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Genetic regulators of a pluripotent adult stem cell system in planarians identified by RNAi and clonal analysis

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Summary

Pluripotency is a central, well-studied feature of embryonic development, but the role of pluripotent cell regulation in somatic tissue regeneration remains poorly understood. In planarians, regeneration of entire animals from tissue fragments is promoted by the activity of adult pluripotent stem cells (cNeoblasts). We utilized transcriptional profiling to identify planarian genes expressed in adult proliferating, regenerative cells (neoblasts). We also developed quantitative clonal analysis methods for expansion and differentiation of cNeoblast descendants that, together with RNAi, revealed gene roles in stem cell biology. Genes encoding two zinc finger proteins, Vasa, a LIM domain protein, Sox and Jun-like transcription factors, two candidate RNA-binding proteins, a Setd8-like protein, and PRC2 (Polycomb) were required for proliferative expansion and/or differentiation of cNeoblast-derived clones. These findings suggest that planarian stem cells utilize molecular mechanisms found in germ cells and other pluripotent cell types, and identify novel genetic regulators of the planarian stem cell system.

Introduction

Pluripotent cells, such as embryonic stem (ES) cells and induced pluripotent (iPS) cells provide attractive opportunities for regenerative medicine. These cells can be propagated long term in cell culture, yet retain the ability to differentiate into all specialized cell types of the body. The possibility of using pluripotent cells to regenerate adult somatic tissues *in vivo*, however, remains a daunting challenge: successful regeneration must balance precise regulation of cell proliferation, lineage commitment, migration, and differentiation, all while avoiding tumor formation.

Model organisms will likely play a crucial role in uncovering important general principles and gene networks that can regulate pluripotency for tissue regeneration. Planarians, an emerging model system for molecular studies of regeneration, are free-living freshwater flatworms that can regenerate any body part – including the head – in about a week (Reddien and Sánchez Alvarado, 2004). Planarians can be reared in large numbers in the laboratory and a recently sequenced genome (Robb et al., 2007), robust histological methods (Pearson et al., 2009), molecular tools (Newmark and Sánchez Alvarado, 2000), and RNAi (Reddien et al., 2005a; Sánchez Alvarado and Newmark, 1999) currently facilitate the identification of genes controlling regeneration. Planarians are triploblastic (possessing

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derivatives of all three germ layers) and contain a population of adult proliferative somatic cells (neoblasts) that have similar morphology (Reddien and Sánchez Alvarado, 2004), can be identified by common expression of PIWI-encoding genes (Reddien et al., 2005b), and have RNA-rich subcellular bodies known as chromatoid bodies (Higuchi et al., 2007). Recently, the neoblast population was demonstrated to contain clonogenic cells (cNeoblasts) that give rise to descendants spanning multiple germ layers, can restore tissue turnover and regenerative ability to irradiated animals, and can even generate entire adult bodies in single-cell transplantation experiments (Wagner et al., 2011). Genetic investigation of planarian stem cell biology, therefore, provides an opportunity to discover gene networks promoting pluripotency, maintenance of pluripotency within adult tissues, and deployment of stem cells for the regeneration of missing cell types.

Here, we took a genome-level approach to profile and functionally characterize a large set of intrinsic regulators of planarian stem cell biology. Motivated by the clonal repopulation of proliferative cells observed in planarians following treatment with low irradiation doses (Wagner et al., 2011), we developed quantitative functional assays for stem cell-initiated clonal expansion and differentiation that permit rapid identification of RNAi phenotypes. Numerous candidate regulatory genes identified by expression analyses were thus assessed and functionally classified by RNAi and clonal analysis. Together, these studies establish a simple yet powerful framework for investigating planarian stem cell regulation, and reveal a large panel of genetic factors utilized by this highly regenerative stem cell system.

Results

Transcriptional Profiling the Proliferative Cell Compartment of Adult Planarians

To identify candidate genetic regulators of planarian stem cells, we performed genome-level microarray studies comparing the expression profiles of untreated animals to animals exposed to a lethal dose (6,000 rads) of γ-irradiation. In planarians, proliferative cells (Dubois, 1949; Reddien et al., 2005b) and their associated transcripts (Eisenhoffer et al., 2008; Rossi et al., 2007) are rapidly and specifically eliminated following lethal irradiation. We generated a comprehensive oligonucleotide array (representing approximately 37,936 sequences) from EST data and predicted genes present in the *Schmidtea mediterranea* genome (Robb et al., 2007; Sánchez Alvarado et al., 2002; Zayas et al., 2005). To specifically profile proliferating cells (neoblasts), rather than their post-mitotic progeny, we identified the first transcripts depleted within 6, 12, 24, and 48 hours after exposure to lethal (6,000 rad) irradiation. As expected, transcripts for *smedwi-1*, *PCNA*, *mcm2*, and *RRM2-1*, which are specifically expressed in proliferative cells (Eisenhoffer et al., 2008; Guo et al., 2006; Hayashi et al., 2010; Wagner et al., 2011), were rapidly depleted within 24 hours of irradiation (Fig. 1A). Transcripts marking post-mitotic cell types, NB.21.11E, NB.32.1G, *AGAT-1, MCP-1,* and *ODC-1* (Eisenhoffer et al., 2008), were not eliminated until 48 hours or later, and transcripts expressed in multiple differentiated cell types remained unaffected (Fig. 1A and Supplemental Fig. 1). We confirmed these trends by whole-mount triple fluorescent *in situ* hybridization (FISH) experiments with animals fixed 24 hours after irradiation (Fig. 1B).

The vast majority of transcripts examined (98.7%) did not show differential expression between untreated and 24 hour-irradiated animals. Of the 578 transcripts that showed differential expression, 90% were downregulated and 67% displayed sequence similarity (BLASTx, E value < 1×10^{-10}) to human genes (Fig. 1C and Supplemental Table 1). Gene Set Enrichment Analysis (Subramanian et al., 2005) revealed that Gene Ontology (GO) terms associated with cell proliferation, e.g., DNA_Replication (GO:0006260) and Cell_Cycle_Process (GO:022402), were enriched within the 24-hour dataset (Fig. 1D; adj. p value < 0.001, and p = 0.0107, respectively). Chromatin_Binding (GO:0003682), RNA-

Binding (GO:0003723) and DNA-Binding (GO:0003677) terms were also enriched (Fig. 1D and Supplemental Table 2), suggesting that genes regulating pluripotency and stem cell maintenance might also be contained within this dataset.

Expression of Candidate Regulatory Genes in Proliferative Cells

We determined the patterns and irradiation sensitivity of expression for identified candidate regulatory genes by whole-mount *in situ* hybridization. We prioritized for analysis genes predicted to encode DNA-, RNA-, or protein-binding domains (see Supplemental Table 3). Proliferative cells (e.g., *smedwi-1*$^{+}$ cells) are irradiation-sensitive, absent from head tips, and distributed throughout the body mesenchyme (Reddien et al., 2005b). We identified 28 genes expressed in irradiation-sensitive patterns similar to the distribution of *smedwi-1*$^{+}$ cells (Fig. 2A). Several of these genes are predicted to encode chromatin-modifying protein domains, including *Smed-mrg-1* (MORF-related), *Smed-nsd-1* (NSD1-like SET domain), *Smed-rbbp4-1* (Retinoblastoma binding protein 4), and *Smed-setd8-1* (SET domain containing 8) (Supplemental Table 3). In addition, three members of Polycomb repressive complex 2 (PRC2) were also identified: *Smed-ezh* (Enhancer of Zeste), *Smed-sz12-1* (Suppressor of Zeste), and *Smed-eed-1* (Embryonic Ectoderm Development), all well-established regulators of cell fate and stem cell activity (Boyer et al., 2006; Ezhkova et al., 2011; Lee et al., 2006; Ringrose and Paro, 2004).

