Identification of rare, transient post-mitotic cell states that are induced by injury and required for whole-body regeneration in Schmidtea mediterranea

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Regeneration requires the coordination of stem cells, their progeny and distant differentiated tissues. Here, we present a comprehensive atlas of whole-body regeneration in Schmidtea mediterranea and identify wound-induced cell states. An analysis of 299,998 single-cell transcriptionomes captured from regeneration-competent and regeneration-incompetent fragments identified transient regeneration-activated cell states (TRACS) in the muscle, epidermis and intestine. TRACS were independent of stem cell division with distinct spatiotemporal distributions, and RNAi depletion of TRACS-enriched genes produced regeneration defects. Muscle expression of notum, follistatin, evi/wls, glypican-1 and junctophilin-1 was required for tissue polarity. Epidermal expression of agat-1/2/3, cyp3142a1, zfkh3 and atp1a1 was important for stem cell proliferation. Finally, expression of spectrinβ and atp12a in intestinal basal cells, and lirrk2, cathepsinB, myosin1e, polybromo-1 and talin-1 in intestinal enterocytes regulated stem cell proliferation and tissue remodelling, respectively. Our results identify cell types and molecules that are important for regeneration, indicating that regenerative ability can emerge from coordinated transcriptional plasticity across all three germ layers.

Robust injury and tissue repair mechanisms provide critical fitness advantages to organisms and regenerative capacity is widely, but heterogeneously, distributed across the animal kingdom. Invertebrate species can regenerate entire body plans from dissociated cells or small tissue fragments12, whereas fish and amphibians have the ability to regenerate appendages and organs after damage or amputation13–15. Even in mammals with relatively limited regenerative potential, numerous organ systems can replace damaged tissue or repopulate ablated cellular compartments16,17. Regeneration of adult tissues requires coordinated signalling between differentiated and proliferating cells. In many tissues, dedicated resident stem cells are maintained by multicellular niches18 and injury results in increased functional plasticity in both niche cells and stem cells, facilitating the replacement of missing cell types and the re-establishment of tissue homeostasis19. During whole-body regeneration, pluripotent adult stem cells can mobilize to sites of injury, proliferate and differentiate in accordance with local patterning cues to specify and rebuild missing tissues11,13,14,18. Although mechanisms coordinating stem cell proliferation and differentiation near the wound site have been described previously13,15–17, less is known about the transcriptional states that occur in distant tissues during regeneration or how these distant cells support tissue repair.

The free-living planarian S. mediterranea is ideal for the study of regenerative plasticity across an animal due to their extraordinary ability to repair and regenerate any organ system from tiny fragments2. T. H. Morgan showed that tissue fragments only 1/279th of the intact animal could regenerate all missing tissues18 and surface area to volume models predict that regeneration requires fragments containing ~10,000 cells19,20. Furthermore, asexual S. mediterranea produce progeny of a fixed size (length, 1.2 mm) by transverse fission, independent of parent length21, indicating that there may be a minimum fragment size that is required for regeneration. The small number of cells that is required for planarian regeneration combined with robust methods for single-cell transcriptomics and RNA interference (RNAi) makes unbiased identification and characterization of regeneration-induced transcriptional states possible. In this Article, we report a comprehensive atlas of successful and unsuccessful planarian regeneration, as well as the identification and characterization of post-mitotic TRACS.

