Int. J. Dev. Biol. 61: 53-63 (2017)
doi: 10.1387/ijdb.160321es

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The pioneer factor *Smed-gata456-1* is required for gut cell differentiation and maintenance in planarians

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ABSTRACT How adult stem cells differentiate into different cell types remains one of the most intriguing questions in regenerative medicine. Pioneer factors are transcription factors that can bind to and open chromatin, and are among the first elements involved in cell differentiation. We used the freshwater planarian *Schmidtea mediterranea* as a model system to study the role of the gata456 family of pioneer factors in gut cell differentiation during both regeneration and maintenance of the digestive system. Our findings reveal the presence of two members of the gata456 family in the *Schmidtea mediterranea* genome; *Smed-gata456-1* and *Smed-gata456-2.* Our results show that *Smed-gata456-1* is the only ortholog with a gut cell-related function. *Smed-gata456-1* is essential for the differentiation of precursors into intestinal cells and for the survival of these differentiated cells, indicating a key role in gut regeneration and maintenance. Furthermore, tissues other than the gut appear normal following *Smed-gata456-1 RNA* interference (RNAi), indicating a gut-specific function. Importantly, different neoblast subtypes are unaffected by *Smed-gata456-1(RNAi),* suggesting that *1) Smed-gata456-1* is involved in the differentiation and maintenance, but not in the early determination, of gut cells; and 2) that the stem cell compartment is not dependent on a functional gut.

KEY WORDS: *gut, gata456, planarian, regeneration, differentiation*

Introduction

One of the most intriguing aspects of regenerative medicine is the ability of adult stem cells to differentiate into multiple cell types. Transcription factors, which drive transcriptional regulation in the cell, play crucial roles in determination and differentiation processes. Pioneer factors, a specific type of transcription factor, can bind to condensed chromatin, thus facilitating the binding of other transcription factors. Pioneer factors are often among the first elements to participate in the induction of determination and differentiation processes (rev. in Zaret and Carroll, 2011). Specifically, the gata456 subfamily of pioneer factors has a conserved role in endoderm determination and gut regionalization, and is active during both embryonic development (Fukushige *et al.,* 1998; Reiter *et al.,* 2001) and adulthood (Beuling *et al.,* 2012; Okumura *et al.,* 2016).

To better understand stem cell determination and differentiation, we investigated the role of gata456 factors in the determination and differentiation of intestinal cells, which have a high rate of renewal, using the highly regenerative freshwater planarian *Schmidtea mediterranea* as a model species (Saló, 2006). Planarians can regenerate any lost body part and continuously renew all tissues during homeostasis, altering body size in accordance with nutrient availability. This continuous cell turnover during planarian regeneration and homeostasis is sustained by an abundant population of adult pluripotent stem cells called neoblasts (Baguñà, 2012), the proliferation, determination and differentiation of which is subject to complex and robust control. Planarians thus constitute an ideal system in which to study the processes of stem cell maintenance and specific fate determination, allowing us to analyse and compare gut cell specification and differentiation in two different scenarios, homeostasis and regeneration.

Our findings reveal the presence of two members of the gata456 family in the *Schmidtea mediterranea* genome; *Smed-gata456-1* and *Smed-gata456-2*. *Smed-gata456-1* is expressed in gut cells

Abbreviations used in this paper: RNAi, RNA interference.

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Supplementary Material (8 figures) for this paper is available at: http://dx.doi.org/10.1387/ijdb.160321es

Submitted: 9 September, 2016; *Accepted:* 12 December, 2016.

ISSN: Online 1696-3547, Print 0214-6282

and is essential for gut regeneration, while *Smed-gata456-2* is involved in re-establishing mediolateral patterning during anterior regeneration. Our results show that *Smed-gata456-1* is crucial for the regeneration, remodelling, and maintenance of gut tissue. In contrast to previous findings reported for *Smed-nkx2.2* and *Smed-egfr-1,* which are also implicated in gut regeneration (Forsthoefel *et al.,* 2012; Barberán *et al.,* 2016), we show that *Smed-gata456-1* is not required for stem cell functionality, suggesting that 1) *Smed-gata456-1* is involved in the differentiation and maintenance, but not the early determination, of gut cells; and 2) the stem cell compartment is not dependent on a functional gut. Moreover, apart from the intestine, no other tissues appear to be affected in animals subjected to *Smed-gata456-1* RNA interference (RNAi), suggesting an exclusive role of *Smed-gata456-1* in the differentiation and maintenance of planarian gut cells.

Results

Smed-gata456-1 *is the planarian ortholog essential for gut regeneration*

By searching the transcriptomic database Planmine (Brandl *et al.,* 2016) we identified in the transcriptome of *S. mediterranea* two gata456 orthologs, which we named *Smed-gata456-1* (previously described as *Smed-gata456* in Wagner *et al.,* 2011) and *Smed-gata456-2* (S. Figs. 1 and 2). These genes correspond to the homologs of *gata456* factors described in the planarian species *Schmidtea polychroa* (*Spol-gata456A* and *Spol-gata456B*) (Martín-Durán and Romero, 2011). The same two homologs are found in four other planarian species, the transcriptomes of which are available in Planmine (see phylogenetic analysis in S. Fig. 1 and sequences in S. Fig. 2).

The expression of *Smed-gata456-1* and *Smed-gata456-2* was determined by whole mount *in situ* hybridization. In intact animals, *Smed-gata456-1* was expressed in the gut and the surrounding parenchyma (S. Fig. 3A). The parenchyma associated expression disappeared 1 day after X ray irradiation, demonstrating that