Consistent with the well-known expression of germ cell-associated factors in planarian mitotic cells (Guo et al., 2006; Reddien et al., 2005b; Salvetti et al., 2005; Shibata et al., 2010; Shibata et al., 1999; Solana et al., 2009) we also identified four genes with sequence similarity to RNA-binding proteins. Two of these genes, *Smed-vasa-1* and *Smed-vasa-2*, encode DEAD-Box 4 helicase domains and are similar to the germline-associated helicase, Vasa. Neoblast expression of Vasa-like PL10 genes (DjvlgA and DjvlgB) and *DjVas-1*, a *vasa* ortholog, have been described in a related planarian species, *Dugesia japonica* (Mochizuki et al., 2001; Rouhana et al., 2010; Shibata et al., 1999). *DjVas-1* is required for regeneration in *D. japonica*, but the cellular basis for this phenotype is unclear (Rouhana et al., 2010). We also identified *Smed-khd-1* (a gene encoding a KH-domain protein) and *Smed-cip29* (similar to Cytokine-Induced Protein 29 kDa) genes, which both encode proteins that might function in RNA metabolism (Sugiura et al., 2007; Valverde et al., 2008; Wang et al., 2002; Yamazaki et al., 2010). Similar expression patterns for a *cip29*-like gene have also been reported for *Dugesia japonica* (Rossi et al., 2007).

To date, the potential role of transcription factors and signaling proteins in planarian stem cell regulation has only received minor attention. We identified seven genes encoding predicted transcription factors expressed in proliferative cells: *Smed-prox-1* (Prospero Homeobox 1), *Smed-tcf15* (Transcription Factor 15), *Smed-soxP-1*, *Smed-soxP-2*, and *Smed-soxP-3* (three genes encoding SRY [sex-determining region Y] box containing proteins), *Smed-junl-1* (*c-jun*-like), and *Smed-egr-1* (Early Growth Response protein), as well as four genes predicted to encode zinc finger domain-containing proteins, *Smed-zfmym-1*, *Smed-zf207-1*, *Smed-fhl-1* (a Four and a Half LIM Domain protein), and *Smed-zfp-1.* Finally, four neoblast-expressed genes encode proteins with similarity to signal transduction proteins: *Smed-nlk-1* (Nemo-like kinase), *Smed-armc1* (Armadillo repeat-containing 1), as well as *Smed-fgfr-1* and *Smed-fgfr-4* (Fibroblast growth factor receptors). Expression of FGFR-like genes in proliferative cells has been reported in *Dugesia japonica* (Ogawa et al., 2002), but no functional roles for these genes in planarian stem cells are described.

Several genes identified by the microarray studies described above were expressed in *smedwi-1*-like, irradiation-sensitive patterns, but appeared enriched in specific subpopulations of cells. Some genes (e.g. *prox-1*, *tcf15*, *armc1*, *fgfr-1*) were expressed at

high levels in irradiation-sensitive cells near the midline of the animal, whereas others (e.g. *egr-1*, *soxP-3*) were found in patterns that extended to the periphery of the body and/or regions slightly anterior to the photoreceptors, similar to previously described post-mitotic cell populations (Fig. 2A) (Eisenhoffer et al., 2008). In addition, expression of many genes was also detected in the pharynx, and/or in the central nervous system (Fig. 2A). Of the 22 transcripts detectable by FISH, all displayed overlapping expression with *smedwi-1* (Fig. 2B and Supplemental Fig. 2), confirming expression within dividing cells. Together, these data significantly extend the list of genes with identified expression in the neoblasts of planarians.

Identification of Genes Required for Recovery from Sublethal Irradiation

To investigate the functions of identified candidate regulatory genes, we first developed a sensitized assay capable of screening for weak (or strong) phenotypic defects in stem cell activity. Animals exposed to a high irradiation dose (6,000 rads) experience complete failure of tissue turnover (Bardeen and Baetjer, 1904; Dubois, 1949) and display stereotypical signs of stem cell loss, including tissue regression, ventral curling, and ultimate lysis (Reddien et al., 2005b). By contrast, delivery of sublethal irradiation doses (e.g. 1,000 – 1,250 rads) initially reduces dividing cell numbers, but such animals can ultimately restore normal proliferative cell levels (Salvetti et al., 2009) and regenerative ability (Wagner et al., 2011). For example, *smedwi-1*$^+$ cell numbers were dramatically reduced seven days after exposure to 1,250 rads, but were restored to approximately normal levels over the next 14 days (Fig. 3A). Accordingly, under mock RNAi conditions, most animals exposed to 1,250 rads survived and remained healthy for over two months of observation (Fig. 3B–D). We reasoned that 1,250-rad irradiated animals would be highly sensitized to defects in stem cell function. In such a background, even subtle RNAi phenotypes might render animals unable to restore tissue turnover and manifest as a lethal (easily scored) phenotype.

Animals were fed dsRNA for the 28 identified gene candidates, exposed to 1,250 rads irradiation, and assessed for the ability to survive. RNAi of 16 genes yielded robust RNAi phenotypes, similar to the effects of lethal irradiation (i.e., head regression, curling, and lysis; Fig. 3E, Supplemental Fig. 3, Supplemental Table 4). *fgfr-1(RNAi)* animals exhibited head regression and curling, then recovered (head regeneration) (Fig. 3E and Supplemental Table 4), possibly explained if RNAi effects wore off. With three phenotypes (*khd-1, zfp-1*, *vasa-1*), lesions developed on the dorsal epidermis, suggesting a mode of tissue failure at least in part distinct from that of lethal irradiation (Fig. 3E and Supplemental Fig. 3A). Sublethal irradiation, therefore, provides a sensitive, readily scalable screening strategy for planarians that can facilitate identification of novel stem cell RNAi phenotypes.

Assessment of Stem Cell Regulation by Single-Colony Analysis

Failed recovery RNAi phenotypes observed following 1,250 rads irradiation (described above) could occur because of defects in a wide range of processes including stem cell self-renewal, lineage commitment, cell proliferation, DNA repair, migration, and/or differentiation. In order investigate identified RNAi phenotypes in greater cellular detail and to distinguish between these possibilities, we developed methods for quantitative analysis of individual planarian stem cell clones. High doses of irradiation (e.g., 6,000 rads) completely eliminate proliferating cells from planarian tissues. Much lower doses (e.g., 1,500–1,750 rads) similarly deplete the vast majority (>99%) of proliferating cells, but permit the survival of rare, isolated neoblasts. These remaining clonogenic neoblasts (“cNeoblasts”) divide to produce, from a single-cell starting point, clonally-derived clusters of cellular descendants (i.e., “colonies”) (Wagner et al., 2011). Between days 7 and 14 post-irradiation colonies exhibited robust (approximately 10-fold) and significant ($p < 0.01$, Student’s t-test) increase of *smedwi-1*$^+$ cell numbers (Fig. 4A–C). In addition to *smedwi-1*$^+$ neoblasts,

expanding colonies also produce post-mitotic differentiating cell types that can be labeled with RNA probes to the NB.21.11E and *AGAT-1* genes (Wagner et al., 2011). NB.21.11E$^+$ and *AGAT-1*$^+$ cells are mesenchymal cell types that are located subepidermally, adjacent to the body-wall musculature (Eisenhoffer et al., 2008). Because these cells have a short (2–4 day) lifespan, they are initially depleted in irradiated animals and are only later produced within expanding *smedwi-1*$^+$ cell colonies. Notably, colonies exhibited a linear relationship between numbers of dividing and differentiating cells (Fig. 4B–C). Single colony analysis can therefore be used to simultaneously and quantitatively assess multiple aspects of stem cell function. First, colony cell number can be readily quantified as a readout of neoblast "expansion" (i.e., self-renewal). Second, the ratio of proliferating to post-mitotic cell types can be used to assess the rate of differentiation within a single colony. Each of these processes (expansion and differentiation) can be measured independently within individual colonies under RNAi conditions, providing a powerful framework for deciphering phenotypes of candidate stem cell regulatory genes.

