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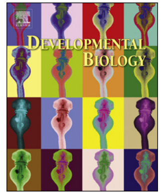

Evolution of Developmental Control Mechanisms

Essential role for the planarian intestinal GATA transcription factor in stem cells and regeneration

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ABSTRACT

The cellular turnover of adult tissues and injury-induced repair proceed through an exquisite integration of proliferation, differentiation, and survival signals that involve stem/progenitor cell populations, their progeny, and differentiated tissues. GATA factors are DNA binding proteins that control stem cells and the development of tissues by activating or repressing transcription. Here we examined the role of GATA transcription factors in *Schmidtea mediterranea*, a freshwater planarian that provides an excellent model to investigate gene function in adult stem cells, regeneration, and differentiation. *Smed-gata4/5/6*, the homolog of the three mammalian GATA-4,-5,-6 factors is expressed at high levels in differentiated gut cells but also at lower levels in neoblast populations, the planarian stem cells. *Smed-gata4/5/6* knock-down results in broad differentiation defects, especially in response to injury. These defects are not restricted to the intestinal lineage. In particular, at late time points during the response to injury, loss of *Smed-gata4/5/6* leads to decreased neoblast proliferation and to gene expression changes in several neoblast subpopulations. Thus, *Smed-gata4/5/6* plays a key evolutionary conserved role in intestinal differentiation in planarians. These data further support a model in which defects in the intestinal lineage can indirectly affect other differentiation pathways in planarians.

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1. Introduction

GATA factors form a family of transcription factors containing zinc finger motifs, which bind to the DNA sequence "GATA" (Merika and Orkin, 1993; Patient and McGhee, 2002). In mammals, six GATA family members (GATA1-6) control cellular differentiation and organogenesis during development and in adults (Chlon and Crispino, 2012; Duncan, 2005), including hematopoiesis (Rodrigues et al., 2005; Weiss and Orkin, 1995), cardiac development (Kawamura et al., 2005; Pikkarainen et al., 2004), mammary gland development (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006), and the differentiation of tissues derived from the endoderm (Aronson et al., 2014; Gao et al., 1998; Zaret, 1999; Zaret et al., 2008). Early during development, GATA factors can control the self-renewal and the differentiation of embryonic stem cells (Capo-Chichi et al., 2010; Serrano et al., 2013; Turbendian et al., 2013), especially differentiation towards the extra-embryonic endoderm (Artus and Chazaud, 2014). GATA factors activity has also been implicated in abnormal proliferation and differentiation in cancer cells (Akiyama et al., 2003; Vicente et al., 2012; Zheng and Blobel, 2010).

GATA factors have been extensively studied in mammalian systems, but the elucidation of their exact roles in stem/progenitor cells and their differentiated progeny is complicated by the overlapping and distinct functions of each family member (Bresnick et al., 2010; Gao et al., 1998; Merika and Orkin, 1993; Patient and McGhee, 2002). Schematically, GATA1, GATA2, and GATA3 are often considered the "hematopoietic" GATA factors, based on their key roles in various aspects of hematopoiesis (Kobayashi-Osaki et al., 2005; Leonard et al., 1993; Orkin, 1992). In contrast, GATA4, GATA5, and GATA6 are expressed in endodermal and mesodermal lineages and have been more implicated in the development of organs derived from these lineages such as the heart, the lung, and the intestine (Bossard and Zaret, 1998; Charron and Nemer, 1999; Liu et al., 2002; Zaret et al., 2008; Zhao et al., 2005).

Planarians are multicellular animals with bilateral symmetry that display a striking capacity to repair injured or lost structures through a robust regeneration process. At any given time, homeostasis is maintained in planarians by dividing cells that generate the cellular progeny that forms adult tissues after terminal differentiation. In amputated or injured animals, a burst of proliferation occurs to form the regenerative blastema, the anatomical place where missing structures

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are recreated (reviewed in Reddien and Sanchez Alvarado (2004), Sanchez Alvarado and Yamanaka (2014), Tanaka and Reddien (2011)). The planarian stem cells, also known as neoblasts, are the only source of new cells in intact and amputated planarians (Betchaku, 1967; Pedersen, 1959; Scimone et al., 2014; van Wolfswinkel et al., 2014). Heterogeneity exists in neoblast populations, but it is likely that at least one subpopulation acts as a true stem cell while other subsets may have more restricted differentiation capacity (Scimone et al., 2014; van Wolfswinkel et al., 2014; Wagner et al., 2011). Based on these properties, planarians are an exceptional model to decipher fundamental mechanisms of stem cell biology and tissue regeneration.

The different biological functions of each GATA factor in mammals are associated with biochemical and molecular complexity that may involve compensatory functions. Therefore, some of this complexity can be resolved by studying GATA factors in animal species in which the GATA family has not expanded to the levels found in mice or humans. For example, in *Caenorhabditis elegans*, intestinal development is largely controlled by one GATA factor (McGhee, 2013; McGhee et al., 2007). *Schmidtea mediterranea* possesses a single homolog for GATA-4, -5, and -6, and phylogenetic analysis has shown *Smed-gata4/5/6* falls within the GATA-4,-5, and -6 clade (Wagner et al., 2011). All six mammalian GATA transcription factors contain a highly conserved DNA binding domain consisting of two zinc fingers with a Cys-X 2-Cys-X 17-Cys-X 2-Cys motif that dictates binding to the GATA nucleotide sequence element (Molkentin, 2000): these two key domains are conserved in *Smed-gata4/5/6* (Supplemental Fig. S1A), suggesting this GATA factor can function as a transcriptional regulator in planarians. Previous RNA-sequencing (RNA-Seq) studies have shown *Smed-gata4/5/6* transcripts are expressed at high levels in the intestine but also in populations of neoblasts (Onal et al., 2012; Resch et al., 2012) (Supplemental Fig. S1B). These observations are consistent with recent studies of single neoblast cells that showed expression of *Smed-gata4/5/6* in the gamma subset of neoblasts (van Wolfswinkel et al., 2014; Wurtzel et al., 2015) (Supplemental Fig. S1C) and a previous study showing *Smed-gata4/5/6* expression in neoblasts interspersed between the intestinal branches (Wagner et al., 2011).

Here we found that disruption of *Smed-gata4/5/6* function in intact and injured worms primarily results in intestinal defects. In addition, however, we observed that the *Smed-gata4/5/6(RNAi)* phenotype does not exclusively affect the intestinal lineage, indicating that *Smed-gata4/5/6* function may play a role in the differentiation of other cell types in planarians. Our data support a model in which intestinal defects due to knock-down of *Smed-gata4/5/6* may indirectly affect neoblast populations and the differentiation of non-intestinal lineages.