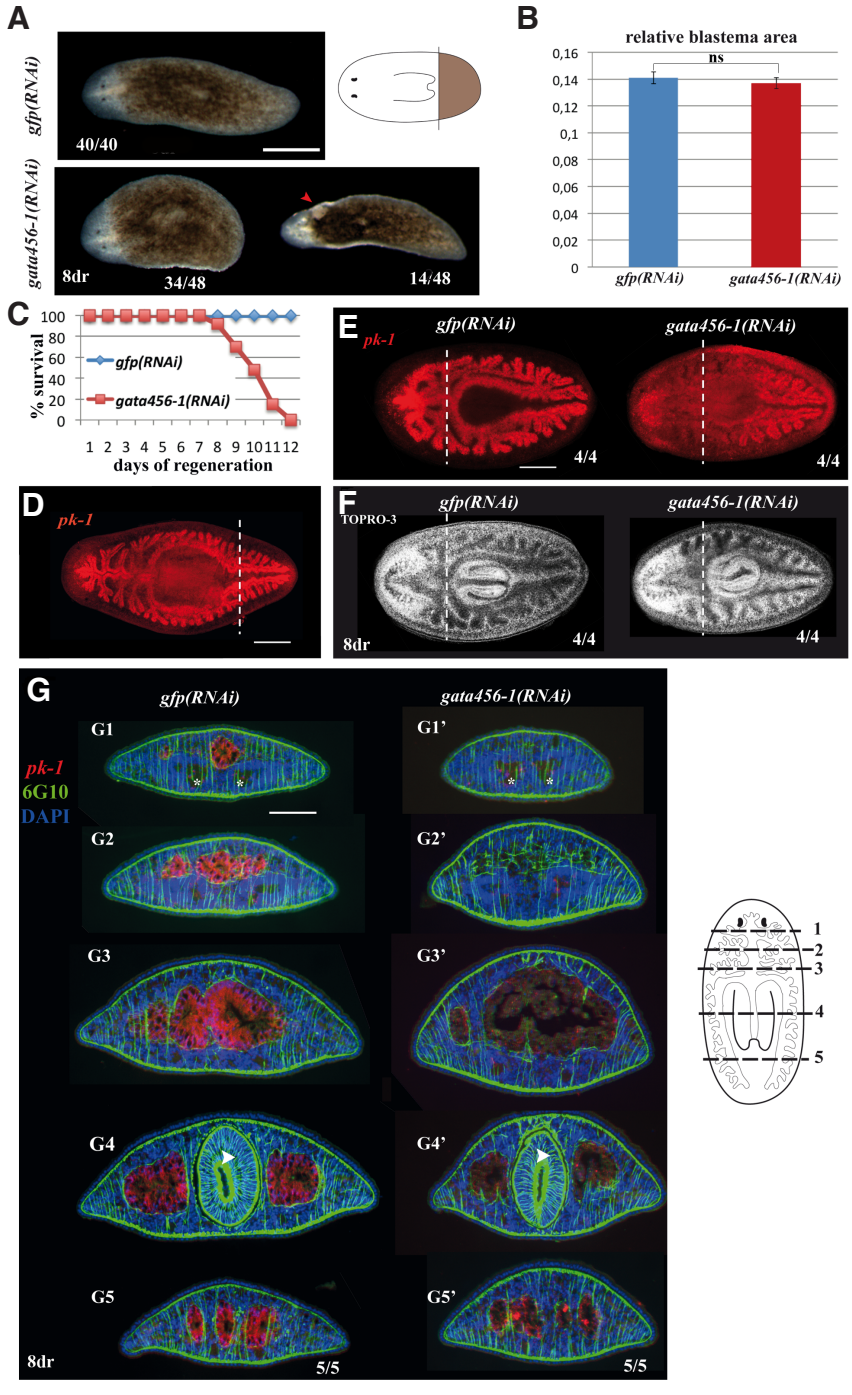

Fig. 1. Gut regeneration and remodelling in regenerating tail fragments is impaired by *Smed-gata456-1(RNAi)*. **(A)** *Live control and* Smed-gata456-1(RNAi) *regenerating tail fragments at 8 days of regeneration. Red arrowhead indicates a lesion.* **(B)** *Quantification of the relative area of the blastema with respect to the total area of the regenerating tails. Error bars represent the standard error of the mean. Data were analyzed by Student's t-test; differences are considered significant at P<0.05.* **(C)** *Graph showing survival of regenerating tails of control (n=45) and* Smed-gata456-1(RNAi) *animals (n=48).* **(D)** *Whole mount fluorescent* in situ *hybridization with the intestinal marker* pk-1, *prior to amputation. White dashed lines correspond to the plane of sectioning.* **(E)** *Whole mount fluorescent* in situ *hybridization for the intestinal marker* pk-1 *in a regenerating tail fragment, showing an absence of labelling in the new tissue of* Smed-gata456-1(RNAi) *animal.* **(F)** *Nuclear TOPRO-3 labelling in animals shown in E. White dashed lines in E and F indicates the estimated limit between the pre-existent and the new tissue, since in tail fragments the new pharynx always appears in the anterior pre-existent tissue.* **(G)** *Fluorescent* in situ *hybridization for* pk-1 *(red) combined with immunohistochemistry using the muscular antibody 6G10 (green) in serial transverse axial sections from the anterior tip (F1-1') to the posterior tip (F5-5') of the animal. Black dashed lines indicate the location of each transverse section. The anterior end is oriented to the left in (A), and in (E-F). The dorsal aspect is oriented to the top in (G). All images correspond to tail fragments after 8 days of regeneration. dr, days of regeneration. Scale bars: 500 μm in (A,D), 200 μm in (E-F), 100 μm in (G).*

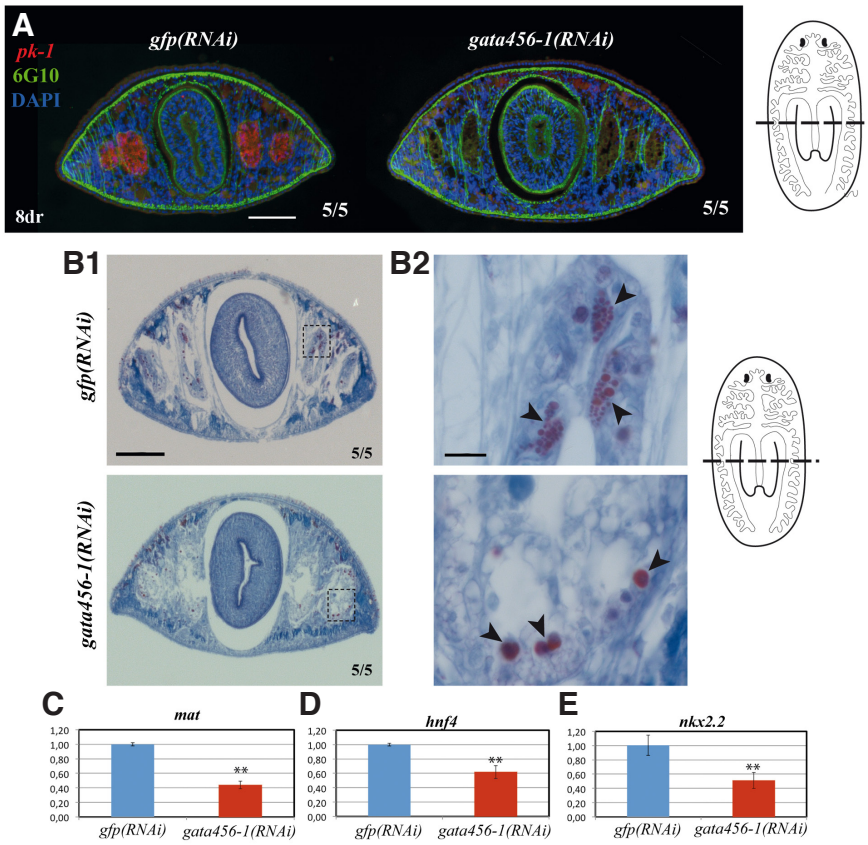

Fig. 2. *Smed-gata456-1(RNAi)* impairs gut remodelling during regeneration. (A) *Fluorescent* in situ *hybridization for* pk-1 *(red) combined with immunohistochemistry with the 6G10 antibody (green) in transverse sections of the pharyngeal region from a bipolar regenerating trunk at 8 days of regeneration, showing an absence of labelling in the pre-existing gut in the* Smed-gata456-1(RNAi*) animal.* **(B)** *Mallory staining of transverse sections. Magnified images in B2 correspond to dashed squares in B1. Arrowheads indicate secretory vesicles of goblet cells.* **(C-E)** *qRT-PCR for the gut markers* Smed-mat, Smed-hnf4, *and* Smed-nkx2.2. *Error bars represent the standard error of the mean. Data were analyzed by Student's t-test. **P<0.001. The dorsal aspect is oriented to the top in (A,B). Scale bars: 100 μm in (A,B1); and 10 μm in (B2). Dashed lines indicate the location of the transverse sections.*

Smed-gata456-1 is expressed in neoblasts (S. Fig. 3A') (Wagner *et al.,* 2011). At 2 days of regeneration, *Smed-gata456-1* was highly expressed in the tips of the regenerating gut, in one anterior and two posterior clusters that corresponded to the anterior and the two posterior gut branches, respectively (S. Fig. 3B). Its homolog *Spol-gata456A* is also specifically present in embryonic gut cells (Martín-Durán and Romero, 2011). *Smed-gata456-2* was expressed in the parenchyma, especially around the anterior gut branch, the pharynx, and between the posterior gut branches (S. Fig. 3C), following an expression pattern similar to that observed for *Spol-gata456b* in *S. polychroa* (Martín-Durán and Romero, 2011).