A single-cell reconstruction of planarian regeneration

To characterize regenerative signalling across a whole animal and determine which signalling mechanisms are dependent on stem cells, we generated a single-cell reconstruction of successful and unsuccessful planarian regeneration. We first determined the smallest S. mediterranea tissue fragment that has the ability to regenerate and the impact of stem cell depletion on regenerative ability. Tissue biopsies ranging from 0.75–1.50 mm in diameter were taken from the tail region of large animals treated with control, lethal or sublethal doses of ionizing radiation (Extended Data Fig. 1a). Biopsies were taken at 3, 7 and 10 days after sublethal irradiation, producing tissue fragments containing increasing numbers of piwi-1+ stem cells (Extended Data Fig. 1a,b). Surprisingly, only biopsies taken from unirradiated animals were competent to regenerate (Extended Data Fig. 1b). Biopsies taken from sublethally irradiated animals, independent of the time since irradiation, had reduced regenerative capacity but survival rates that were comparable to unirradiated (Extended Data Fig. 1a,b). On the basis of these results, we chose to characterize regenerative signalling in three different biological contexts—(1) biopsies from unirradiated animals competent to regenerate; (2) biopsies

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taken from sublethally irradiated animals in which stem cells were depleted; and (3) biopsies from lethally irradiated animals in which stem cells were ablated (Fig. 1a).

Split-pool ligation-based single-cell RNA sequencing (SPLIT-seq) was used to capture single-cell transcriptomes from 1.00 mm biopsies taken from unirradiated, sublethally irradiated

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**Fig. 1** A single-cell reconstruction of successful and unsuccessful planarian regeneration. **a**, Schematic of the experimental design of single-cell reconstruction. d.p.a, days post-amputation; d.p.i, days post-irradiation; R, rads. **b**, Uniform manifold approximation and projection (UMAP) embedding of captured single-cell transcriptomes, coloured by tissue annotation. **c,d**, Scaled mean gene expression in single-cell reconstruction (**c**) and gene expression patterns determined using whole-mount in situ hybridization (**d**) of select tissue-specific markers (see Extended Data Figs. 3–5 for additional markers and tissue-subcluster specificity). **e**, The scaled proportion of cells from each tissue subcluster in the sampled conditions, normalized to the sample in which the subcluster had maximum representation. UI, unirradiated; SL, sub-lethally irradiated; L, lethally irradiated. For **d**, scale bars, 500 µm.
and lethally irradiated animals over 2 weeks of regeneration (Fig. 1a and Extended Data Fig. 1c). Fluorescently tagged linker molecules and image cytometry were used to visualize SPLiT-seq barcoding reagents in dissociated planarian cells (Extended Data Fig. 2a,b) and to determine the optimal dye-based sorting conditions to ensure that only intact barcoded cells were sequenced (Extended Data Fig. 2c–f). Biopsies were then dissociated, barcoded and sequenced at 0, 1, 2, 4, 7, 10 and 14 d post amputation (d.p.a.), resulting in 21 samples from 3 regeneration time courses. After filtering for transcriptome quality, the final dataset contained 299,998 single-cell transcriptomes with an average of 14,285 transcriptomes per sample, 1,981 unique molecular identifiers (UMIs) per cell and 429 genes per cell (Extended Data Fig. 2g,h and Supplementary Table 1). Clustering of cell states identified 89 global clusters, most of which could be assigned to known tissue classes on the basis of previously reported marker genes, comparison to transcriptional profiles from published planarian atlases^{2,3} and expression patterns of enriched genes (Fig. 1b–d, Extended Data Figs. 3–5 and 6a,b, and Supplementary Table 2). Once assigned to known tissue classes, cell states were split into tissue-level data subsets and reanalysed to identify additional subcluster diversity (Extended Data Figs. 2–5 and Supplementary Tables 3–14), producing 211 tissue subclusters.

To validate the reconstruction, we quantified the relative abundance of all tissue subclusters during successful and unsuccessful regeneration (Figs. 1e and Fig. 2c and Extended Data Fig. 6c–l). Unsurprisingly, stem cell clusters marked by high expression of the stem cell marker pwt-1 (ref. ^{22}) were enriched in biopsies taken from unirradiated animals (Fig. 2a–d) and RNAi-mediated knockdown of genes enriched in irradiation-sensitive subclusters resulted in strong tissue maintenance and regeneration defects, as expected for regulators of stem cell function^{23–25} (Fig. 2d–j and Supplementary Table 1). Clustering of cell states identified 89 global clusters, most of which could be assigned to known tissue classes on the basis of previously reported marker genes, comparison to transcriptional profiles from published planarian atlases^{2,3} and expression patterns of enriched genes (Fig. 1b–d, Extended Data Figs. 3–5 and 6a,b, and Supplementary Table 2). Once assigned to known tissue classes, cell states were split into tissue-level data subsets and reanalysed to identify additional subcluster diversity (Extended Data Figs. 2–5 and Supplementary Tables 3–14), producing 211 tissue subclusters.