We first tested the effectiveness of colony analysis by studying genes with previously reported RNAi phenotypes. Animals were fed a single dose of dsRNA 4 days after exposure to 1,500 rads irradiation, and fixed on days 7 and 14. With this protocol, colonies initiate prior to dsRNA administration, and consequences of RNAi on these colonies are thereafter assessed. We devised criteria for several colony phenotype classes. "Colony loss" phenotypes, for example, were designated when RNAi caused complete disappearance of colony cells between days 7 and day 14 post-irradiation. "Failed expansion" phenotypes were classified as those in which both *smedwi-1*$^+$ and NB.21.11E$^+$ day-14 colony cell counts were significantly decreased (t-test, $p < 0.05$) relative to control RNAi colonies. "Failed differentiation" phenotypes were designated when post-mitotic to proliferative cell count ratios deviated significantly (ANCOVA, $p < 0.01$) from those of control RNAi colonies (see Materials and Methods). We first analyzed, as a control, the RNAi phenotype of *Smed-rpa-1*, a gene encoding a protein similar to human Replication Protein A1 (RPA1). RPA1 is required for DNA replication, and *Smed-rpa1* RNAi leads to decreased mitotic activity and tissue failure in adult planarians (Reddien et al., 2005a). Animals fed dsRNA for *Smed-rpa1* experienced complete disappearance of colony cells (i.e., "colony loss") between days 7 and 14 post-irradiation (Fig. 4D). Comparable results were obtained with additional cell cycle genes *Smed-rplp0* (similar to human Ribosomal Protein, large, P0), *Smed-cdc23* (similar to human CDC23), and *Smed-cyclinL1* (similar to human Cyclin L1) (Supplemental Fig. 4A–C), indicating that RNAi of genes required for cell division disrupts continued presence of colony cells, as expected. Furthermore, these results demonstrate that a single RNAi dose can be highly effective for triggering colony phenotypes.

We next analyzed the RNAi phenotype of *Smed-bruli*, a gene similar to the germline-associated RNA-binding protein, Bruno (Guo et al., 2006). *bruli* is expressed in dividing cells in adult planarians, and *bruli* RNAi leads to gradual neoblast loss, possibly reflecting a stem cell self-renewal defect (Guo et al., 2006). Such a hypothesis predicts that following *bruli* RNAi, *smedwi-1*$^+$ colonies should continue to produce differentiating cells, but fail to expand. Indeed, *bruli* RNAi resulted in colonies with a "failed expansion" colony phenotype in which both *smedwi-1*$^+$ cell (t-test, $p = 0.016$) and NB.21.11E$^+$ cell ($p = 0.0065$) numbers were significantly reduced by day 14, relative to controls, but possessed normal ratios of *smedwi-1*$^+$ to NB.21.11E$^+$ cells (Fig 4D and Supplemental Table 5). These results are consistent with a role for *bruli* in stem cell self-renewal.

smedwi-2 and *smedwi-3* encode two Piwi homologs that, like *bruli*, are expressed in dividing cells of adult planarians (Palakodeti et al., 2008; Reddien et al., 2005b). Previous studies demonstrated that RNAi of *smedwi-2* or *smedwi-3* leads to failure of regeneration and tissue homeostasis. Furthermore, RNAi of *smedwi-2* (and to a lesser extent *smedwi-3*)

ultimately depletes proliferative cells (Palakodeti et al., 2008; Reddien et al., 2005b). We observed a "colony loss" phenotype following *smedwi-2* RNAi (Fig. 4D). *smedwi-3* RNAi produced a similar effect, although some colonies, which had failed to expand, were still present on day 14 (Fig. 4D). Colony assays, therefore, demonstrate clear intrinsic defects in stem cell division/expansion for both *smedwi-2* and *smedwi-3(RNAi)* animals.

To assess stem cell differentiation, we examined *Smed-CHD4* and *Smed-p53*, two genes with previously described roles in the production of post-mitotic cell types (Pearson and Sánchez Alvarado, 2010; Scimone et al., 2010). As predicted, RNAi of *CHD4* and *p53* each resulted in "failed differentiation" colony phenotypes, with significant reductions ($p \leq 0.0107$) in NB.21.11E$^+$ and *AGAT-1*$^+$ cell numbers on day 14, and significant deviations in ratios of *smedwi-1*$^+$ to NB.21.11E$^+$ (or *AGAT-1*$^+$) cells (Fig. 4D, Supplemental Fig. 4D–E, Supplemental Table 5). In addition to differentiation defects, *CHD4(RNAi)* and *p53(RNAi)* planarians have been reported to ultimately experience exhaustion of proliferative cell activity. *p53* RNAi colonies also displayed significantly lower numbers of *smedwi-1*$^+$ cells ($p = 0.0018$), confirming a combined defect in both colony expansion and differentiation (Fig. 4D, Supplemental Table 5). By contrast, *CHD4(RNAi)* colonies displayed approximately normal numbers of *smedwi-1*$^+$ cells on day 14, indicating an initial persistence of colony expansion activity even while differentiation was diminished (Fig. 4D and Supplemental Table 5). Taken together, these RNAi experiments demonstrate the power of colony analysis for examination of stem cell phenotypes. In particular, *bruli* and *CHD4* RNAi phenotypes also indicate that expansion and differentiation of colony cells are distinct biological processes that can be independently assessed and phenotypically decoupled.

In analyses of any stem cell system, distinguishing primary defects associated with gene loss of function from indirect changes in stem cell behavior (e.g., as a consequence of tissues becoming defective) is a major challenge. Prior strategies for studying stem cell function in planarians often involve slow-unfolding phenotypes that can confound interpretation. The study of RNAi phenotypes by colony analysis in planarians resolves many of these issues because stem cell defects can be assessed before substantial tissue degeneration has occurred. This approach using planarians and cNeoblast colonies is sensitive, direct, and allows rapid determination of defects immediately following initial gene inhibition.

RNAi and Clonal Analysis Identify Regulatory Roles for Neoblast Genes

We next assessed the functions of genes associated with "failed recovery" RNAi phenotypes following sublethal irradiation (described above) using colony analysis. One gene, *setd8-1,* exhibited a "colony loss" phenotype (Fig. 4E), indicating that this gene is required for the persistence of cell division in growing colonies. Accordingly, human SETD8/PR-SET7 (an H4K20me1 lysine methyltransferase) is known to promote a silent chromatin state and is required for cell cycle progression (Nishioka et al., 2002; Oda et al., 2009).

Nine genes had "failed colony expansion" RNAi phenotypes (Fig. 4E and Supplemental Table 5). Included in this class were *zmym-1, khd-1, cip29, soxP-1, fhl-1*, and *junl-1*. Interestingly, RNAi of all three genes encoding components of the Polycomb PRC2 complex, *ezh*, *sz12-1*, and *eed-1*, also resulted in failed colony expansion phenotypes, indicating that Polycomb proteins might also play a key role in the regulation of planarian stem cell self-renewal.

We uncovered roles for two genes in the process of differentiation. Following RNAi of *Smed-zfp-1* or *Smed-vasa-1*, day 14 colonies displayed significantly distorted ratios ($p < 0.0001$) of proliferative to post-mitotic cells (Fig. 4E, Supplemental Fig. 4, and Supplemental Table 5). The *zfp-1* RNAi phenotype was particularly strong, with day 14 colonies completely devoid of both NB.21.11E$^+$ and *AGAT-1*$^+$ cells (Fig. 4E, Supplemental

Fig. 4F). In addition to defects in post-mitotic cell numbers, RNAi of *zfp-1* or *vasa-1* also led to reduced numbers of *smedwi-1*$^+$ cells in day 14 colonies (Fig. 4E, Supplemental Table 5). We utilized *zfp-1* to test colony formation with an independent method; following transplantation of *zfp-1* RNAi cells into irradiated non-RNAi hosts, similar colony abnormalities were observed (Supplemental Fig. 4H). *zfp-1* and *vasa-1* are similar to *p53* in that RNAi phenotypes include aspects of both failed colony expansion and failed differentiation. However, unlike *p53*, which is expressed predominantly in post-mitotic cells (Pearson and Sánchez Alvarado, 2010), both *vasa-1* and *zfp-1* are primarily expressed within the proliferative *smedwi-1*$^+$ population (Fig. 2A-B). Together, these data suggest that *vasa-1* and *zfp-1* function within proliferative cells, prior to significant expression of *p53,* to promote the process of differentiation. Furthermore, these data demonstrate that *vasa-1*, a gene similar to the germ cell-promoting VASA helicase, is integral to the process of somatic cell differentiation in planarian adults.