To characterize their function, we knocked down *Smed-gata456-1* and *Smed-gata456-2* expression by injection of double-stranded RNA (dsRNA). Inhibition of *Smed-gata456-1*, confirmed by qRT-PCR (S. Fig. 4A), resulted in normal early regeneration, with the formation of an anterior blastema and eyes in a regenerating tail (Fig. 1A). The relative size of the blastema at 8 days of regeneration shows no difference between *Smed-gata456-1(RNAi)* and the corresponding controls (Fig. 1B). However, beginning as early as day 7 of regeneration, small lesions gradually appeared in some animals, predominantly in the anterior region, followed by death of the animal the following day. All *Smed-gata456-1(RNAi)* animals died between 8 and 12 days of regeneration (Fig. 1C), i.e., 12 to 16 days after the last dsRNA injection. Regenerating trunk and head fragments showed a similar phenotype, although head fragments died earlier, after 5 to 7 days of regeneration (S. Fig. 4B-E). These results indicate that *Smed-gata456-1(RNAi)* does not interfere with early regeneration processes but is necessary for survival in later stages.

To examine the possible role of *Smed-gata456-1* in the gut, we analysed the intestine in regenerating tail fragments in order to clearly differentiate *de novo* gut regeneration from remodelling of the pre-existing gut. Whole mount fluorescent *in situ* hybridization with the intestinal marker *Smed-pk-1* (Fraguas *et al.,* 2014) allow to visualize the planarian gut, which is composed of one anterior and two posterior gut branches that fuse in the region anterior to the pharynx (Fig. 1D, Forsthoefel *et al.,* 2011). Analysis of *pk-1* expression revealed that *Smed-gata456-1(RNAi)* animals are unable to regenerate the single anterior branch (Fig. 1E), although nuclear TOPRO-3 staining revealed apparently normal anterior regeneration (Fig. 1F). Moreover, *pk-1* expression was absent in the central region of the animal, indicating that the two pre-existing gut branches failed to properly remodel and maintain their structure (Fig. 1E). Analysis of *pk-1* in transverse sections, combined with 6G10 immunohistochemistry to label muscle fibres (Ross *et al.*, 2015) and delimit the muscular intestinal plexus, also revealed an absence of gut labelling in the sections corresponding to the newly regenerated anterior portion of the animal (Fig. 1G1'-1G2'), and a faint, non-structured signal in the pre-existing posterior tip (Fig. 1G5'). Examination of the muscle fibres surrounding the gut suggested that, although the pre-existing posterior gut branches are remodelled to form the single anterior gut branch in the pre-pharyngeal region (Fig. 1G3'), this anterior branch cannot elongate through the anterior tip of the animal (Fig. 1 E,G'). Nuclear DAPI staining revealed the depletion of intestinal nuclei after *Smed-gata456-1(RNAi)* (S. Fig. 5). Taken together, these results indicate that gut regeneration is blocked and gut remodelling impaired in *Smed-gata456-1(RNAi)* animals.

Inhibition of *Smed-gata456-2* resulted in aberrant regeneration of the anterior region of the animal. These animals regenerated two fused cephalic ganglia in the midline with a single cyclopic eye (S. Fig. 6 A-D). These defects in anterior regeneration were not due to alterations in early proliferation, which was normal in the anterior postblastema after both 6 and 48 hours of regeneration (S. Fig. 6E). Whole mount fluorescent *in situ* hybridization with the intestinal marker *pk-1* revealed both anterior (S. Fig. 6F) and posterior (S. Fig. 6G) gut regeneration, although the length of the anterior gut branch in relation to the prepharyngeal region was slightly shorter than in controls (S. Fig. 6H).

In summary, planarians possess two *Smed-gata456* orthologs. Only *Smed-gata456-1* is expressed in gut cells, and plays an essential role in gut regeneration and remodelling, while *Smed-gata456-2* is implicated in anterior mediolateral patterning.

Smed-gata456-1 *is necessary for gut remodelling and maintenance*

To better understand the role of *Smed-gata456-1* in the gut, we analysed gut remodelling in bilaterally regenerating trunks at 8 days of regeneration and in intact animals using fluorescent *in situ* hybridization with *pk-1* and 6G10 immunohistochemistry. Analysis of regenerating planarians showed no *pk-1* labelling in the gut of the pharyngeal region, which corresponds to pre-existing tissue (Fig. 2A). Even though the enteric muscle, as visualized by 6G10 immunohistochemistry, appeared unaffected (Fig. 2A), the gastrodermis of *Smed-gata456-1(RNAi)* animals in the pre-existing pharyngeal region was histologically disorganized (Fig. 2A). Disruption of the pre-existing gut was also observed in Mallory-stained sections after *Smed-gata456-1(RNAi)* (Fig. 2B), in which the gut tissue appears blotted and the brown secretory vesicles characteristic of goblet cells were found dispersed throughout the gut tissue (Fig. 2B). The disorganization of gut tissue during planarian regeneration was confirmed by the observed down-regulation in *Smed-gata456-1(RNAi)* animals of *Smed-mat* (Wagner *et al.,* 2011), a marker of differentiated gut tissue, as determined by qRT-PCR (Fig. 2C), and of *Smed-hnf4* (Wagner *et al.,* 2011) and *Smed-nkx2.2* (Forsthoefel *et al.,* 2012), both in differentiated and in gut precursor cells (Fig. 2 D-E). These results confirm the destruction of the pre-existing gut in regenerating animals and point to a role of *Smed-gata456-1* in gut cell differentiation and survival.

In intact, non-regenerating animals *Smed-gata456-1(RNAi)* generated lesions and the death of the animals