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Regulators of tissue homeostasis were identified in irradiation-sensitive stem cell clusters.

Fig. 2 | Regulators of tissue homeostasis were identified in irradiation-sensitive stem cell clusters. a, UMAP embedding of a global dataset with stem cells highlighted. b, UMAP embedding of all stem cells, coloured by time after amputation. nUMI, number of unique molecular identifiers. c, The scaled proportion of cells from each stem cell subcluster across sampled conditions, normalized to the sample in which the subcluster had the maximum representation. d, Scaled mean expression of stem-cell-enriched genes by tissue and sample. e, z projection of a confocal stack of the stem-cell-enriched gene 60kDa HSP. f, Schematic of the RNAi screen design. d.p.f., days post feeding. g, Representative images of RNAi-treated animals 3 d.p.f. h, The survival of RNAi-treated animals shown in f, n = 19 or n = 20 as noted in g. i, Four feeding cycles. j, Representative images of homeostatic (21 d.p.f., n = 10) and regeneration phenotypes (14 d.p.a., n = 20) (Fig. 2d–j and Supplementary Table 1) of the selected high-expression cell states. k, UMAP embedding of all stem cells, coloured by time after amputation. l, Scaled mean expression of stem-cell-enriched genes by tissue and sample. m, Relative abundance of excretory tissues in planaria has been shown to be highly dependent on culture conditions and water salinity; we therefore chose to focus on the muscle, epidermal and intestinal cell states for characterization. In an effort to characterize TRACS that are likely to be upstream of stem cell activation, we focused on those that occurred in both the unirradiated regeneration time course and in biopsies in which stem cells were depleted or ablated (Fig. 3d–f (black arrows)). These subclusters were muscle subcluster 16 (M16; Figs. 3f and 4a), epidermal subclusters 2 and 20 (E2 and E20; Figs. 3e and 4b) and intestinal subclusters 9 and 12 (I9 and I12; Figs. 3d and 4c).

M16 was highly enriched at early time points after amputation (Fig. 4a) and analysis of enriched genes compared to other muscle clusters identified notum as a highly specific M16 marker (Fig. 4d). Interestingly, notum is known to be activated in muscle at anterior wounds and is required for polarity re-establishment during whole-body regeneration^{26}. Indeed, visualization of notum+collagen+ cells in vivo confirmed their induction 24 h after amputation at anterior-facing wounds, and quantification of notum+collagen+ cells indicated that M16 represents ~50% muscle cells near the wound site (Fig. 4e,f and Extended Data Fig. 7a,b). In addition to notum, M16 was marked by the expression of several regulators of polarity re-establishment after injury (Extended Data Fig. 8e), including follistatin27,28, evi29,30 and glypican-1 (ref. ^{31}). Thus, M16 is a wound-induced muscle cell state that is transiently induced 24 h after amputation and marked by dynamic expression of position control genes (PCGs).