Genes with Colony RNAi Phenotypes are Required for Tissue Homeostasis and Regeneration

Because homeostasis and regeneration RNAi data can be predictors of colony phenotypes (Fig. 4D), we tested whether RNAi phenotypes initially identified by colony analyses would manifest in adult planarians in the absence of irradiation. *Smed-soxP-1* encodes SRY-box-containing (Sox) transcription factor (Supplemental Fig. 5) and displayed a failed expansion RNAi phenotype in colony assays. Long-term experiments in unirradiated animals revealed that *soxP-1(RNAi)* animals experience progressive loss of *smedwi-1*$^+$ cells, resulting in near complete depletion after 41 days of RNAi (Fig. 5A). Animals amputated after 40 days of *soxP-1* RNAi either lysed (9/20) or failed to regenerate (11/20) (Fig. 5B), and animals exposed to continuous *soxP-1* RNAi in the absence of injury developed epidermal lesions, experienced regression of head tissue (Fig. 5C), and all ultimately died (n > 30 animals/sample) (Fig. 5D). Together these data indicate *soxP-1* is necessary for long-term maintenance of adult stem cell activity in the absence of irradiation. Notably, SOXP-1 represents the first known transcription factor expressed broadly in the neoblast population and required for its maintenance.

We investigated additional genes displaying expansion RNAi phenotypes by long term RNAi timecourse experiments. Flow cytometry-based quantification of the X1 cell population (~90% of which express *smedwi-1*) is an established, reliable method for assessing the presence of proliferating cells (Hayashi et al., 2006; Palakodeti et al., 2008; Reddien et al., 2005b; Scimone et al., 2010). We predicted that similar to *soxP-1* and *bruli* (Guo et al., 2006), RNAi of *ezh*, *eed-1*, *sz12-1*, *fhl-1*, or *cip29* would lead to a loss of proliferating cells, but at a rate much slower than that of a gene required for cell division (e.g., *rpa1*). Indeed, the proportion of X1 cells present in planarian tissues gradually decreased over a 4–6 week RNAi timecourse for *ezh*, *eed-1*, *sz12-1*, and *cip29*, similar to the decline measured in *bruli* RNAi animals (Fig. 6A). Proliferative cells were depleted much more rapidly following *rpa1* RNAi (Fig. 6A). RNAi of *fhl-1* did not result in decline of cell proliferation (Fig. 6A), indicating that not all genes required for colony expansion will necessarily display defects during normal homeostasis. Loss of proliferative cell activity in *ezh*, *eed-1*, *sz12-1*, or *cip29* RNAi animals was correlated with reduced size of regenerative blastemas, and faint or absent photoreceptors when compared to control RNAi animals (Fig. 6B). These data indicate impairment, although not a complete elimination, of regenerative ability. These results confirm that *ezh*, *eed-1*, *sz12-1*, and *cip29* do indeed promote the long-term maintenance of proliferative cell activity and regenerative ability in adult planarians, even under normal homeostasis conditions.

We next tested whether *zfp-1* and *vasa-1*, two genes for which RNAi caused failed colony expansion and differentiation, might similarly be required for tissue maintenance in non-

irradiated animals. For both *zfp-1* and *vasa-1(RNAi)* animals, successful head regeneration was observed 7 days after amputation, although regenerated tissues subsequently regressed (Supplemental Fig. 6). 21 days of continuous RNAi treatment resulted in dramatic reductions in the numbers of *smedwi-1*$^+$, NB.21.11E$^+$, and *AGAT-1*$^+$ cells in head regions of some *vasa-1(RNAi)* animals, as well as dramatic reductions in NB.21.11E$^+$ and *AGAT-1*$^+$ cell numbers in all *zfp-1(RNAi)* animals examined (Fig. 6C). Similar to phenotypes observed following 1,250 rads irradiation, lesions appeared on the dorsal surface of *vasa-1(RNAi)* animals, and *zfp-1(RNAi)* animals experienced tissue regression and ventral curling (Fig. 6D). These signs of tissue failure were accompanied by complete lethality within 30 days (Fig. 6E). Together, these data indicate that failures of colony expansion and differentiation observed following *zfp-1* or *vasa-1* RNAi reflect a general requirement of these genes in stem cell-mediated maintenance of adult tissues.

Discussion

Planarians Provide an Ideal *in vivo* System for Gene-Function Studies of Somatic Pluripotency

Investigations into the molecular basis of pluripotency have long focused on germ cell regulation and embryonic cells in culture. Genetic screens in *D. melanogaster* and *C. elegans*, for example, have identified regulatory factors important for germline development and maintenance, and early embryonic fate decisions (Rongo and Lehmann, 1996; Seydoux and Braun, 2006). Recent efforts investigating regulation of embryonic stem (ES) cells and induced pluripotent (iPS) cells additionally provide a window into the molecular underpinnings of early events in mammalian development (Young, 2011).

Increasingly, it has become evident that several genes associated with germ cell biology are also expressed in multipotent, adult, somatic cell types across disparate animal phyla including cnidarians, sponges, lophotrochozoans, and echinoderms (Juliano et al., 2010). These organisms provide a novel physiological context – adult tissue maintenance and regeneration – and useful evolutionary positions for studying the pluripotent state. Planarians, with the availability of robust culturing methods, a sequenced genome, molecular tools, and RNAi, are well suited for this task. Planarians possess remarkable regenerative abilities and are also now known to contain pluripotent stem cells in the adult animal (Wagner et al., 2011). The neoblast population therefore presents an attractive, experimentally accessible system for gene function studies of somatic pluripotency.

Sublethal Irradiation and Clonal Analysis Enable Rapid Elucidation of Stem Cell Phenotypes

Limitations of currently available methods prompted us to develop novel functional assays for studying stem cell regulation in planarians. One of the most striking properties of stem cells is the ability to reconstitute and restore tissues of an irradiated host animal (Osawa et al., 1996; Spangrude et al., 1988; Till and McCulloch, 1961). Such a capacity for cNeoblasts has been recently shown by single-cell transplantation into lethally irradiated hosts (Wagner et al., 2011), and by sublethal irradiation experiments (Salvetti et al., 2009; Wagner et al., 2011). Here we demonstrated the feasibility of using sublethal irradiation to screen for “failed recovery” phenotypes as a first step in identifying genes regulating planarian stem cell activity. Additionally, we determined that individual stem cell colonies undergo a stereotyped process of expansion and post-mitotic cell production. Single colony analysis can therefore be used to quantitatively assess these features under RNAi conditions.

Phenotypes described by colony assays can mirror those elucidated during homeostasis and regeneration (and vice versa). For example, we assessed the requirements of planarian

CHD4 and *p53* homologs in colonies. RNAi of either *CHD4* or *p53* was previously described to affect the numbers of differentiating neoblast-descendent cells (Pearson and Sánchez Alvarado, 2010; Scimone et al., 2010), and RNAi of either gene caused a robust defect in colony differentiation. cNeoblast colonies provide several key advantages over previously available functional assays in planarians: they are quantitative in nature, they tend to generate rapid phenotypes (prior to significant somatic tissue damage which can confound phenotype interpretation), they involve a synchronized starting point, and they assess gene function under conditions of rapid symmetric stem cell expansion, a process which might only occur intermittently during normal homeostasis. Together, these methods represent a significant step forward in the ability to study stem cell phenotypes in planarians. Using clonal analysis and RNAi, we categorized several known and novel genetic factors with “failed differentiation” or “failed expansion” phenotypes in planarians (Fig. 7).