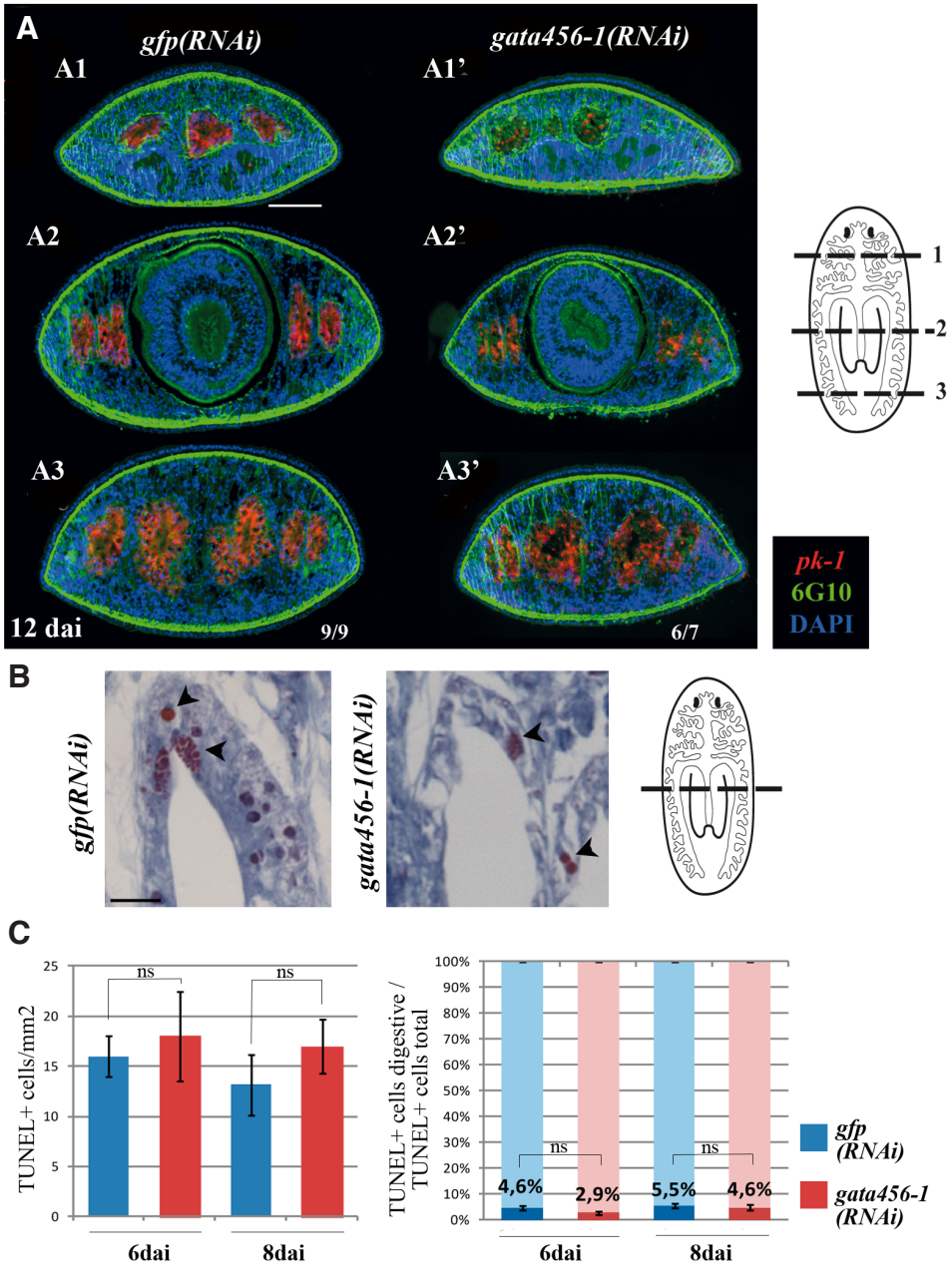

Fig. 3. *Smed-gata456-1(RNAi)* impairs gut maintenance during homeostasis. (A) *Fluorescent* in situ *hybridization for* pk-1 *(red) combined with immunohistochemistry with the 6G10 antibody (green) in an axial series of transverse sections from intact animals 12 days after the last injection.* **(B)** *Mallory staining of transverse sections from intact animals 12 days after the last injection. Arrowheads indicate secretory vesicles of goblet cells.* **(C)** *Quantification of TUNEL + cells in intact animals 6 and 8 days after the last dsRNA injection. Left, total number of TUNEL+ cells /mm² (n=6-7 sections corresponding to 3 animals at the 6 days time point and n=13-14 sections corresponding to 3 animals at the 8 days time point); right, percentage of TUNEL+ cells found in the gastrodermis (n=10-15 sections corresponding to 3 animals at the 6 and 8 days time points). Error bars represent the standard error of the mean; differences are considered significant at P<0.05. The dorsal aspect is oriented to the top in (A,B). Scale bars: 100 μm in (A); and 10 μm in (B). Dashed lines indicate the location of the transverse sections. dai, days after injection.*

between 12 and 16 days after the last dsRNA injection (S. Fig. 7 A-B). Analysis of gut tissue by *pk-1 in situ* hybridization revealed downregulation of *pk-1* expression after *Smed-gata456-1(RNAi)* (Fig. 3 A-A'). This loss of intestinal labelling occurred gradually in an anterior to posterior direction, and was stronger in the anterior (Fig. 3A1') versus the central (Fig. 3A2') and posterior (Fig. 3A3') regions of the animal. Observation of nuclear DAPI staining in those animals demonstrates the gradual loss of gut cells in *Smed-gata456-1(RNAi)* animals (S. Fig. 7C). Mallory staining further demonstrates the disorganization of the gastrodermis in *Smed-gata456-1(RNAi)* animals 12 days after the last dsRNA injection (Fig. 3B). These results indicate that *Smed-gata456-1* is also required for gut cell maintenance during homeostatic tissue renewal.

To check whether the disruption of the gut tissues was caused by the death of pre-existent gut cells, we analyzed the apoptotic levels in *Smed-gata456-1(RNAi)* animals and their corresponding controls through quantification of caspase-3 enzymatic activity (González-Estévez *et al.,* 2007) and TUNEL (Pelletieri *et al.,* 2010). We analyzed animals 6 and 8 days after the last dsRNA injection, since during this period the gut phenotype is set up (S. Fig. 8A). Our results demonstrate that *Smed-gata456-1(RNAi)* animals do not show an increase in the total caspase-3 activity or in the number of TUNEL positive cells with respect to the controls (S. Fig. 8B, 3C). Furthermore, our results demonstrate that the percentage of apoptotic cells in the gut compared to the total number of apoptotic cells is maintained in *Smed-gata456-1(RNAi)* animals with respect to the controls (Fig. 3C). Thus, the disappearance of gut cells in *Smed-gata456-1(RNAi)* planarians is not due to an increase in apoptosis.

Our results suggest that the gut impairment is caused by problems in cell replacement due to improper terminal differentiation of gut cells. However, the early and severe disruption of the gut indicates that *Smed-gata456-1* could have a direct role in the maintenance or survival of differentiated gut cells. According to the Mallory staining (Fig. 2B, 3B), gut cells of *Smed-gata456-1 (RNAi)* animals could be dying through a caspase-independent mechanism as necrosis.

Taken together, these results demonstrate an essential role of *Smed-gata456-1* in the regeneration, remodelling, and maintenance of the planarian gut, both in regenerating and in intact animals. The underlying mechanism could be both in blocking terminal differentiation and maintenance of the gut cells.

Tissues other than the gut are not affected in Smed-gata456-1(RNAi) *planarians*

To determine whether the effects of *Smed-gata456-1* inhibition are gut-specific, we analysed other tissues in *Smed-gata456-1(RNAi)* animals, including the brain, eyes, epidermal precursors, excretory system, and pharynx. Nuclear staining with TOPRO-3 and DAPI revealed normal regeneration of the cephalic ganglia in *Smed-gata456-1(RNAi)* animals (Fig. 1F, asterisks in Fig. 1G1 and 1G1'). This result was confirmed by whole mount fluorescent *in situ* hybridization and qRT-PCR for the neural marker *Smed-pc2* (Collins *et al.,* 2010) (Fig. 4A,B). Moreover, normal eye regeneration was observed in *in vivo* analyses (Fig. 1A), and by double fluorescent *in situ* hybridization with *Smed-opsin* (Sánchez-Alvarado and Newmark, 1999), which labels photoreceptor cells, and *Smed-tph* (Fraguas *et al.,* 2011), which labels pigment cells (Fig. 4C). Quantification of both cell types revealed no difference between *Smed-gata456-1(RNAi)* animals and controls (Fig. 4D). qRT-PCR of the excretory system marker *Smed*-CA (Fig. 4E) (Scimone *et al.,* 2011) and the epidermal precursors *Smed-prog-1*