2. Results

2.1. Loss of Smed-gata4/5/6 function disrupts homeostasis in planarians

Whole-mount in situ hybridization (WISH) showed high levels of *Smed-gata4/5/6* expression in the digestive tract of the worms (Fig. 1A), as previously described (Wagner et al., 2011), and reminiscent of what is seen in the mammalian gut (Beuling et al., 2011; Bossard and Zaret, 1998; Dusing and Wiginton, 2005). These observations suggested that *Smed-gata4/5/6* may play a role in the differentiation and/or the maintenance of intestinal structures in *S. mediterranea*.

To investigate the role of *Smed-gata4/5/6*, we knocked down its expression in intact animals by RNA interference (RNAi). We developed a feeding schedule that consists of four feedings every 2 days (Fig. 1D). The effective downregulation of *Smed-gata4/5/6* mRNA was independently confirmed by WISH and RT-qPCR experiments (Fig. 1B and C). *Smed-gata4/5/6(RNAi)* animals developed dorsal lesions twelve days after the final feeding (12dpf, Fig. 1D), which eventually led to animal lethality (Fig. 1E). These observations indicate that *Smed-gata4/5/6* is required for the long-term maintenance of adult tissue and homeostasis in planarians.

To investigate the cellular basis of these observations, we first examined whether the *Smed-gata4/5/6(RNAi)* would affect the proliferation of neoblasts. However, under these conditions, we observed no significant differences in the mitotic activity of control and experimental animals as measured by immunostaining for phospho-Histone H3 (PH3) expression, a marker of mitosis (Fig. 1F). Under physiological conditions, cell turnover is a balanced combination of cell division and cell death (Pellettieri et al., 2010). No changes in mitotic activity suggested that tissue loss in *Smed-gata4/5/6(RNAi)* animals could result from an increase in cell death. Indeed, quantification of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) showed a significant increase in TUNEL-positive cells in *Smed-gata4/5/6(RNAi)* animals compared to controls at 12dpf (Fig. 1G).

The increase in cell death after *Smed-gata4/5/6(RNAi)* may be a consequence of dysfunctional neoblast response to cellular turnover demands or structural defects in the intestine where *Smed-gata4/5/6* is expressed. Throughout the initial stages of the experiment, we did not observe animal impairment to search for food nor differences in size between *Smed-gata4/5/6(RNAi)* and control worms (data not shown). Nonetheless, to investigate the possibility of abnormalities in intestinal morphology that develop overtime, we fed control and *Smed-gata4/5/6(RNAi)* worms with liver paste mixed with fluorescent-conjugated dextran, which labels the intestinal phagocytes and allow intestine visualization in situ (Forsthoefel et al., 2011). This experiment did not reveal any visible difference between the two groups at an early time point (Supplemental Fig. S2A). However, when we performed WISH for the intestinal marker, *smedinx-9*, at late stages of the experiment (12dpf), when animals were unable to eat, we found a significant loss of expression and intestinal integrity (Supplemental Fig. S2B). These experiments suggest that deterioration of the intestine may be linked to cell death and animal survival as some *Smed-gata4/5/6 (RNAi)* animals began to die at this time point.

Altogether, these experiments indicate that *Smed-gata4/5/6* is necessary for the long-term maintenance of intestinal function and overall survival of planarians under homeostatic conditions. Based on these studies in intact animals, we sought to investigate the role of *Smed-gata4/5/6* and to explore its mechanisms of action under conditions where neoblasts are challenged.

2.2. Smed-gata4/5/6 is critical for the regeneration of planarians after amputation

We performed RNAi feeding, 4 feedings every 2 days, and amputated planarians four days after the final feeding. We analyzed the animals 7 days post-amputation (7dpa) and observed similar defects as with the uninjured worms, but exacerbated, including visible epithelial lesions. Head, trunk, and tail fragments all showed some impaired blastema formation and increased mortality upon *Smed-gata4/5/6* knock-down (Fig. 2A and B).

We decided to focus our analyses on the phenotypes of regenerating trunks because they have to regenerate both a tail and a head. Decreased blastema growth was highly significant in both the anterior and the posterior parts of regenerating trunks in knock-down animals (Fig. 2C). All 7dpa mutant trunk fragments analyzed lacked photoreceptor pigmentation (Fig. 2A and data not shown, see below). Next, to determine the role of *Smed-gata4/5/6* in neoblast populations during regeneration, we quantified neoblast mitotic activity with PH3 at 7dpa. We found that cell divisions were significantly decreased in *Smed-gata4/5/6(RNAi)* animals compared to controls (Fig. 2D),