E2 and E20 (Fig. 4b) represented distinct cell types in the epidermal lineage and had distinct temporal dynamics. E2 expressed markers of mature epidermal cells and was marked by dynamic expression of hadrian (Fig. 4d). Visualization of hadrian+ cells confirmed that they were enriched in the surface epithelium at 24–48 h after amputation (Fig. 4e,g and Extended Data Fig. 7a). By contrast, E20 strongly expressed markers of late epidermal progenitors, such as agat1, agat2 and agat3 (refs. ^{32–34}). E20 was marked by increased expression of actin3 (Fig. 4d,e,h and Extended Data Fig. 9e) and included a smaller subset of epidermal cells. Visualization and quantification of agat1+actin3+ cells confirmed that these cells increased 24–48 h after amputation and remained slightly elevated at 4–7 d.p.a. (Fig. 4e,h and Extended Data Fig. 7a). Whereas hadrian+ epidermal cells were observed both near to and far from the wound site, agat1+actin3+ cells were enriched adjacent to the wound and in blastema tissue (Extended Data Fig. 7a).

Similar to the TRACS that we identified in the epidermis, I9 and I12 (Fig. 4c) were related to distinct cell types in the intestine and had unique temporal dynamics. The planarian intestine has three main cell types: secretory goblet cells, absorptive phagocytes/...
enterocytes and the recently identified outer or basal intestinal cells, 19 expressed markers of basal/outer intestinal cells (tdo2 and HAL) and was marked by increased expression of lectin2b (Fig. 4d,e,i and Extended Data Fig. 10e). By contrast, 112 expressed markers associated with intestinal enterocytes, and increased expression of methyltransferases (polybromo-1 and mettrans) or
regulators of endocytosis (*myosin1E*) and lysosomal degradation (*cathepsinB*, *cathepsinL*) after amputation. In particular, I12 was marked by the uncharacterized planarian gene 26267, which is typically expressed only in peripheral phagocytic cells (Fig. 4d,e) and

Extended Data Figs. 7a and 10e). Owing to its expression during injury-induced gastrovascular remodelling, we refer to gene 26267 as Injury Remodelling Activated 1 Gene (*ira-1*). Visualization and quantification of *prcn*-*lectin2b*+ cells confirmed that the percentage
Fig. 4 | Five amputation-specific states were identified, visualized and quantified in the muscle, epidermis and intestine. a–c, UMAP embedding of all muscle (a), epidermal (b) or intestinal (c) cells, coloured by the time after amputation. d, UMAP feature plots showing the expression of amputation-enriched genes in relevant tissue. Scale bars show scaled gene expression. e, Visualization of amputation-induced genes (yellow) in surface epithelium or co-expressed with tissue specific marker (magenta) for each amputation-specific cell state following amputation. f–j, Quantification of TRACS markers as a percentage of the relevant tissue or cell type after amputation for MC16 (f; n = 3, n = 5, n = 3, n = 6 and n = 3 biologically independent animals); EC2 (g; n = 4, n = 5, n = 7, n = 4 and n = 4 biologically independent animals); EC20 (h; n = 5, n = 6, n = 5, n = 6 and n = 4 biologically independent animals); IC9 (i; n = 3, n = 4, n = 5, n = 6 and n = 4 biologically independent animals); and IC12 (j; n = 3, n = 5, n = 4, n = 4 and n = 3 biologically independent animals). P values are indicated in the plots and were determined using two-sided unpaired t-tests (f–j) compared with the 10 d.p.a. time point, with no adjustments for multiple comparisons. Data are mean ± s.e.m. For e, scale bars, 20 µm.
of prcn⁺ and also lectin2b⁺ intestinal cells increased 2–4 d.p.a. (Fig. 4e,i and Extended Data Fig. 7a). Interestingly, we observed a high level of variability in the proportion of outer basal cells expressing lectin2b between individual animals (Fig. 3i) and, when lectin2b was highly expressed, it was expressed in basal cells throughout the intestine, rather than enriched near the wound site (Extended Data Fig. 9a). By contrast, I12 was a very rare cell state among enterocytes (Fig. 4j). Visualization and quantification of prcn⁺ira-1⁺ cells confirmed that these cells made up only ~4% of intestinal cells 1–4 d.p.a. (Fig. 3e,j) and were enriched in gut branches near the wound or adjacent to the regenerating pharynx (Extended Data Fig. 7a).