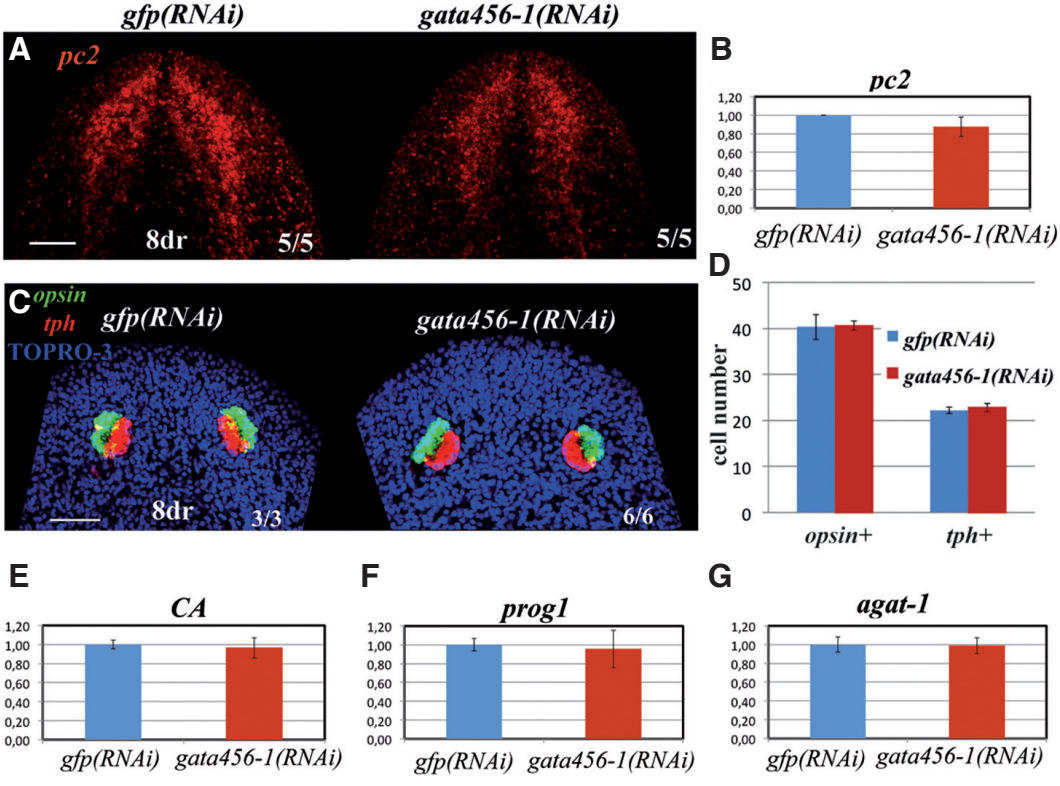

Fig. 4. Non-intestinal tissues are unaffected by *Smed-gata456-1(RNAi)*. (A-B) *Whole mount fluorescent* in situ *hybridization* **(A)** *and qRT-PCR* **(B)** *for the neural marker* pc2. **(C)** *Whole mount double fluorescent* in situ *hybridization for the eye markers* opsin *(green), a photoreceptor cell marker, and* tph *(red), a pigment cell marker.* **(D)** *Quantification of* opsin*-positive cells and* tph*-positive cells* **(E-F)** *qRT-PCR of markers of the excretory system* **(E)** *and epidermal precursors* **(F, G)**. *All experiments were performed on bipolar regenerating trunks after 8 days of regeneration. dr, days of regeneration. Error bars represent the standard error of the mean. Data were analyzed by Student's t-test; differences are considered significant at P<0.05. Scale bars: 100 μm in (A), 50 μm in (C).*

(Fig. 4F) and *Smed-agat1* (Fig. 4G) (Eisenhoffer *et al.,* 2008; van Wolfswinkel *et al.,* 2014) also showed no significant differences between *Smed-gata456-1(RNAi)* animals and controls. Finally, despite the observed disruption of the intestinal tissue, in *Smed-gata456-1(RNAi)* regenerating tails a new pharynx was formed within the pre-existing tissue (Fig. 1F, arrowhead in Fig. 1G4 and 1G4'). Thus, apart from the gut, all tissues analysed (eyes, CNS, excretory system, pharynx, and epidermal lineage) showed normal regeneration following *Smed-gata456-1(RNAi)*, indicating that *Smed-gata456-1* is specifically required for gut regeneration and maintenance.

The neoblast population is unaffected by Smed-gata456-1(RNAi)

Stem cells are one possible source of the defects observed in gut regeneration and maintenance in *Smed-gata456-1(RNAi)* animals. Alterations in the proliferation of neoblasts or their determination and differentiation into gut cells could contribute to the phenotype observed in these animals. Furthermore, it is possible that these gut defects in turn affect stem cell function (Forsthoefel *et al.,* 2012).

To identify potential neoblast alterations, we analysed expression of the general neoblast markers *Smedwi-1* and *Smed-h2b* (Guo *et al.,* 2006). qRT-PCR of *Smedwi-1* revealed no alterations following *Smed-gata456-1(RNAi)* (Fig. 5A). Moreover, fluorescent *in situ* hybridization in transverse sections with a *Smed-h2b* probe revealed a normal distribution of neoblasts, both in the newly regenerated region (Fig. 5 B1-B1'), which lacks a regenerated gut, and in the pre-existing region (Fig. 5 B2-B2'), in which gut branches are remodelled and a new pharynx formed. Thus, neoblasts were distributed in the parenchyma and absent from the gastrodermis. Finally, we investigated whether the lack of neoblast affectation in *Smed-gata456-1(RNAi)* animals was accompanied by normal proliferation. Immunohistochemistry for phospho-histone H3 (H3P) revealed no significant differences in mitosis with respect to controls (Fig. 5C), neither during early regeneration nor at later stages. Based on these findings, we conclude that *Smed-gata456-1(RNAi)* has no effect on neoblast number, distribution, or proliferation.

Neoblasts have been described as a heterogeneous population of stem cells, and can be characterized by different attributes such as membrane antigens (Moritz *et al.,* 2012) or their ability to give rise to distinct cell lineages (van Wolfswinkel *et al.,* 2014). The latter study describes two main sub-classes of neoblasts: sigma-neoblasts, which have a broad spectrum of possible fates, and zeta-neoblasts, which are restricted to the epidermal lineage. qRT-PCR of *Smed-soxP-2*, a sigma-neoblast marker, and of *Smed-zfp-1*, a zeta-neoblast marker, revealed no significant alterations in *Smed-gata456-1(RNAi)* animals with respect to controls (Fig. 5D and E). Furthermore, analysis of the specific transcription factor *Smed-prox-1* to quantify levels of a sub-group of sigma-neoblasts, the gamma-neoblasts, which are defined as gut precursor cells, revealed no differences between *Smed-gata456-1(RNAi)* animals and controls (Fig. 5F).