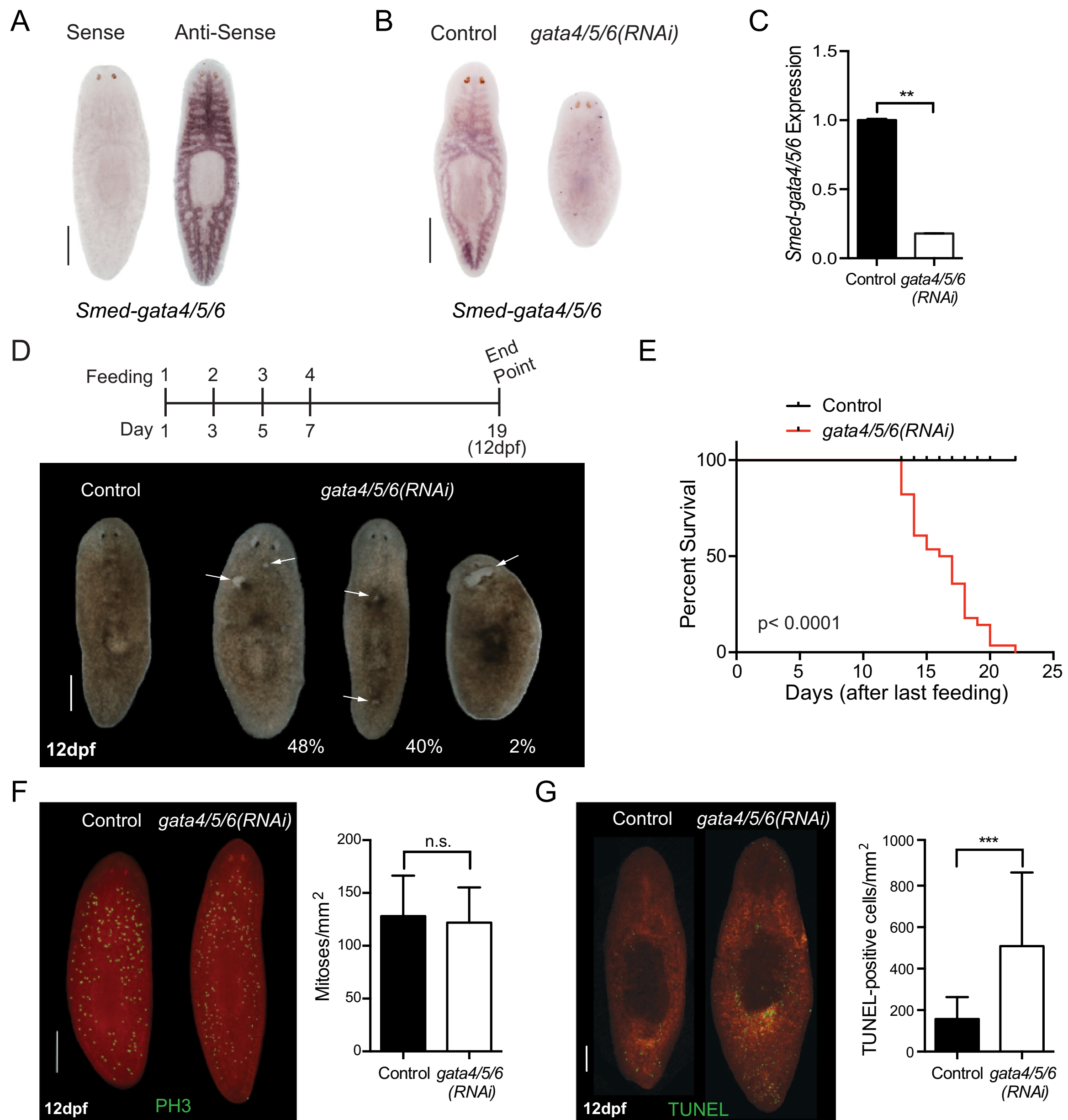


Fig. 1. *Smed-gata4/5/6* loss in homeostatic animals increases cell death. (A) Whole-mount in situ hybridization (WISH) for *Smed-gata4/5/6* expression (anti-sense probe) in uninjured animal. The sense *Smed-gata4/5/6* probe is used as a control. Scale bar: 500 μm. (B) Representative WISH for *Smed-gata4/5/6* in a control and *Smed-gata4/5/6(RNAi)* animal, 12 days after final RNAi feeding (anti-sense probe). Scale bar: 500 μm. (C) RT-qPCR analysis of *Smed-gata4/5/6* levels in control and *Smed-gata4/5/6(RNAi)* animals. Analysis based on 2 biological replicates ($n=10$), each replicate containing 5 animals pooled. Ct values are normalized to internal control GAPDH and relative to controls. Two-tailed unpaired Student's *t*-test p-value=0.0091, values represent average and error bars s.e.m. (D) Representative live images of intact RNAi animals 12 days after the final feeding. *Smed-gata4/5/6(RNAi)* animals develop lesions on both the anterior and posterior as indicated by white arrows. Scale bar: 500 μm. ($n>50$) The feeding time line is shown above. (E) Kaplan-Meier survival of *Smed-gata4/5/6(RNAi)* animals ($n=30$, med. survival=16.5 days). p-value < 0.0001 by log-rank test for significance. (F) Representative images of whole-mount immunostaining using anti-PH3 antibody in intact animals 12 days after final feeding (left) and quantification of mitoses (right) (two-way ANOVA, p-value=0.5433). Three independent experiments, $n=30$. Results represent average and error bars s.e.m. Scale bar: 500 μm. (G) TUNEL assay quantification of control and *Smed-gata4/5/6(RNAi)* intact animals 12 days after final feeding (two-way ANOVA, ***: p-value=0.0002). Two independent experiments, $n \geq 19$. Results represent average and error bars s.e.m.

suggesting that disruption of *Smed-gata4/5/6* impairs the neoblast response to the demands of tissue regeneration. A previous study has shown that two early bursts of neoblast proliferation take place in planarians just after amputation, a systemic response at ~6h and a local response at ~48 h (Wenemoser et al., 2012). In *Smed-gata4/5/6 (RNAi)* animals, we found no observable changes in these early proliferative events (Fig. 2E).

At 7dpa we also found a significant increase in TUNEL-positive cells in *Smed-gata4/5/6(RNAi)* animals compared to controls (Fig. 2F). When we performed the TUNEL assay on 7dpa transverse sections, we observed cell death throughout the animal, and not only in the intestine of *Smed-gata4/5/6(RNAi)* animals (Supplemental Fig. S3). To determine if *Smed-gata4/5/6(RNAi)* leads to cell death during the early stages of regeneration, we performed the TUNEL assay at two early time points, 1 and 3 days post-amputation (1dpa and 3dpa, respectively) and found no significant differences in *Smed-gata4/5/6(RNAi)* animals as compared to controls (Fig. 2G).

Together, the late decrease in proliferation and the late increase in cell death observed in *Smed-gata4/5/6(RNAi)* worms suggested that *Smed-gata4/5/6* may not directly affect neoblasts but that loss of *Smed-gata4/5/6* may indirectly affect the differentiation of neoblast populations during the late stages of injury response.