**TRACS occur in the absence of cell divisions**

TRACS could be induced in pre-existing post-mitotic cells or could arise as a product of stem cell divisions. Indeed, all of the TRACS that we characterized in the muscle, epidermis and intestine occurred at different rates in unirradiated biopsies compared with those taken from irradiated animals (Extended Data Figs. 8a–d, 9a–d and 10a–d). Trajectory inference methods can be used to...
predict the connectivity between cell states in single-cell sequencing datasets; we therefore used partition-based graph abstraction (PAGA\textsuperscript{40}) to predict the connectivity between our observed cell states and the stem cell compartment. Amputation-specific cell states did not connect directly to the stem cell progenitor subcluster (Fig. 5a–c). Instead, the connectivity path from the stem cell compartment (black arrows) to M16 (Fig. 5a), E2 and E20 (Fig. 5b), and I12 (Fig. 5c) passed through several cell states that were not amputation induced or present only at later stages of regeneration. I9 was connected to the stem cells through I1 (Fig. 5c)—another wound-induced cell state (Extended Data Fig. 10d). Thus, PAGA analysis of subcluster connectivity indicated that most TRACS were more likely to arise from pre-existing post-mitotic tissues than from proliferating stem cells.

To test experimentally whether the generation of TRACS required stem cell divisions, we treated animals with 0.15% colchicine after amputation (Fig. 5d), which results in a mitotic arrest and accumulation of H3P\textsuperscript{+}/piwi\textsuperscript{-1}\* cells\textsuperscript{46} (Extended Data Fig. 7c,d). Despite this mitotic arrest, TRACS in the muscle, epidermis and intestine were induced at comparable or higher levels after amputation (Fig. 5e,f). Notum\* muscle cells were slightly reduced in colchicine-treated animals, as predicted by our single-cell sequencing analysis (Extended Data Fig. 8a–d), but this reduction was not statistically significant. While E20 was reduced in sequenced biopsy samples taken from irradiated animals (Extended Data Fig. 9a–d), agat\textsuperscript{-1}\*actin\* cells (E20) became more plentiful in colchicine-treated animals (Fig. 5e,f). The irradiation sensitivity of E20 in our single-cell reconstruction was therefore most likely due to the loss of agat\textsuperscript{-1}\* progenitors before amputation, rather than a failure to induce the cell state. These results reveal that TRACS are more related to pre-existing post-mitotic tissues than to stem cells and do not require stem cell divisions to occur in vivo. Thus, we identified subsets of differentiated cells in the muscle, epidermis and intestine that transiently change their function after amputation, independent of stem cell proliferation.

**Muscle TRACS express genes required for polarity**

Muscle cells constitutively express regionally restricted PCGs, which are required to maintain and regenerate the adult body plan\textsuperscript{47}. As such, the re-establishment of positional information in muscle cells is an important step that is required for the regeneration of missing tissues in planaria\textsuperscript{46–48,51,52}. As M16 was marked by increased expression of the polarity gene notum, we postulated that the cell state could represent wound-induced muscle and express additional genes that are important for tissue polarity. Genes enriched in M16 relative to other muscle subclusters were highly expressed in the muscle and were wound induced in all three single-cell regeneration time courses and in a bulk RNA-sequencing (RNA-seq) dataset\textsuperscript{49} of planarian regeneration (Extended Data Fig. 8e,f). M16-enriched genes included several previously identified wound-induced genes (follistatin, notum, glypican-1 and evi/wls) that produce regeneration defects after RNAi depletion. A targeted RNAi screen of M16-enriched genes recapitulated previously reported regeneration defects for follistatin, notum, evi/wls and glypican-1 (refs. 34–38). Furthermore, grp78, Ca-trans ATPase and tubulin-\(\beta\) were identified as regulators of tissue homeostasis (Extended Data Fig. 8g–j) and RNAI inhibition of junctionophilin-1 resulted in small blastemas and increased incidence of cyclopia, reminiscent of evi/wls-RNAI-treated animals (Fig. 6a–c). Junctionophilin-1 was expressed in the body wall musculature and was enriched adjacent to the wound site (Fig. 6a and Extended Data Fig. 8k). To determine whether junctionophilin-1 was indeed a regulator of tissue patterning, we compared the regeneration defects of junctionophilin-1-RNAI-treated animals with those produced after notum, follistatin, glypican-1 and evi/wls RNAI depletion. Visualization of the central nervous system (PC2) and intestinal branches (gata4/5/6) 14 d.p.a. in RNAI-treated animals revealed absent or small anterior cephalic ganglia after all RNAI treatments, including junctionophilin-1 (Fig. 6d). We next visualized the notum\* anterior poles and wnt1\* posterior poles in RNAI-treated animals (Fig. 6e). Follistatin- and glypican-1-RNAI-treated animals had an altered number or distribution of notum\* cells at the regenerated anterior pole, while evi/wls- and junctionophilin-1-RNAI-treated animals had a normal number and distribution of notum\* cells (Fig. 6f–h). Importantly, all evaluated RNAI conditions except for evi/wls knockdown resulted in an expanded range of wnt1\* cells at the posterior pole and follistatin-RNAI-treated animals also had an increased number of wnt1\* cells (Fig. 6i–k).