Regulation of Neoblasts Involves Molecular Features Shared with other Stem Cell Types

We identified three planarian genes *Smed-ezh, Smed-sz12-1*, and *Smed-eed-1* (encoding predicted proteins similar to Polycomb Repressive Complex 2 (PRC2) components) that are expressed in proliferative cells and required for colony expansion and long-term maintenance of proliferative cell activity. In *C. elegans*, Mes proteins including MES-2 (a homologue of *Drosophila* PRC2 protein, Enhancer of Zeste) are required for X-chromosome dosage compensation and are essential for germ cell development (Fong et al., 2002; Garvin et al., 1998). Polycomb-mediated regulation of pluripotency, furthermore, is found in mammalian embryonic stem cells in which PRC2 complex components bind and deposit repressive H3K27me3 histone marks at promoters of developmental regulators (Boyer et al., 2006; Lee et al., 2006). In mouse ES cells, PRC2 inhibition leads to upregulation of genes normally expressed during differentiation, though these ES cells can still propagate *in vitro* and remain pluripotent (Boyer et al., 2006; Chamberlain et al., 2008). Similarly, loss of PRC2 in adult cells such as hair follicles of the epidermis and skeletal muscle satellite cells leads to transcriptional derepression and stem cell dysfunction (Ezhkova et al., 2011; Juan et al., 2011). In these *in vivo* contexts, however, stem cell failure is associated with loss of proliferative activity (Ezhkova et al., 2011; Juan et al., 2011). In planarians, loss of proliferative cells following RNAi of PRC2 genes therefore resembles *in vivo* phenotypes observed for adult stem cells. Future studies of PRC2 function in planarians will therefore provide an important *in vivo* context for further investigating the role of this complex in pluripotent cells.

We also identified a requirement for *Smed-soxP-1*, a gene encoding a Sox transcription factor, in the process of colony expansion, however we could not classify the Sox subfamily to which SOXP-1 belongs. The discovery of *soxP-1* is significant because this encodes the first known transcription factor expressed broadly in neoblasts and required for their maintenance. Sox transcription factors regulate cell fate in a wide range of contexts from sex determination (Sinclair et al., 1990) to pluripotency (Avilion et al., 2003; Boyer et al., 2005). It will be interesting to compare the functions of SOXP-1 and to those of Sox proteins in other organisms, such as Sox2 in ES cells.

Polycomb and Sox proteins are not exclusively utilized in pluripotent cells, and are routinely deployed in other physiological contexts (e.g., adult stem cell regulation (Ezhkova et al., 2011; Juan et al., 2011) and tissue patterning (Denell, 1978)). By contrast, expression and function of conserved RNA-binding germline-associated genes (e.g., *vasa*, *bruno*, *piwi*) might be more restricted to instances of pluripotent cell activity (Juliano et al., 2010). Cancer cells, which share many common features with pluripotent cells, do in some cases express germline-associated genes and/or require them for tumorigenesis (Janic et al., 2010; Simpson et al., 2005). In planarian adults, homologs of germline regulators (e.g., *bruli*, *smedwi-2*, *smedwi-3, DjPum*, and *Spoltud-1*) are expressed within proliferating cells and

required for their maintenance (Guo et al., 2006; Palakodeti et al., 2008; Reddien et al., 2005b; Salvetti et al., 2005; Solana et al., 2009). Here, we determined that *smedwi-2*, *smedwi-3*, and *bruli* are all required for proliferation and/or expansion of cNeoblast descendants. We also defined a functional role for *Smed-vasa-1,* a gene highly similar to *Drosophila* and human Vasa proteins, in planarian stem cell regulation. *Drosophila* Vasa contains a DEAD-box RNA helicase domain (Lasko and Ashburner, 1988), and is required for accumulation and proper translational regulation of germ cell mRNAs, oocyte patterning, and differentiation (Styhler et al., 1998; Tomancak et al., 1998). Similarly, we demonstrated that *Smed-vasa-1* is required for proper differentiation and expansion of somatic stem cell colonies, and for maintenance of tissue integrity in unirradiated animals. Interestingly, the mode of tissue failure in *Smed-vasa-1* RNAi animals (lesions) is reminiscent of other phenotypes related to loss of differentiation activity (e.g., *p53* or *zfp-1* RNAi animals). These findings, in particular those for *Smed-vasa-1*, support the idea that pluripotent stem cells and germ cells can share common molecular features.

Novel Genetic Regulators of Pluripotent Stem Cell Activity

In addition to the genes we characterized with previously studied functions in other organisms, we also identified phenotypes for a number of genes with understudied roles in stem cell biology (Fig 7). For example, we identified novel functional roles in stem cell regulation for three genes encoding zinc finger proteins (*zmym-1, fhl-1,* and *zfp-1*), one additional transcription factor (*junl-1*), and two genes encoding putative RNA-binding proteins (*khd-1* and *cip29*) (Sugiura et al., 2007; Valverde et al., 2008). Given the extensive role of RNA-binding proteins in germ cell and planarian stem cell biology, further study of *khd-1* and *cip29* might reveal additional molecular characteristics of pluripotency and the undifferentiated state. Our expression analyses also identified additional candidate regulatory genes expressed in neoblasts, including those encoding additional transcription factors (*soxP-2, soxP-3*, *prox-1, tcf15,* and *egr-1*), which were not associated with detected RNAi phenotypes. Furthermore, RNAi of some genes (*rtel1*, *fgfr-4*, *znf207-1,* and *mrg-1*) caused tissue maintenance defects after sublethal irradiation but did not cause detected defects in colony assays. Future in depth studies of identified phenotypes and exploration of potential redundancy with those genes without phenotypes are likely to reveal additional important regulatory aspects of the planarian stem cell system. An additional interesting future direction will involve exploring the expression of genes identified here and any candidate heterogeneity that might exist in the neoblasts. Given that a sequenced genome and now suitable functional assays provide an avenue for large-scale unbiased screens, *Schmidtea mediterranea* presents a clear, tractable *in vivo* model system for future molecular dissection of stem cell and regenerative biology.

Experimental Procedures

Planarian Culture and Irradiation

Strain ClW4 was maintained as described (Newmark and Sánchez Alvarado, 2000), including Gentamycin. Irradiation experiments involved ≥seven days starved, size-matched animals and dual Gammacell-40 137 Cesium sources delivering 79–82 rads/min. Irradiated animals were washed every 3–4 days.

RNAi and Molecular Biology

cDNAs (Sánchez Alvarado et al., 2002) and libraries from regenerating animals were used. Genes were cloned into pGEM (Promega). pPR244 usage and bacterial growth for RNAi were as described (Reddien et al., 2005a). Bacteria were resuspended in 66% homogenized beef liver (in water) at 1/300th the initial bacterial culture volume. Negative RNAi controls used *unc-22*, a *C. elegans* gene lacking significant similarity to the *S. mediterranea* genome.

Flow cytometry with RNAi phenotypes was performed as described (Scimone et al., 2010). Microarray and Gene Set Enrichment Analysis (GSEA) methods can be found in Supplemental Material.

Histology

RNA probes were in vitro transcribed by T7 polymerase (Promega) using DIG-, FITC- (Roche), or DNP- (Perkin Elmer) modified ribonucleotides, purified by ethanol precipitation with 7.5M ammonium acetate, and resuspended in deionized formamide. Tyramide-conjugated fluorophores were generated from AMCA, Fluorescein, Rhodamine (Pierce), and Cy5 (GE Healthcare) N-Hydroxysuccinimide (NHS) esters. *in situ* hybridizations were performed as described (Pearson et al., 2009). For double/triple-color fluorescence in situ hybridization (FISH), HRP-inactivation was performed between labelings in 4% formaldehyde, 45 min.