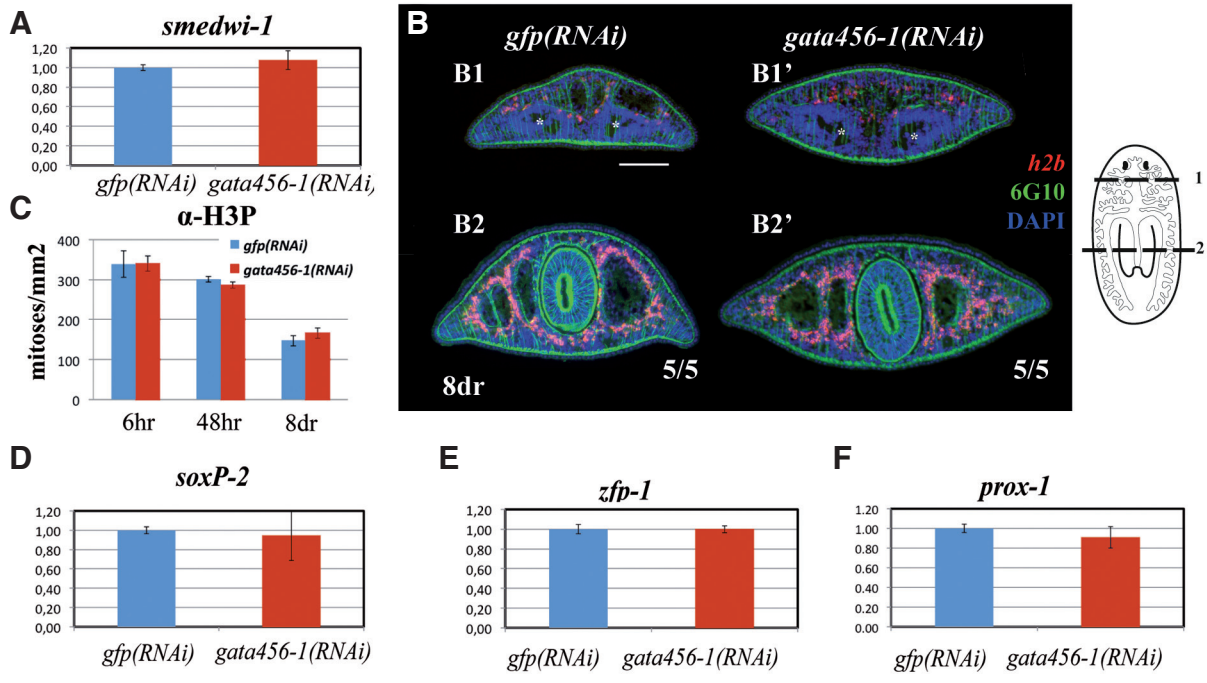

Fig. 5. Normal stem cell distribution and function after *Smed-gata456-1(RNAi)*. (A) *qRT-PCR for the neoblast marker* smedwi-1. **(B)** *Fluorescent* in situ *hybridization for the neoblast marker* h2b *(red) combined with immunohistochemistry with the muscle antibody 6G10 (green) in transverse sections of newly regenerated tissue* **(B1-B1')** *and the pre-existing region* **(B2-B2')** *of a regenerating tail at 8 days of regeneration. The dorsal aspect is oriented to the top.* **(C)** *Quantification of mitotic cells by anti-H3P immunohistochemistry in the whole animal, at different stages of regeneration (n=6 controls and n=6* Smed-gata456-1(RNAi) *animals at 6 hours of regeneration; n=5 controls and n=6* Smed-gata456-1(RNAi) *animals at 48 hours of regeneration; n=8 controls and n=4* Smed-gata456-1(RNAi) *animals at 8 days of regeneration).* **(D-F)** *qRT-PCR for markers of different neoblast classes. Error bars represent the standard error of the mean. Data were analyzed by Student's t-test; differences are considered significant at P<0.05. Experiments were performed in bipolar regenerating trunks* **(A, D-F)** *and regenerating tails* **(B)** *after 8 days of regeneration, and in regenerating tails at different stages of regeneration* **(C)**. *hr, hours of regeneration; dr, days of regeneration Scale bar: 100 μm. Dashed lines indicate the location of transverse sections.*

Taken together, these findings indicate that neither inhibition of *Smed-gata456-1* nor disruption of the gastrodermis causes any alteration in neoblast number, type, distribution, or proliferation.

Discussion

Smed-gata456-1 plays an exclusive role in gut cell differentiation and maintenance

It has been proposed that gut regeneration involves both the differentiation of neoblasts and the migration of differentiated cells from the pre-existing gut to the regenerating zone (Forsthoefel *et al.,* 2011). Thus, in regenerating tails that must regenerate the rest of the animal, the gut near the wound site undergoes significant remodelling and actively contributes to the regeneration of the new gut. In *Smed-gata456-1(RNAi)* animals the posterior gut branches fused into a single branch in the region anterior to the newly regenerated pharynx. However, no *pk-1* labelling was observed. This result could indicate a problem during the process of differentiation of new gut cells as well as in the cell fate maintenance of the migrating gut cells. Furthermore, the overall morphology of the presumptive intestinal region, as visualised by immunohistochemistry with the muscle marker 6G10 and the nuclear marker DAPI, also appeared to be altered. This initial remodelling of the gut branches, which fused in the region anterior to the newly regenerated pharynx (Fig. 1G3'), and the subsequent failure in gut elongation, is similar to the phenotype induced by *Smed-nkx2.2(RNAi)* (Forsthoefel *et al.,* 2012), and may be explained by the contribution of pre-existing tissue to the newly generated tissue. The same mechanism of pre-existing gut cells migration may also explain the faster loss of *pk-1* labelling observed in the pharyngeal region of regenerating tails as compared with the posterior tip.

The disruption observed in the gut of *Smed-gata456-1(RNAi)* animals during homeostasis, as well as the lack of gut remodelling in the pre-existent region of regenerating animals also suggests a role of *Smed-gata456-1* in the differentiation of gut cell precursors (gamma-neoblasts) into differentiated gut cells. However, it has been shown that blockade of gut cell differentiation trough *Smed-egfr-1(RNAi)* allows the maintenance of the old gut for several weeks (Barberán *et al.,* 2016). The rapid (8 days after last dsRNA injection in intact animals) and severe disruption of the pre-existing gut following *Smed-gata456-1* inhibition suggest that *Smed-gata456-1* plays an additional role in the maintenance or survival of differentiated gut cells. Quantification of caspase-3 activity and TUNEL staining revealed that apoptosis was not the cause of cell death in *Smed-gata456-1(RNAi)* animals. The disruption of the gastrodermis revealed by Mallory staining, characterized by soft and swollen tissue with randomly dispersed secretory vesicles of goblet cells, suggests necrotic destruction of gut cells.

The general disruption of the gut observed after *Smed-gata456-1(RNAi)* was accompanied by the appearance of lesions all over the animal between 11 and 15 days after the last injection, both in regenerating and non-regenerating animals (Fig. 1A, Fig. S4 B,D and 7A), followed by death one day later (Fig. 1C, Fig S4 C,E and 7B). In contrast to *Smed-nkx2.2(RNAi)* animals (Forsthoefel *et al.,* 2012), the lesions observed affected the entire body and not just the region over the gut. It is possible that disruption of the gut triggers the rupture of digestive vesicles, provoking the digestion of planarian tissues, which gives rise to the observed lesions and ultimately leads to death. That markers of epidermal cell lineages (*Smed-prog-1, Smed-agat-1*) were unaltered in *Smed-gata456-1(RNAi)* animals supports the view that these lesions are caused not by the impairment of epidermal differentiation but rather by a direct mechanism such as ectopic digestion. Furthermore, the decrease in caspase-3 activity observed in *Smed-gata456-1(RNAi)* intact animals 8 days after the last dsRNA injection, indicates that lesions are not produced by a caspase-3 dependent mechanism as programmed cell death.

Although the gut disruption and the lesions were observed throughout the body of *Smed-gata456-1(RNAi)* animals, a graded affectation along the antero-posterior axis was evident in both regenerating and intact animals. Thus, lesions were more prevalent in the anterior portion, and intestinal *pk-1* labelling was weaker in the anterior region of non-regenerating animals. This graded affectation is also observed following inhibition of other genes involved in gut regeneration. For example, *Smed-nkx2.2(RNAi)* results in partial depletion of neoblasts exclusively in the anterior portion (Forsthoefel *et al.,* 2012), while the reduction in size and number of secondary, tertiary, and quaternary gut branches observed after *Smed-egfr-1(RNAi)* is more severe in the anterior region (Barberán *et al.,* 2016). This phenomenon may be in part attributable to the physiological gradient theory, that proposes a quantitative difference in metabolic activity along the A-P axis (Child, 1929; Blackstone, 2006), as suggested by the higher cell turnover in anterior-most regions (Eisenhoffer *et al.,* 2008).

Overall, our results suggest a role of *Smed-gata456-1* in the differentiation and maintenance of gut cells in *Schmidtea mediterranea*. This dual function is also observed in other models such as *Drosophila melanogaster*, in which dGATAe is required for late differentiation and to maintain the correct shape and expression profile of adult enterocytes (Murakami *et al.,* 2005; Buchon *et al.,* 2013). The expansion of the gata456 family (Gillis *et al.,* 2008; Tang *et al.,* 2014) leaves open the possibility that different members of this family have other gut-related functions, such as early determination of gut cells. In *D. melanogaster* another member of the family, srp/dGATAb, acts as a selector gene in endoderm determination (Reuter, 1994), while in *Caenorhabditis elegans end-2* is necessary for the early determination of the endoderm (Zhu *et al*., 1997) and *elt-2* is essential for terminal differentiation of the gut (Fukushige *et al*., 1998). However, although further experiments are required, the second member of the *gata456* family found in planarians (*Smed-gata456-2*) does not appear to have any direct function in gut tissues.