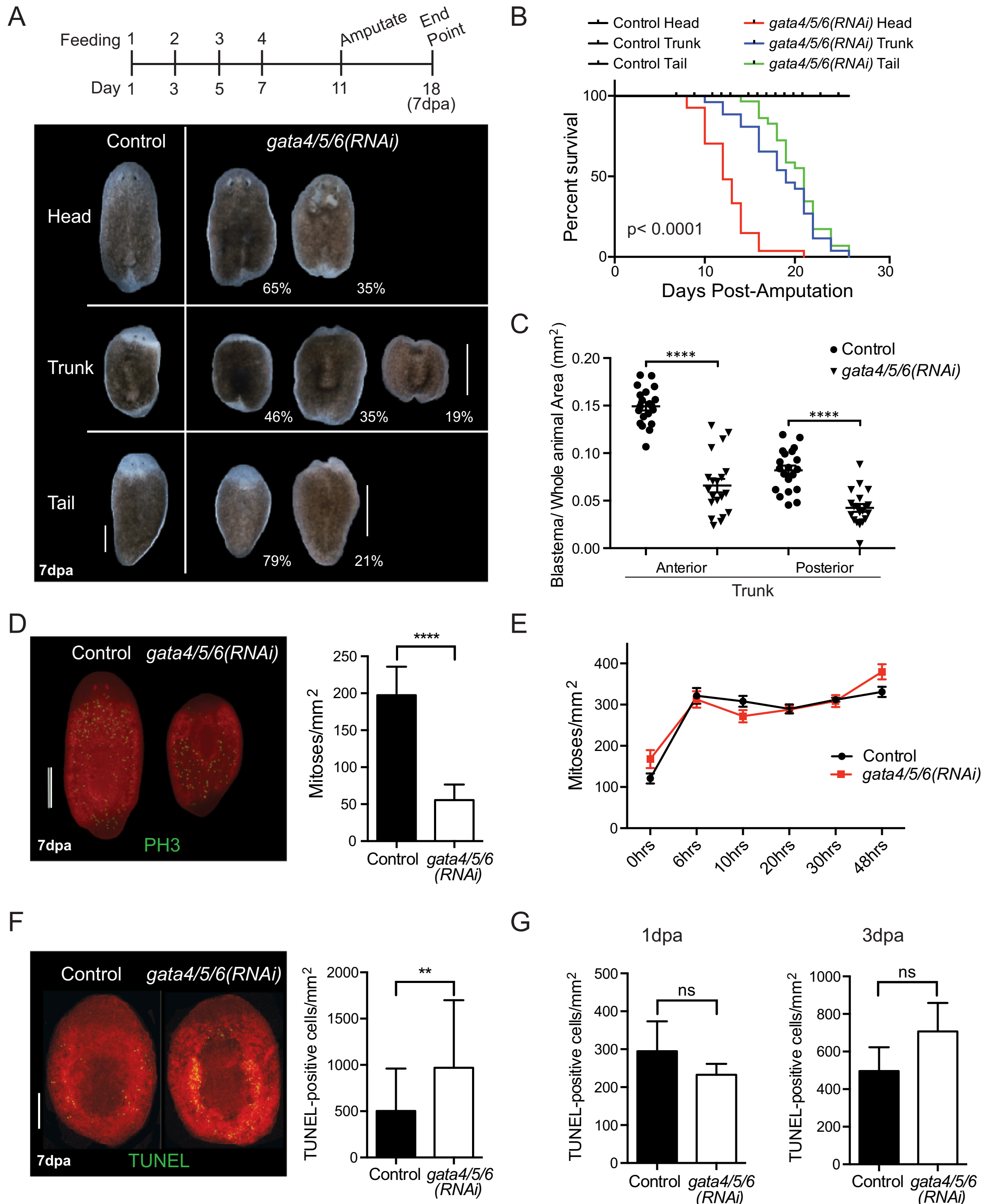


Fig. 2. *Smed-gata4/5/6* loss perturbs regeneration, decreases mitotic activity, and increases cell death. (A) Representative live images of regenerating RNAi head, trunk, and tail worms 7 days post-amputation. Scale bars: 500 µm. (n > 50) The feeding time line is shown above. (B) Kaplan-Meier survival of *Smed-gata4/5/6(RNAi)* animals (n=30, head-med. survival=12 days, trunk-med. survival=19 days, tail-med. survival=21 days). p-value < 0.0001 by log-rank test for significance. (C) Loss of *Smed-gata4/5/6* prevents blastema growth. Represented are the anterior and posterior blastema areas of the trunks. A ratio of the blastema area over the whole animal area was used to take into account the initial size of the regenerating fragment (two-way ANOVA, ****: p-value < 0.0001). Two independent experiments, $n=20$. (D) Representative images of whole-mount immunostaining using anti-PH3 antibody in regenerating animals 7 days post-amputation. Quantification of mitoses in the trunk of control and *Smed-gata4/5/6(RNAi)* animals (two-way ANOVA, p-value < 0.0001). Three independent experiments, $n \geq 25$. Results represent average and error bars s.e.m. Scale bar: 500 µm. (E) Graph of early mitosis peaks after amputation. In controls two peaks of mitotic activity occur, first at 6 h and second at 30–48 h post-amputation. Two independent experiments, $n=10$ per time point. Results represent average and error bars s.e.m. (F) Representative images of apoptosis (TUNEL-positive cells) in RNAi animals 7 days post-amputation. Quantification of TUNEL-positive cells in control and *Smed-gata4/5/6(RNAi)* worms 7 days post-amputation (two-way ANOVA, ****: p-value < 0.0001, **: p-value=0.0010, *: p-value=0.0239). Two independent experiments, $n \geq 19$. Results represent average and error bars s.e.m. Scale bar: 100 µm. (E) Graphs of early apoptosis after amputation. Quantification of TUNEL-positive cells at 1 d and 3 days post-amputation (two-way ANOVA). Two independent experiments, $n=10$ per time point. Results represent average and error bars s.e.m.

2.3. Smed-gata4/5/6 is critical for the differentiation of specific cell lineages in planarians during regeneration

To investigate a potential role for *Smed-gata4/5/6* in the differentiation and remodeling of different organs, we examined markers for various lineages. First, we performed WISH for *smedinx-9*, an intestinal marker (Oviedo and Levin, 2007). Qualitative evaluation of the *smedinx*-9 staining revealed that the intensity of the signal was diminished 7dpa in *Smed-gata4/5/6(RNAi)* animals; the gross morphology of the intestine within the pre-existing tissue appeared to remain without changes (Fig. 3A). Strikingly, however, 7dpa *Smed-gata4/5/6(RNAi)* animals did not develop new intestinal branches within the newly formed blastema, in contrast to control animals (Fig. 3A). These observations provide support for a key role for *Smed-gata4/5/6* in intestinal differentiation.

More surprisingly, we found defects after *Smed-gata4/5/6(RNAi)* in the terminal differentiation of the ocelli, as noted above, with a complete absence of photoreceptor pigmentation in all regenerating tail and trunk fragments, even at late time points in survival studies (Fig. 2A, and data not shown). Using Synapsin as a marker for the differentiation of the central nervous system, we identified differentiated neuronal cells in the regenerating foremost anterior region of trunks and posterior fragments both in controls and knock-down worms but we also observed fusion defects between the two CNS tracts in the knock-down animals in

