of the animal. Mean and s.d. values were computed and statistical comparisons were performed using an unpaired Student’s *t*-test. Error bars in graphs are s.d.

**Acknowledgements**

We thank Jordana Henderson, Amy Hubert and Kelly Ross for helpful comments on the manuscript, Kayla Muth for assistance with RNAi experiments, Claire Cowles for artwork design, and the anonymous reviewers whose constructive criticisms improved this work.

**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

M.W.C. and R.M.Z. designed and interpreted the experiments and wrote the manuscript. M.W.C., D.D.R.B., S.V.N. and B.N.S. conducted the experiments and analyzed the data. B.J.P. performed phylogenetic analysis. M.W.C., B.J.P. and R.M.Z. discussed the results and edited the final version of the manuscript.

**Funding**

M.W.C. acknowledges support from the San Diego Chapter of the Achievement Rewards for College Scientists Foundation and the Inamori Foundation. D.D.R.B. was supported by a Canadian Institutes of Health Research Student Fellowship [GSD-121763]. This research was supported by a Natural Sciences and Engineering Research Council of Canada grant [402264-11] and Ontario Institute for Cancer Research New Investigator Award [IA-026] to B.J.P. and a California Institute for Regenerative Medicine grant [RNZ-00940-1] to R.M.Z.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi:10.1242/dev.098616/-/DC1

**References**