Single-Colony Analysis

Doses causing 30–50% of irradiated animals to lack any colony were optimized, such that single or sparse colonies were examined in the remaining animals. 1,750 and 1,500 rads were used for 2–4mm and < 2mm animals, respectively. Colonies that merged or were in close proximity (<50–100μm) were not analyzed. Proliferative to post-mitotic cell ratios were assessed by linear regression (best-fit slope and 95% confidence intervals). Student's t-test (two-tailed, unequal variance) was used to determine significant changes in cell numbers. Analysis of covariance (ANCOVA) was used to test for significant changes in cell-type ratios (relative to control RNAi ratios) by comparing slopes of linear regression lines fit to day 14 colony data plots (see Supplemental Table 5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Expression profiling identifies regulatory genes expressed in neoblasts
- Clonal stem cell assay and RNAi identify stem cell expansion/differentiation genes
- Planarian stem cells share molecular features with embryonic and germ cells

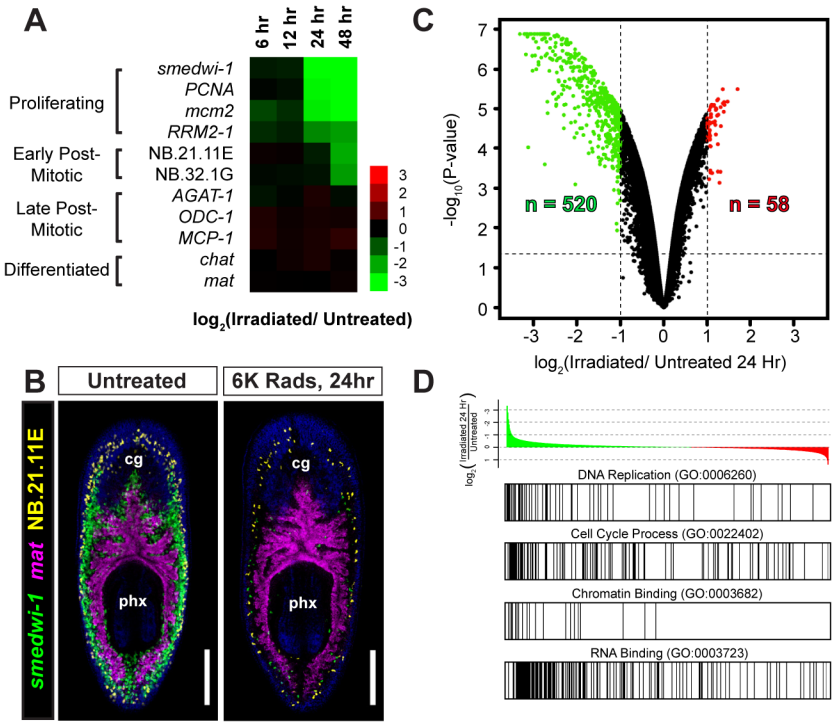

Figure 1. Identification of Irradiation-Sensitive Transcripts in Adult Planarians by Microarrays

(A) Heat map illustrating mRNA depletion kinetics following 6,000 Rads γirradiation. Markers for proliferating cells (*smedwi-1*, *PCNA*, *mcm2*, *RRM2-1*), post-mitotic cells (NB. 21.11E, NB.32.1G, *AGAT-1*, *ODC-1*, and *MCP-1*), neurons (*chat*), and intestine (*mat*) are shown. (B) Whole-mount triple-fluorescence *in situ* hybridization (FISH) shows the anatomical distribution of proliferative cells (neoblasts) with untreated and 24-hour irradiated animals. Shown are projections through z-stacks of multiple confocal planes in the interior of entire animals. Pharynx (px) and cephalic ganglia (cg) are indicated. Ventral views, anterior up. Scale bars, 200 μm. (C) Volcano plot showing transcripts depleted (green) or upregulated (red) 24 hours after irradiation. See Supplemental Table 1. (D) Gene set enrichment analysis (GSEA) with annotated gene list pre-ranked by log2 ratio (24-hour irradiated/untreated). Example gene sets enriched among irradiation-depleted transcripts are shown. See Supplemental Table 2.

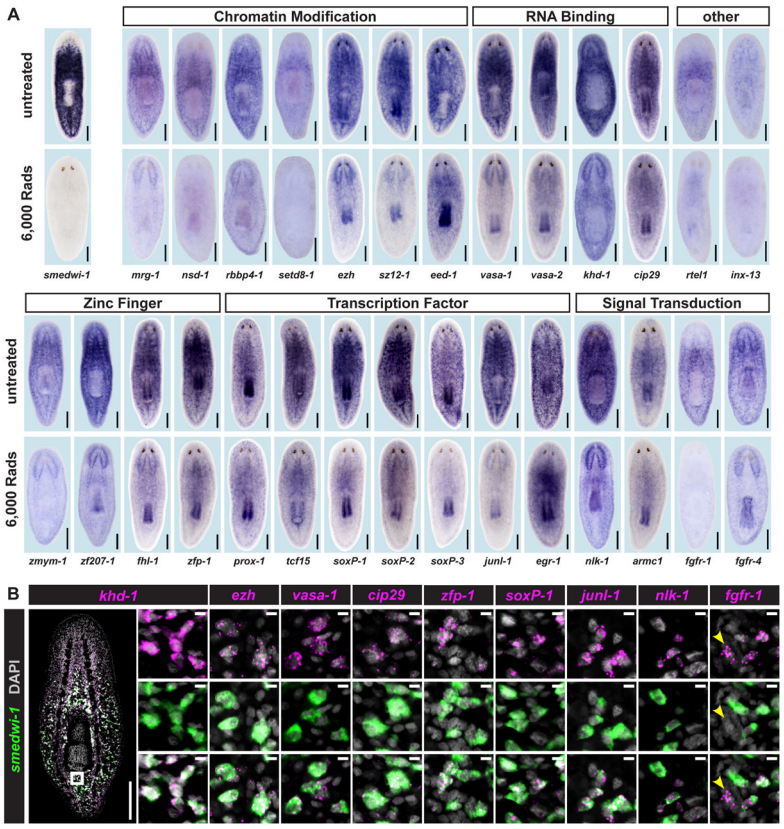

Figure 2. Irradiation-Sensitive Transcripts are Expressed in *smedwi-1*$^+$ Proliferating Cells

(A) Whole-mount *in situ* hybridization (ISH) in untreated animals and animals fixed 5 days after 6,000 Rads γ-irradiation. Genes were annotated by BLASTx and PFAM (See also Supplemental Table 3). (B) Expression of genes identified by microarray, analyzed by double FISH with a *smedwi-1* RNA probe (proliferative cell marker). Zoomed images are single confocal planes from tail regions. Most cells detected by FISH co-expressed *smedwi-1*; cells with little/no *smedwi-1* gene expression are labeled by arrowheads. Some transcripts (e.g., *ezh* and *cip29*) are expressed at low levels with background signal (scattered magenta dots) also visible. See also Supplemental Figure 2. Shown are representative ventral views, anterior up. Scale bars 200 μm, 10 μm (zoomed images).