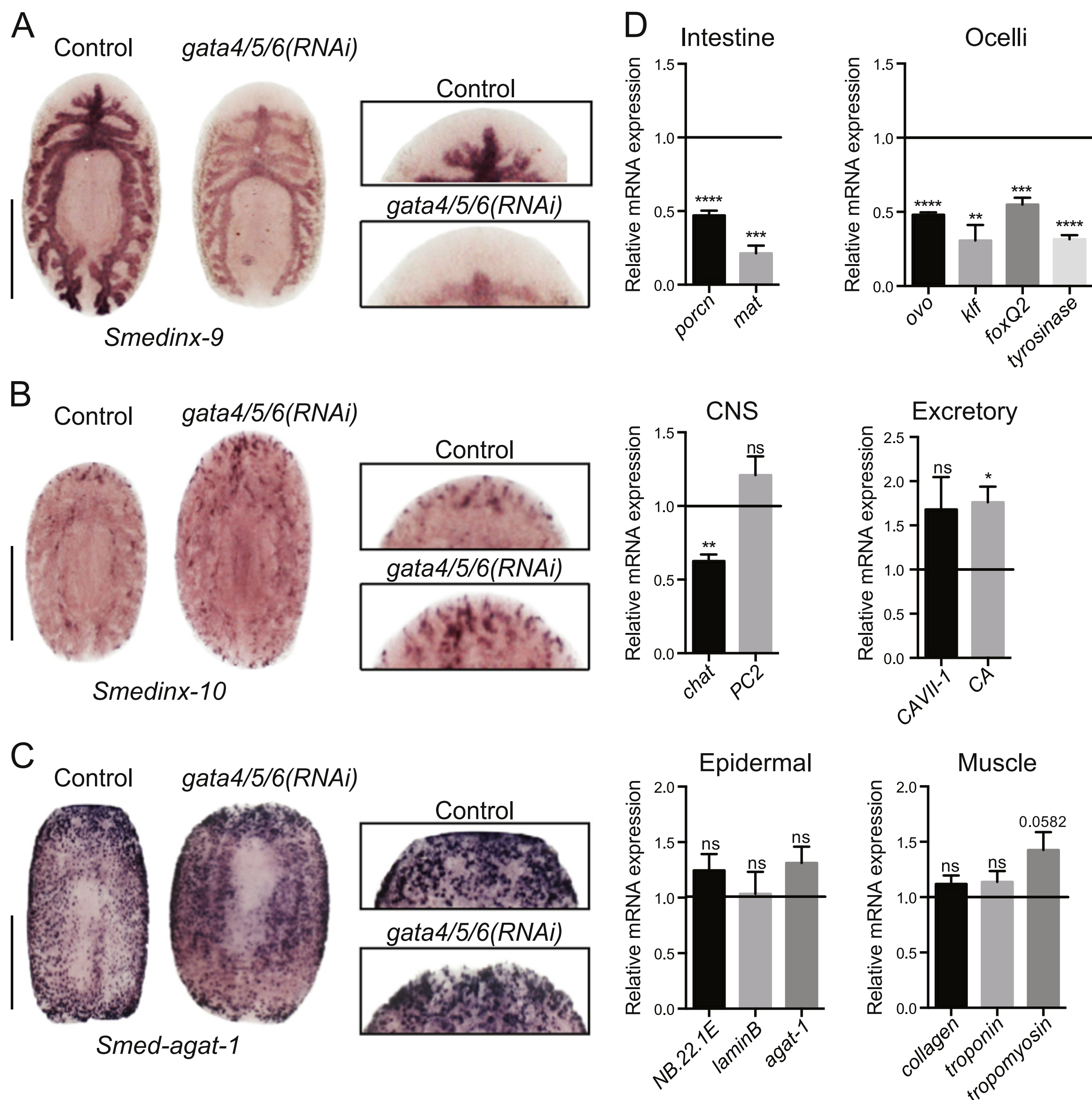
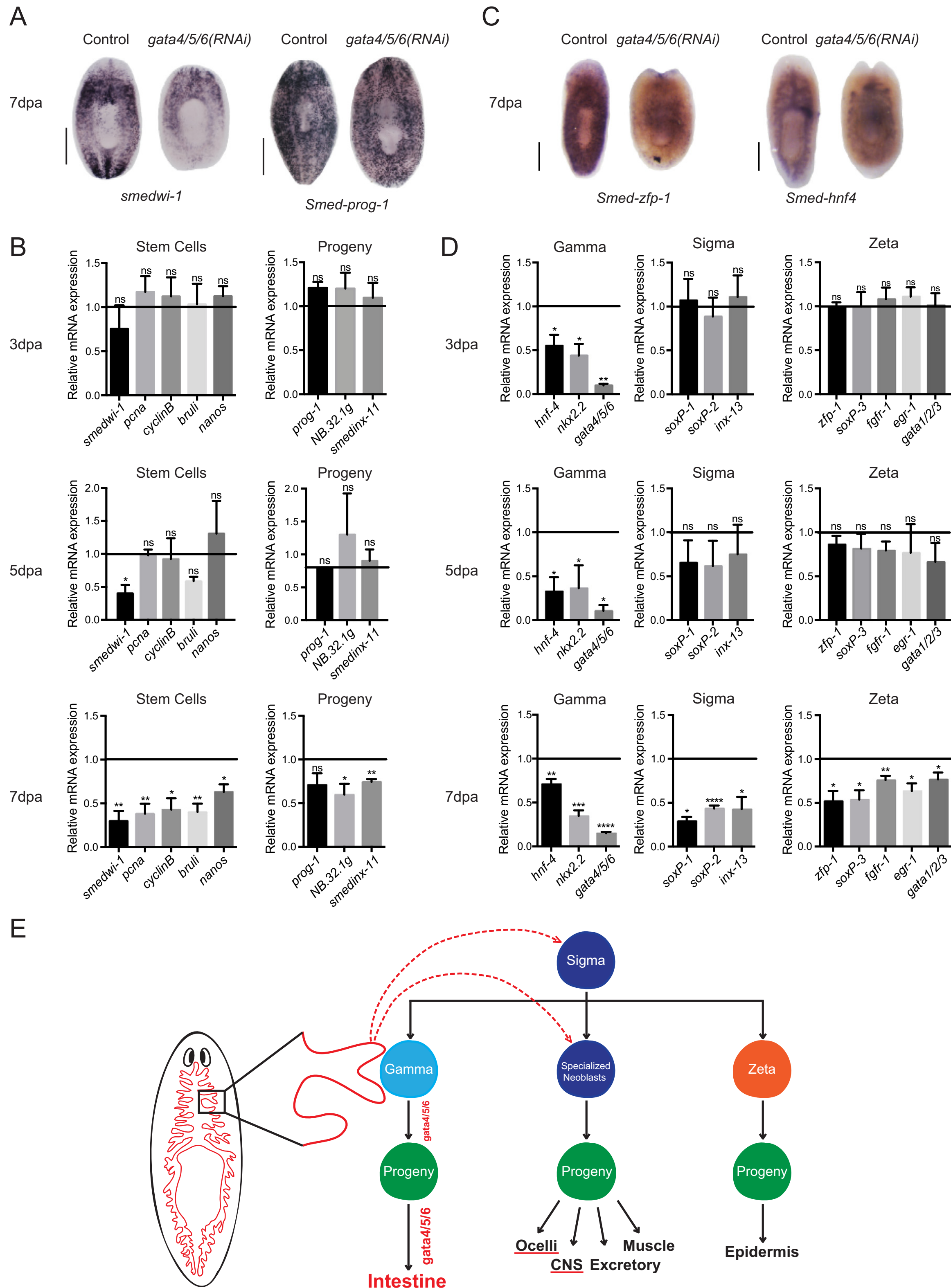