Despite the severe affliction of the gut in *Smed-gata456-1(RNAi)* animals, none of the other tissues analysed in the present study, including the nervous system, excretory system, and the eyes, showed any alterations. This was particularly obvious in regenerating *Smed-gata456-1(RNAi)* tails, in which the brain and even the pharynx were regenerated *de novo* in animals that failed to regenerate the anterior gut branch (Fig. 1 E-F). Quantification of specific non-gut-related markers further demonstrated that structures other that the gut were unaffected. This observation contradicts a recent study (Flores *et al.,* 2016) in which *Smed-gata456-1* silencing resulted in similar disruption of gut regeneration and maintenance, accompanied by lesions and death of the animal, but also caused defects in the regeneration of the nervous system and eyes. One potential explanation for these conflicting findings is the different protocols used for RNAi; Flores and coworkers used dsRNA feeding, which is less effective than injection and produces

general and sustained activation of proliferation due to food ingestion. This effect is evident in the longer survival time, measured from the first day of RNAi treatment, reported by Flores *et al.,* The timing of the experiment should be also considered; our experiments involved a 7-day interval between the first dsRNA injection and amputation, as compared with the 11-day interval used by Flores *et al.,* The milder and longer RNAi treatment used by Flores *et al.,* may thus have resulted in earlier, but less apparent gut disruption, ultimately causing general disruption of the different cell compartments and tissues that could be observed before death. In our experiments, the rapid tissue deterioration observed in the gut was not observed in any other of the other tissues analysed, which showed normal early regenerative behaviour.

The lack of neoblast affectation following Smed-gata456-1(RNAi) *questions the existence of a gut neoblast niche*

Previous findings demonstrating an increase in proliferation shortly after feeding (Baguñà, 1976,) suggest that this rapid response may be mediated by close communication between intestinal cells and neoblasts. Thus, the planarian gut may act as a neoblast "niche", sending proliferation, differentiation, or self-renewal signals to neoblasts. Forsthoefel and coworkers reported that inhibition of *Smed-nkx2.2* decreases proliferation after affecting the gut (Forsthoefel *et al.,* 2012). However, those authors proposed that this decrease in proliferation may involve elements downstream of the inhibited gene, rather than the gut itself. By contrast, Barberán and coworkers show that inhibition of *Smed-egfr-1*, which blocks the differentiation of new gut cells and leads to progressive decay of the gut, results in neoblast accumulation and increased proliferation (Barberán *et al.,* 2016). Although these two findings appear contradictory, both are in agreement with the proposed close correlation between neoblasts and the gut. In our experiments, we observed no alterations in the stem cell compartment in *Smed-gata456-1(RNAi)* animals, in which the gut compartment appears to be specifically affected, and no defects in neoblast distribution nor proliferation were observed. Furthermore, these animals displayed no alterations in the different neoblast subpopulations. In the case of gamma-neoblasts, although *prox-1* levels were normal, qRT-PCR for the markers *Smed-hnf4* and *Smed-nkx2.2* revealed clear downregulation following *Smed-gata456-1(RNAi)*. Given that *prox-1* is specifically expressed in gamma neoblasts, and *Smed-hnf4* and *Smed-nkx2.2* are expressed in both gamma neoblasts and in differentiated gut cells (Wurtzel *et al.,* 2015), the observed downregulation of *Smed-hnf4* and *Smed-nkx2.2* may be a result of the defects observed in the gut itself. Although *Smed-gata456-1* is expressed in gamma neoblasts (van Wolfswinkel *et al.,* 2014), it may only be required for the differentiation of these cells to intestinal cells, but not for their maintenance. It is also possible that *Smed-gata456-1* inhibition affects communication between the two compartments, and hence that stem cells do not sense the disruption of the gut. In contrast to our results, Flores and colleagues reported that *Smed-gata456-1* inhibition causes downregulation of all neoblast types, particularly gamma-neoblasts. The same argument proposed above for the differences observed regarding central nervous system and eye regeneration may explain the apparent disparity between the two studies. The dsRNA injection used in the present study compared to the dsRNA feeding used by Flores *et al.,* produces a rapid and severe gut deterioration that leaves no time to be sensed by the other compartments. Moreover, the RNAi carried out by injection does not produce a sustained increase in proliferation like the one by feeding, which could as well produce a different response of the neoblast. Thus, neoblast affectation observed in Flores *et al.,* may be explained by an indirect effect due to the experimental procedure. Our findings suggest that the primary role of *Smed-gata456-1* is in gut cell differentiation and maintenance.

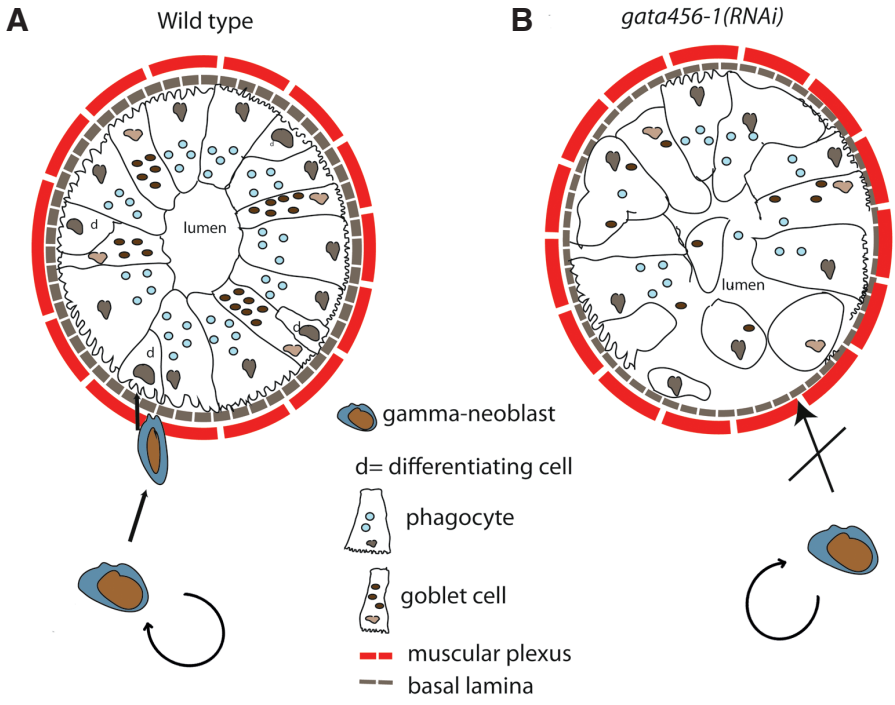

Fig. 6. Schematic showing role of *Smed-gata456-1* in intestinal differentiation and maintenance. (A) *Normal gut homeostasis. The planarian gut contains two main cell types: absorptive phagocytes (which engulf food) and goblet cells (which secrete digestive enzymes (Garcia-Corrales and Gamo, 1986). Both cell types are found interspersed within a columnar epithelium that sits upon a basal lamina, which in turn is surrounded by enteric muscles. Dividing neoblasts are absent from gut tissues, although dividing gut precursor cells (gamma-neoblasts) are observed in the parenchyma close to the gut. Gamma-neoblasts can cross the enteric muscle to enter the columnar intestinal epithelium and differentiate to maintain gut cell turnover.* **(B)** *After* Smed-gata456-1(RNAi), *gamma-neoblast differentiation into gut cells is blocked, and maintenance of pre-existing gut cells is impaired. This results in disorganization of the gastrodermis.*

Conclusion

In summary, we demonstrate that in *Schmidtea mediterranea* the pioneer factor *Smed-gata456-1* has a specific and evolutionarily conserved role in endoderm differentiation and maintenance, while *Smed-gata456-2* is involved in the establishment of anterior mediolateral patterning during regeneration. *Smed-gata456-1* is

required for gut cell differentiation from gamma neoblasts and for the maintenance or survival of differentiated gut cells (summarized in Fig. 6), and is thus essential for gut regeneration and maintenance. *Smed-gata456-1(RNAi)* results in severe and specific affectation of the gut, ultimately resulting in death, with no effects on the neoblast compartment, even the gamma neoblast subpopulation. These findings suggest that in planarians *gata456-1* is not involved in the early specification of neoblasts to gut precursors. Furthermore, the gut-specific function of *Smed-gata456-1* is confirmed by the lack of affectation of other tissues in *Smed-gata456-1(RNAi)* animals. Taken together, our results call into question the proposed existence of a gut neoblast niche.