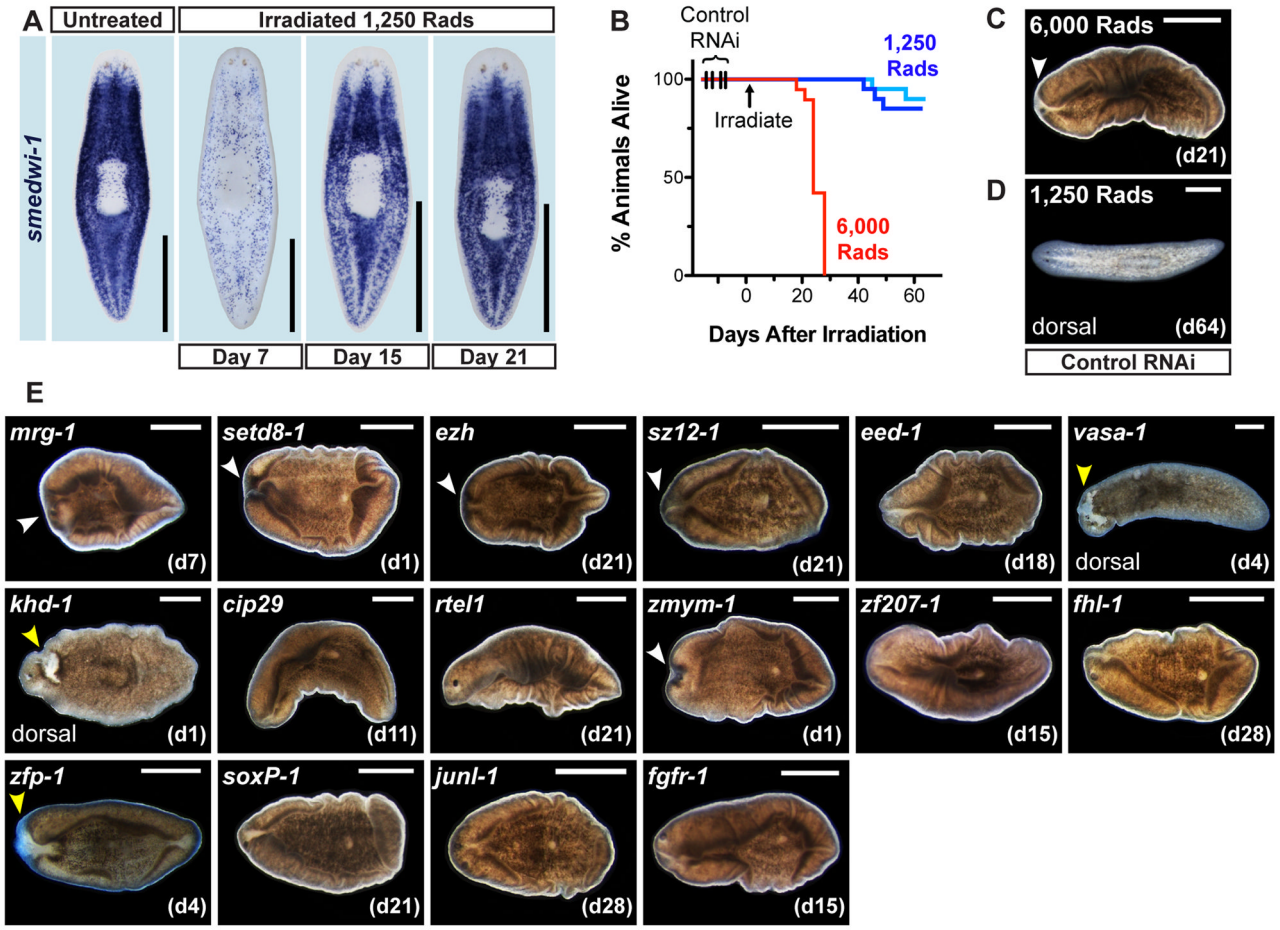

Figure 3. Identification of RNAi Phenotypes after Sublethal Irradiation

(A) Proliferative cell repopulation detected by *smedwi-1* ISH in animals fixed 7, 15, and 21 days after 1,250 Rads γ-irradiation. Ventral views, anterior up. (B) Survival curves of control RNAi animals exposed to 1,250 Rads (two independent experiments) and 6,000 Rads (n = 19–20 animals per sample). (C–E) Representative views of irradiated RNAi animals, anterior left. Images are ventral views unless otherwise noted. (C) 6,000 Rad-irradiated animals experienced head regression (white arrowheads) and ventral curling by day 21. (D) Most sublethally irradiated (1,250 Rad) animals, by contrast, were visibly normal on day 64. (E) Representative live images of animals with RNAi phenotypes after 1,250 rad exposure. Images are from the first day post-irradiation (noted in parenthesis) when tissue failure appeared; only cases where >50% of animals displayed defects are shown. White arrowheads: tissue regression. yellow arrowhead: epidermal lesions. Scale bar, 500 μm. See also Supplemental Figure 3 and Supplemental Table 4.

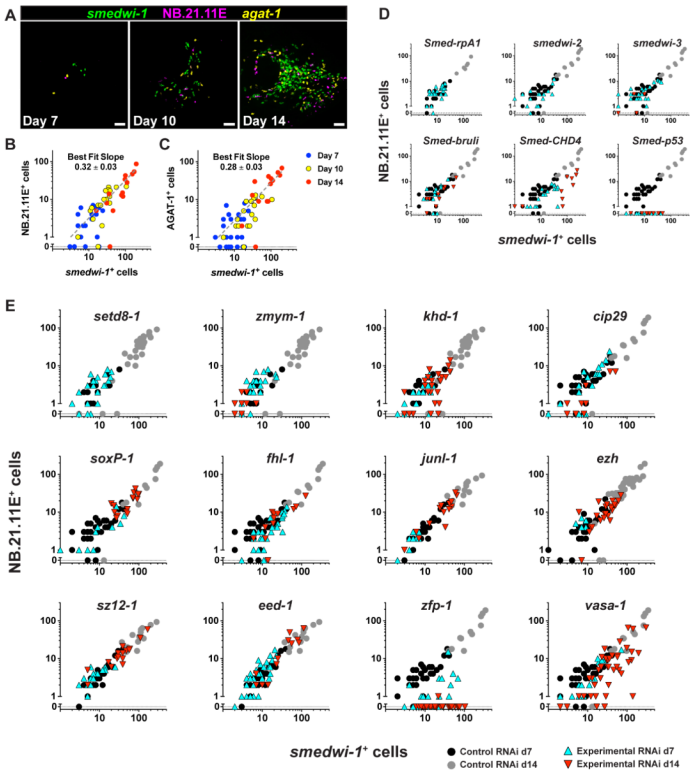

Figure 4. Genes Required for Proliferative Cell Expansion and Differentiation Identified by RNAi and Clonal Analysis

(A) Representative colonies from animals exposed to 1,750 Rads analyzed by triple FISH. Ventral views, adjacent to the pharynx, anterior left. Proliferating cells (*smedwi-1*$^+$), and two post-mitotic cell types (NB.21.11E$^+$ and *AGAT-1*$^+$) are labeled. Scale bars, 50 μm. (B–C) Log-scale plots of raw cell count data. Each dot represents an individual colony. *smedwi-1*$^+$ cell numbers per colony are plotted against numbers of NB.21.11E$^+$ (B) and *AGAT-1*$^+$ cells (C). (D–E) Animals irradiated 1,500 – 1,750 Rads fed one RNAi food dose displayed reduced numbers of *smedwi-1*$^+$ and/or NB.21.11E$^+$ cells per colony. RNAi was administered by feeding 4 days after irradiation except for *junl-1*, *ezh*, *sz12-1*, and *eed-1*, which were fed seven days prior to irradiation. See also Supplemental Figure 4. For statistical analysis of colony phenotypes, see Supplemental Table 5.

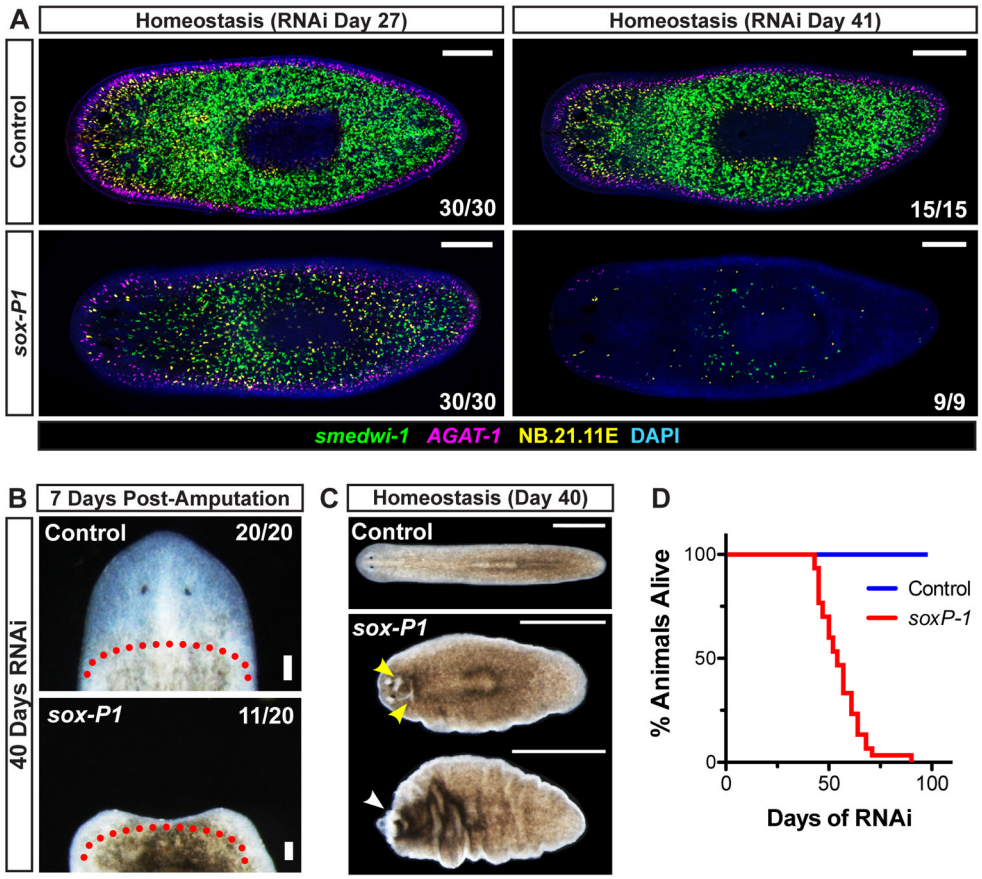

Figure 5. *Smed-soxP-1* is Required for Maintenance of *smedwi-1*$^+$ Cells, Tissue Homeostasis, and Regeneration