Fig. 3. Analysis of differentiated cell lineages in *Smed-gata4/5/6(RNAi)* animals. (A) Representative WISH for *smedinx-9*, an intestine marker, in control and *Smed-gata4/5/6 (RNAi)* animals (anti-sense probe). A magnification of the blastema (inset) shows the absence of intestinal branching in the newly formed tissue in knock-down worms 7 days post-amputation. ($n=5$) Scale bar: 250 μm. (B) Representative WISH for *smedinx-10*, an excretory marker (flame cells), in control and *Smed-gata4/5/6(RNAi)* animals (anti-sense probe). A magnification of the blastema (inset) shows that new flame cells are established in the newly-formed tissue 7 days post-amputation. ($n=5$) Scale bar: 250 μm. (C) Representative WISH for *Smed-agat-1*, a general differentiation marker, in control and *Smed-gata4/5/6(RNAi)* animals (anti-sense probe). A magnification of the blastema (inset) shows that differentiated cell types are found in the new tissue 7 days post-amputation. ($n=5$) Scale bar: 500 μm. (D) RT-qPCR analysis showing mRNA expression changes in differentiation tissue marker transcripts following *Smed-gata4/5/6(RNAi)* 7 days post-amputation. Bar graphs show fold change in expression relative to controls after normalization to GAPDH. Controls represented by horizontal line set at 1. Two-tailed unpaired Student's *t*-test (****: p-value <0.0001, ***: p-value <0.001, **: p-value >0.01*: p-value >0.05, ns: not significant) values represent average and error bars s.e.m. Analysis for regenerating trunks based on 3 biological replicates ($n=15$), each replicate containing 5 animals pooled.

the most severe cases (unresolved cleft in the blastema) (Supplemental Fig. S4). Thus, low levels of *Smed-gata4/5/6* prevent the development of photoreceptors and sometimes lead to developmental defects in the CNS.

In contrast, WISH for *smedinx-10*, a marker for the excretory system (flame cells) (Oviedo et al., 2010), showed no visible defects in the knock-down animals, with clear expression of this marker in the newly regenerated tissue (Fig. 3B). Similarly, when we examined the expression of a L-arginine:glycine amidinotransferase (*Smed-AGAT-1*), which is expressed broadly in subepidermal mesenchymal tissue (Eisenhoffer et al., 2008; Wagner et al., 2011), we found no qualitative difference in the expression



of this marker between *Smed-gata4/5/6(RNAi)* animals and controls 7dpa (Fig. 3C).

To quantitatively assess changes in gene expression in differentiated tissues, we performed RT-qPCR experiments for genes associated with the intestine, the muscles, the epidermis, the nervous and excretory systems. These experiments confirmed a significant inhibition of intestinal and photoreceptor differentiation in *Smed-gata4/5/6(RNAi)* worms, variable changes in the CNS, and no significant changes in the expression of most markers belonging to the excretory system, muscles, and the epidermis (Fig. 3D).

Together, these experiments identified a key role for *Smed-gata4/5/6* in intestinal regeneration and selective roles of this transcription factor in non-intestinal tissues in injured animals during the later stages of regeneration.

2.4. Absence of Smed-gata4/5/6 affects several neoblast populations

Based on our observations of significantly decreased mitotic cells in regenerating *Smed-gata4/5/6(RNAi)* animals at late time points (Fig. 2D) and the defects described above in differentiated cell lineages (Fig. 3), we sought to examine the role of *Smed-gata4/5/6* in neoblasts and their early progeny. We first compared the expression of neoblasts and early progeny markers (*smedwi-1* and *Smed-prog1*, respectively) by WISH following *Smed-gata4/5/6 (RNAi)* in 7dpa trunks. We found a decrease in *smedwi-1* expression and no visible change in *Smed-prog-1* (Fig. 4A). RT-qPCR analysis for general neoblast and early progeny markers showed no or few significant changes at 3dpa and 5dpa but significant decreases in these markers by 7dpa (Fig. 4B); this effect was not observed in uninjured animals (data not shown). Thus, the effects of *Smed-gata4/5/6(RNAi)* on neoblasts likely begin around day 4–5 of regeneration.

A recent study of the planarian stem cell compartment identified two major classes of neoblasts, the zeta-class and sigma-class, which are further divided into subclasses (van Wolfswinkel et al., 2014). The gamma subclass, a branch of sigma, was predicted to be involved in the development of the planarian gut, and *Smed-gata4/5/6* is expressed in this subpopulation (van Wolfswinkel et al., 2014; Wurtzel et al., 2015) (Supplemental Fig. S1C). The phenotypes of *Smed-gata4/5/6(RNAi)* worms and our molecular analyses raised the possibility that gamma neoblasts might be affected by loss of *Smed-gata4/5/6*. Indeed, RT-qPCR analysis of two other markers of the gamma neoblast population showed a significant decrease in expression as early as 3dpa, suggestive of an early loss of these populations in *Smed-gata4/5/6(RNAi)* worms (Fig. 4D). In contrast, loss of zeta and sigma neoblast expression markers was only significant following *Smed-gata4/5/6(RNAi)* in regenerating 7dpa trunks (Fig. 4D). Qualitative analysis by WISH following *Smed-gata4/5/6(RNAi)* in 7dpa trunks also showed a decrease in *Smed-hnf4* (gamma) and *Smed-zfp-1* (zeta) expression (Fig. 4C). Together, these observations indicate that *Smed-gata4/5/6* is a key regulator of intestinal differentiation in *Schmidtea mediterranea*, from gamma neoblasts to differentiated intestinal cells, and suggest that intestinal defects induced by loss of this intestinal transcription factor may indirectly affect other neoblast populations and the differentiation of other lineages.

3. Discussion

Here we examined the role of the planarian homolog of mammalian GATA-4, -5, and -6 transcription factors in organismal homeostasis and differentiation. *Smed-gata4/5/6(RNAi)* is detrimental to long-term neoblast maintenance, regeneration, differentiation of specific lineages, and ultimately survival. Our data further indicate that, in addition to its main role in intestinal differentiation from the gamma subclass of neoblasts, *Smed-gata4/5/6* may be indirectly implicated in the differentiation of other cell lineages (Fig. 4E).