Materials and Methods

Organisms

The planarians used in this study belong to an asexual biotype of *Schmidtea mediterranea* (asexual BCN-10 clonal line). Animals were maintained at 20°C in artificial water (Cebrià and Newmark, 2005), fed with organic veal liver, and starved for at least one week before experiments.

Molecular phylogenetic analysis

Conserved dual zinc-finger domains of GATA sequences from different species were aligned using the MAFFT server (http://mafft.cbrc.jp/alignment/server/). Neighbour-joining distance-based analyses were conducted using MEGA version 6 (Tamura *et al.,* 2013), and the support given by bootstrap percentiles of 1000 replicates.

RNAi analysis

Double-stranded RNA (dsRNA) for *Smed-gata456-1* (JF802198) and *Smed-gata456-2* (KX827244) was synthesized by *in vitro* transcription (Roche), as described previously (Sánchez Alvarado and Newmark, 1999), with a length of 469 and 419 bp, respectively. dsRNA microinjections were performed as described previously (Sánchez Alvarado and Newmark, 1999), following the standard protocol of 3 injections of 32 nl of dsRNA at a concentration of 1000 ng/μl, on 3 consecutive days. Control animals were injected with dsRNA corresponding to GFP, a gene not found in the genome of *S. mediterranea*. For regeneration experiments, *Smed-gata456-1(RNAi)* animals were amputated pre- and postpharyngeally 4 days after the last injection and allowed to regenerate. Successful RNAi was confirmed by qRT-PCR. In *Smed-gata456-2(RNAi)* experiments, animals received one round of injections on 3 consecutive days, followed by amputation on the day 4, and a second round beginning 4 days after amputation.

Irradiation

Intact planarians were X-irradiated at 96 Gy (1.6 Gy/minute) with a X ray cabinet Maxi Shot 200 (Ylon Int.) at the facilities of the Scientific and Technological Center of the University of Barcelona (CCiTUB). One day after irradiation they were processed as explained in the whole mount *in situ* hybridization protocol.

Quantitative real-time PCR

Quantitative real-time PCR experiments were performed as described previously (Solana *et al.,* 2012). All experiments were performed using 3 biological and 3 technical replicates, using a pool of 5 animals for each biological replicate. The animals used for analysis were trunk regenerating fragments at 8 days of regeneration with no wounds. Results were normalized with respect to the *ura4* gene. The following primers were used (5'→3'): agat1-1F, TCCATCCAGAACCGATTGAT; agat1-1R, CTCCCAAGTCATGGTGGACT; gata456-1-1F, TGATCCCTGGAGCTGTAAA; gata456-1-1R, TTGCTAGCTCCACAGTTCAC; hnf4-1F, ACAAGCTATCAATCTCGGCT; hnf41R, GCTGCTGACAATTCCTGA; mat-1F, GACCTGGCCACATAGAAAA; mat-1R, CCATGTAAACTCTGGTCTTCC; nkx2.2-1F, ATTCAGCGTACGGATCACT; nkx2.2-1R, TTCCATTGATATCGGGGT; pc2-1F, ACGATTTCACTGCATTGC; pc2-1R, TATCTGATTTGCACGCGT; prog1-1F, TCCGTTTTTCACGTCATCTG; prog1-1R, TTGCGTTCGCGTATATTGAA; prox-1-1F, CCATCAAACAGTTCCCAGT; prox-1-1R, GTTTGGCCTTTCGTAAATG; smedwi-1-1F, GATGGGGCTAATCCAAATCC; smedwi-1-1R, AATTCGTAGGAGATGCTGTACC; soxp2-1F, CCAGCAATTTTCCCAAAG; soxp2-1R, CCCCTTCTGAATCATCCAT; ura4-1F, TTCACGTTGTCGATCTAGCC; ura4-1R, CGAATATCCTCTGCCAGTGC; zfp1-1F, AAATTTTCCCGTGCCTG; zfp1-1R, TGATCTTTGAGTGAAGCTGGT.

Whole-mount immunohistochemistry

Immunostaining was carried out as described previously (Ross *et al.,* 2015). Animals were sacrificed in 2% HCl in milli-Q water and fixed in 4% formaldehyde in PBS/0.3% Triton. The following primary antibodies were used: α-phospho-histone H3 (H3P), to detect mitotic cells (1:500; Cell Signaling Technology); α-arrestin (VC-1), to detect planarian photoreceptor cells (1:15000; Sakai *et al.*, 2000, kindly provided by Dr. Hidefumi Orii and Prof. Kenji Watanabe); and α-SYNORF-1, to detect the pan-neural marker synapsin (1:20; Developmental Studies Hybridoma Bank). The secondary antibodies used were Alexa 568-conjugated goat α-rabbit (1:1000; Molecular Probes) and Alexa 488-conjugated goat α-mouse (1:400; Molecular Probes).

Whole-mount In situ *hybridization*

Colorimetric whole mount *in situ* hybridization was performed using an *In situ* Pro hybridization robot (Abimed/Intavis) as previously described (Molina *et al.,* 2007). Fluorescent whole mount *in situ* hybridization was performed manually as previously described (King and Newmark 2013).

Immunohistochemistry, in situ *hybridization, Mallory staining and TUNEL assay on paraffin sections*

In situ hybridization combined with immunohistochemistry using paraffin sections was performed as described previously (Barberán *et al.,* 2016). Mallory staining was performed as previously described (Sluys 1989). TUNEL staining was performed in paraffin sections as described previously (Almuedo-Castillo *et al.,* 2014) and following manufacturer's recommendations (ApopTag Red *In situ* Apoptosis Detection Kit, Merck-Millipore).

Imaging

Colorimetric whole mount *in situ* hybridization samples were observed through a Leica MS16F stereomicroscope and images captured with a Jenoptik ProgRes C3 camera. FISH images were obtained by confocal laser scanning microscopy using a Leica SPE confocal microscope. Sections were imaged using a Zeiss Axiophot microscope with a Leica DFC300FX camera for Mallory staining and a Jenoptik ProgRes MF camera for fluorescent images. Images were processed using Fiji and Photoshop CS3 (Adobe) software.

Analysis of caspase-3 activity

Quantification of caspase-3 activity was performed as described previously (González-Estévez *et al.,* 2007).

Data availability

The sequence of *Smed-gata456-2* has been deposited in GenBank with accession number KX827244.

Genbank accession numbers

Smed-gata456-2 *KX827244.*

Competing interests

The authors declare no competing of financial interests.

Authors' contributions

AGS and ES conceived and designed the study. AGS conducted most of the experiments. NDS and TA carried out the cell death and the phylogenetic analysis, respectively. AGS drafted the manuscript with contributions from

all authors. TA and ES edited the paper. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank all members of the Saló and Cebrià labs for their support and discussion, Dr. Francesc Cebrià and Dr. Iain Patten for editorial advice, Dr. Owen Howard for English language editing, and Dr. Hidefumi Orii and Prof. Kenji Watanabe for the VC-1 antibody.

Funding

This work was supported by grants BFU2011-22786 and BFU2014-56055P from the Ministerio de Economía y Competitividad, Spain (to ES), and grants 2009SGR1018 and 2014SGR-2016 from the Agència de Gestió d'Ajuts Universitaris i de Recerca (to ES). AGS is a recipient of a FPU fellowship from the MECD, Spain, and NDS is a recipient of a APIF fellowship from the Universitat de Barcelona.

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