(A) Animals were assessed for proliferative (*smedwi-1*$^+$) and post-mitotic (*AGAT-1*$^+$ and NB.21.11E$^+$) cell types after several weeks of RNAi. Shown are representative confocal planes, anterior left. (B–C) Regenerative ability and tissue homeostasis were assessed after 40 days of RNAi. (B) Representative live images of head regions 7 days post-amputation are shown, anterior up. Approximate amputation plane is indicated by dotted line. (C–D) Tissue maintenance and animal survival after continuous RNAi feedings every 3–4 days ($n \geq 30$ animals/sample). (C) Representative live images of whole animals undergoing tissue failure, anterior left. Epidermal lesions and head regression are indicated by yellow and white arrowheads, respectively. (D) Survival curves. Scale bars 200 μm (A), 100μm (B), 1mm (C).

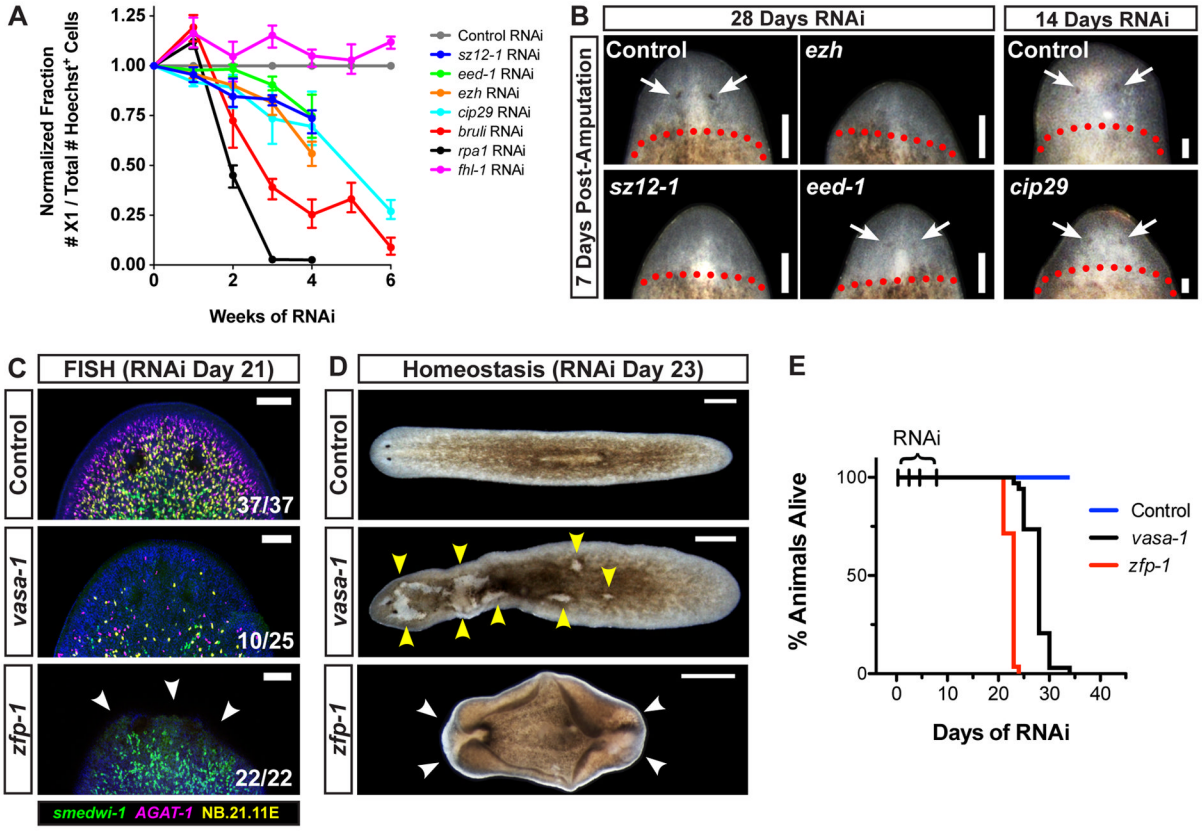

Figure 6. Genes Necessary for Colony Expansion and Differentiation are Required for Tissue Maintenance

(A) Animals fed RNAi food for several weeks were assessed for proliferative cell presence by flow cytometry. Numbers of proliferating X1 cells, represented as a fraction of total Hoechst$^+$ cells, were normalized to internal RNAi controls. Three biological replicates were used per time point. Shown are means; error bars denote data range. (B) Regenerative ability after 14 or 28 days of continuous RNAi feeding. Shown are head regions, anterior up, seven days after decapitation. Dotted line, approximate amputation planes. Arrows, photoreceptors. (C–E) RNAi animals were assessed for tissue maintenance defects. (C) Confocal projections from animals after 21 days of RNAi. Dorsal views of head regions, anterior up. Arrows, tissue regression sites. (D–E) Tissue maintenance and animal survival after RNAi (n = 29–34 worms/sample). (D) Images after 23 days of RNAi. Anterior, left. Yellow arrows, lesions. White arrows, tissue regression and ventral curling. (E) Survival curves. Scale bars 100 μm (B–C), 500 μm (D).

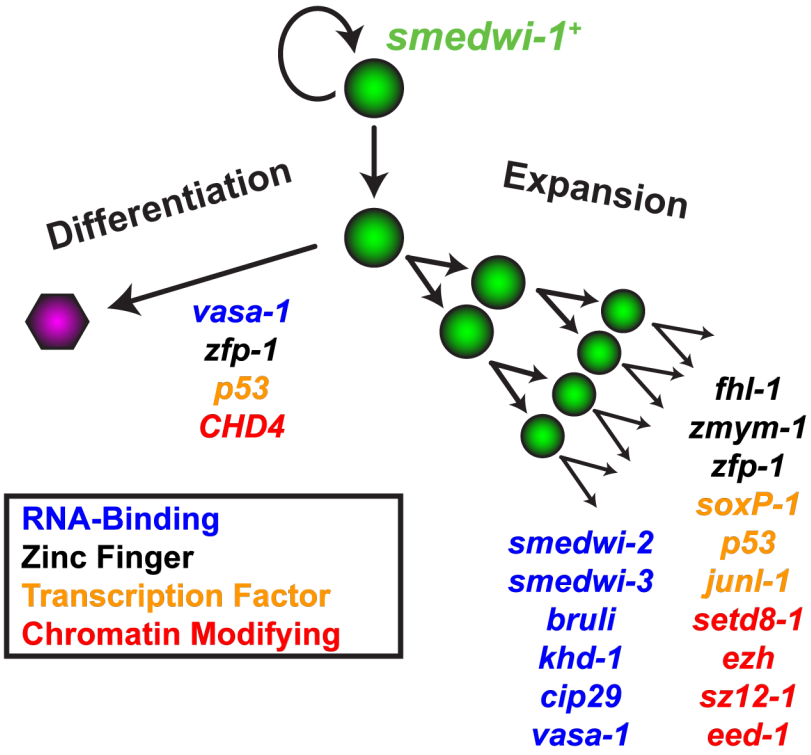

Figure 7. RNA-Binding, Transcription, and Chromatin Modifying Factors Regulate Clonogenic Expansion and Differentiation of Planarian Stem Cells

Genes expressed within the proliferative cell compartment are required for expansion and differentiation activity associated with clonogenic cells (cNeoblasts). Several of these genes (e.g. *p53, zfp-1, vasa-1*) are involved in both proliferative cell expansion and differentiation.