GATA transcription factors are involved in embryonic development, differentiation, and adult tissue maintenance (Chlon and Crispino, 2012; Duncan, 2005). In vertebrates, six GATA factors are conserved and are separated into two major subfamilies. In contrast, *C. elegans* and Drosophila have one GATA1/2/3-like factor and multiple endoderm/mesoderm GATA4/5/6 related GATA factors (Aronson et al., 2014). In *S. mediterranea* we found two GATA factors orthologous to vertebrates, *Smed-gata1/2/3* and *Smed-gata4/5/6*, indicating that this planarian lacks the functionally redundant GATA4/5/6-like transcription factors often seen in lower Metazoa. Intriguingly, both planarian GATA factors maintain complete dual zinc finger domains, unlike *C. elegans* where all GATA4/5/6 subgroup factors lack the N-terminal zinc finger (Gillis et al., 2008). Initial *Smed-gata1/2/3* RNAi experiments did not yield visible phenotypes (data not shown), which led us to focus on *Smed-gata4/5/6* for this study; in addition, double RNAi experiments with *Smed-gata1/2/3* and *Smed-gata4/5/6* did not visibly enhance the phenotypes of *Smed-gata4/5/6(RNAi)* worms (data not shown), suggesting that *Smed-gata1/2/3* does not compensate for the loss of *Smed-gata4/5/6*. Additionally, *Smed-gata1/2/3* is expressed in zeta neoblasts (van Wolfswinkel et al., 2014). The functional role of *Smed-gata1/2/3* will need to be investigated in future studies.

Smed-gata4/5/6(RNAi) prevented the development of new intestinal branches in planarians, showing that the intrinsic function of this transcription factor and its vertebrate homologs in intestinal development is conserved (Bossard and Zaret, 1998; Charron and Nemer, 1999; Liu et al., 2002; Zaret et al., 2008; Zhao et al., 2005). These data reveal selective contribution of a transcription factor during simultaneous regeneration of adult tissues, which further validates *S. mediterranea* as a relevant model organism to study developmental pathways in the context of the

Fig. 4. Loss of *Smed-gata4/5/6* rapidly eliminates the gamma sub-class of neoblasts and affects both the zeta-class and sigma-class. (A) Representative WISH for *smedwi-1* and *Smed-prog-1,* markers for the stem cells and progeny, respectively. ($n=5$) Scale bars: 500 μm. (B) Representative WISH for *Smed-zfp-1* and *Smed-hnf-4,* markers for the zeta-class and gamma-class, respectively. ($n=5$) Scale bars: 500 μm. (C) RT-qPCR analysis showing mRNA expression changes in stem cell and progeny marker transcripts of control and *Smed-gata4/5/6(RNAi)* animals. Bar graphs show fold change in expression relative to controls after normalization to GAPDH. Controls represented by horizontal line set at 1. Two-tailed unpaired Student's *t*-test (****: p-value < 0.0001,***: p-value < 0.001, **: p-value > 0.01, *: p-value > 0.05, ns: not significant) values represent average and error bars s.e.m. Analysis for 3dpa and 7dpa regenerating trunks based on 3 biological replicates ($n=15$), each replicate containing 5 animals pooled. Analysis for 5dpa regenerating trunks based on 2 biological replicates ($n=10$), each replicate containing 5 animals pooled. (D) RT-qPCR analysis showing mRNA expression changes in gamma-class, zeta-class, and sigma-class marker transcripts following *Smed-gata4/5/6* RNAi. Bar graphs show fold change in expression relative to controls after normalization to GAPDH. Controls represented by horizontal line set at 1. Two-tailed unpaired Student's *t*-test (****: p-value < 0.0001, ***: p-value < 0.001, **: p-value > 0.01, *: p-value > 0.05, ns: not significant) values represent average and error bars s.e.m. Analysis for 3dpa and 7dpa regenerating trunks based on 3 biological replicates ($n=15$), each replicate containing 5 animals pooled. Analysis for 5dpa regenerating trunks based on 2 biological replicates ($n=10$), each replicate containing 5 animals pooled. (E) Model of *Smed-gata4/5/6* action in *S. mediterranea* (based on van Wolfswinkel et al. (2014)). Gamma neoblasts are required for the development and maintenance of the intestine, and *Smed-gata4/5/6*, which is expressed at low levels in these neoblasts and higher levels in differentiated intestinal cells, is intrinsically essential for this process. The most likely mechanism to explain the late defects in other neoblast populations (e.g. sigma and zeta subtypes) and other differentiation lineages (e.g. ocelli and CNS) in *Smed-gata4/5/6 (RNAi)* animals would be that intestinal defects disrupt these neoblasts and differentiation pathways indirectly (possible disruption of a niche, or lack of unidentified proliferation/survival factors). See text for a discussion on alternative models.

whole organism. Recent single-cell transcriptional profiling and RNA-Seq studies indicate that *Smed-gata4/5/6* may not be expressed in all intestinal cells and possibly not all gamma neoblasts (Fig. S1C) (van Wolfswinkel et al., 2014; Wurtzel et al., 2015). Further experiments will be required to address whether *Smed-gata4/5/6* is responsible for the generation of all cells within the digestive system; it is also possible that the role of *Smed-gata4/5/6* may be dependent on whether the animals are under homeostatic conditions or responding to injury.

Photoreceptor pigmentation and, in severe cases, CNS development were also affected by loss of *Smed-gata4/5/6*. Emerging evidence indicates that GATA4 and GATA6 are expressed in the CNS and that GATA4 may play a role in the proliferation and the survival of astrocytes (Agnihotri et al., 2009; Kamnasaran and Guha, 2005). Similarly, *Smed-gata4/5/6(RNAi)* caused an increase in cell death, supporting previous reports indicating GATA4 and GATA6 regulate anti-apoptotic signaling (Agnihotri et al., 2009; Rong et al., 2012; Suzuki, 2011). A careful analysis of data from Wurtzel and colleagues (Wurtzel et al., 2015) shows some expression in a "neural" cluster (Fig. S1C); expression of *Smed-gata4/5/6* in some "neural" cells might be the cause of some direct effects on neuronal differentiation in the knock-down animals. However, similar RNA-Seq data from Molinaro and colleagues (Molinaro and Pearson, 2016) in cells under homeostatic conditions indicate that a subtype of neoblasts that may contribute specifically to neuronal lineages and these cells do not express *Smed-gata4/5/6*. These analyses are clearly preliminary and do not exclude a direct effect, but, overall, would suggest that the phenotypes observed in non-intestinal lineages in *Smed-gata4/5/6(RNAi)* animals are indirect.

Another open question is why the phenotypes we describe here with *Smed-gata4/5/6(RNAi)* are different from those observed with *Smed-nkx2.2(RNAi)*, even though *Smed-nkx2.2* may be another key regulator of intestinal differentiation expressed in gamma neoblasts and differentiated intestinal cells (Forsthoefel et al., 2012). Loss of *Smed-nkx2.2* has similar effects on intestinal differentiation as loss of *Smed-gata4/5/6* but more rapid effects on overall neoblast proliferation (Forsthoefel et al., 2012). Analysis of single-cell RNA-Seq shows that the pattern of expression of the two genes is similar but not identical, which may explain these differences (for example, only 17/28 intestinal cells express both genes in Wurtzel et al. (2015) – data not shown). It is also possible that different protocols and knock-down efficiency result in different phenotypes.

In conclusion, single-cell RNA-Seq studies strongly indicate that *Smed-gata4/5/6* is expressed mostly in the intestinal lineage, from gamma neoblasts to differentiated intestinal cells (Molinaro and Pearson, 2016; Scimone et al., 2016, 2014; van Wolfswinkel et al., 2014; Wurtzel et al., 2015). Together with our observations that phenotypes in non-intestinal lineages arise late during the response to injury, this supports a model in which *Smed-gata4/5/6* plays a critical role in intestinal differentiation and wherein the differentiation of other lineages may be affected indirectly by intestinal defects (Fig. 4E). This model would fit with a conserved role of this GATA transcription factor in intestinal development and would provide a simple explanation for its role in other lineages. Other less-likely models may explain our data, including functional interactions between gamma neoblasts and other neoblast subpopulations but the inability to perform lineage-tracing assays in planarians severely limit possible investigations of the functional interactions between neoblast subclasses and different differentiation lineages in this model. In our favored model where the intestine serves as a structural and/or functional niche that normally supports the differentiation of other lineages from neoblasts, we do not understand why some lineages are more affected or more rapidly affected than others. Notably, a primary defect in the CNS has been shown to result in secondary defects in the planarian gut (Cebria and Newmark, 2007), and the converse is therefore possible. Future studies will seek to identify the mechanisms underlying such non-cell autonomous roles for the intestine in the development of other differentiation pathways in planarians.

4. Material and methods

4.1. Protein sequence and phylogenetic analysis

Smed-gata4/5/6 was found annotated in the NCBI database with the use of BLAST (GenBank accession # JF802198). Protein sequence alignments with other species and a predictive evolutionary model were created using CLUSTALW and MEGA6 software (www.megasoftware.net), respectively.

4.2. Planarian culture and RNAi

The asexual CIW4 strain of *Schmidtea mediterranea* was used in all experiments and maintained as previously described (Oviedo et al., 2008a). For RNA interference assays (RNAi), HT115 bacteria containing cDNA was cloned in to the pPR244 vector to make dsRNA as previously described (Recombinant DNA procedures approved under APB# 712-JS0510) (Reddien et al., 2005). Briefly, bacteria were grown to an OD600 of 0.6 and induced with 1 mM isopropyl-β-thiogalactopyranoside for 2 h, centrifuged and mixed with liver paste. Animals were fed every 2 days for 4 feedings. Amputation was performed four days after the final feeding. The control RNAi plasmid used contains the *C. elegans* gene *unc-22* (Addgene plasmid 1690). For dextran feeding assays, animals were fed a dextran-liver paste mixture 4 days after the last feeding and imaged 3 days later. 100μl of liver paste was mixed with 2 μl (1 mg/mL) 10,000 MW dextran conjugated to Alexa 546 (Molecular Probes), and fed to the planarians (Forsthoefel et al., 2011).

4.3. RNA analysis by RT-qPCR and in situ hybridization

RNA was extracted with TRIzol (Invitrogen). RT-PCR and quantitative Real-Time PCR were performed using the ProtoScript cDNA synthesis kit (New England BioLabs) and the PerfeCTa SYBRGreen FastMix (Quanta Biosciences), respectively. All reactions were performed in triplicates and run on an ABI 7900 HT Fast Real Time PCR System (Applied Biosystems). Fold change in expression of *Smed-gata4/5/6(RNAi)* animals shown relative to controls (*unc-22(RNAi)* animals) after all CT values are normalized to the internal control *Smed-GAPDH*. See Supporting Information Table S1 for primer sequences. Animals were fixed and whole-mount in situ hybridization (WISH) was performed as previously described (King and Newmark, 2013; Pearson et al., 2009).

4.4. Immunostaining, TUNEL assay, and image processing

Planarians were fixed and immunostaining was performed as previously reported (Oviedo et al., 2008b). Antibodies were used at the following dilutions: 1:250 anti-phospho-histone H3 (phosphorylated Serine 10 on histone H3, Millipore), 1:75 anti-SYNORF1 (Synapsin, Developmental Studies Hybridoma Bank), 1:800 anti-rabbit DyLight 594 conjugated and 1:500 anti-mouse HRP. Tyramide development was performed as previously described (Cowles et al., 2012). TUNEL assay on whole worms was performed as previously described (Pellettieri et al., 2010). Counted foci were normalized to the area (mm^2) using NIS element software (Nikon). Digital images were captured using a Nikon AZ-100 multizoom microscope and NIS Elements AR 3.2 software. Area measurements and scale bars were calculated on NIS Elements AR 3.2 software.

For TUNEL assay on sections, animals were fixed according to (King and Newmark, 2013). Briefly, animals were killed in 5% N-acetyl cysteine in PBS for 5 min, then fixed in 4% formaldehyde in PBSTx (0.3% Triton X-100) for 30 min, and washed 2 × in PBSTx. Animals were then dehydrated in 50% methanol in PBSTx followed by 100% methanol and stored for <2weeks at $-20\,°C$ in 100% methanol before being processed and embedded in paraffin. Blocks were serially sectioned at 10 µM. For staining the ApopTag® Red Apoptosis Detection Kit (Millipore cat. S7165) protocol was followed. Immunostaining for anti-Smed-6G10 (Developmental Studies Hybridoma Bank) was performed as previously reported (Ross et al., 2015). Counted foci were normalized to the area (mm^2) using Image J. Digital images were captured using a Keyence All-in-one Fluorescent Microscope BZ-X700 series and BZ-X Analyzer software. Scale bars and area measurements were calculated on BZ-X Analyzer software and Image J, respectively. Adobe Photoshop was used to adjust the brightness and contrast, and merge images.

4.5. Statistical analyses

Statistical analyses, two-way ANOVA and Student's *t*-Test, were performed using Prism6 (GraphPad).

Author contribution

NF: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing.

NO: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing.

JS: Conception and design, data analysis and interpretation, manuscript writing.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.08.015.

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