Together, our results indicate that M16 represents a subset of muscle cells that are adjacent to the anterior wound site that coexpress genes important for anterior pole specification and inhibition of posterior tissue specification by wnt1. While several genes expressed in M16 have previously been identified as wound-induced PCGs, data on their coexpression in a subset of wound-adjacent muscle cells were lacking. Moreover, by characterizing several genes expressed within this cell state, we were able to identify additional regulators of homeostasis and polarity re-establishment.

**Epidermal genes regulate stem cell proliferation**

In both vertebrate and invertebrate systems, the wounded epidermis expresses and secretes molecules that promote wound closure and tissue regeneration\textsuperscript{53,54,55,56}. In planaria, the molecules that regulate the differentiation of piwi\textsuperscript{-1}\* progenitors into mature epidermal cells are some of the most well characterized among all tissue lineages. zpf1\* zeta neoblasts progress through a prog2\* early progenitor state and an agat-3\* late progenitor state, then transition through a zpuf6\* or vim1\* stage before becoming mature rootletin\*+, PRSS12\* or laminin\* epidermal cells\textsuperscript{39–41}. While zpf1\* zeta neoblasts and prog2\* early progenitors did not significantly alter transcription after amputation, both agat-3\* late epidermal progenitors and mature epidermal cells had associated TRACS—epidermal subcluster 20 (EC20) and EC2, respectively (Fig. 3).

**Fig. 6 | Muscle TRACS express genes that are required for tissue polarity.** a. UMAP feature plots showing muscle expression of M16-enriched genes that produced penetrant RNAi phenotypes. Scale bars show general gene expression. b. Representative images of homeostatic (21 d.p.f., n = 10 animals) and regeneration phenotypes (14 d.p.a., n = 20 animals) in RNAi-treated animals. Scale bar, 500\(\mu\)m. c. Scoring of regeneration phenotypes. d. Selected images of whole-mount double fluorescence in situ hybridization (dfISH) visualization of CNS (PC2) and intestine (gata4/5/6) in RNAi-treated animals. Scale bars, 500\(\mu\)m. e. Schematic of visualized anterior and posterior pole cells in regenerating fragments. f. Selected images and raw x-y position of notum\* anterior pole cells visualized using whole-mount in situ hybridization. Scale bars, 150\(\mu\)m. AP, anterior–posterior; ML, mediolateral. g. The number of notum\* cells in RNAi-treated animals. n = 4 (unc-22), n = 2 (follistatin), n = 3 (glypican-1), n = 2 (evi/wls) and n = 4 (junctionophilin-1) biologically independent animals. h. The distribution of notum\* cells in RNAi-treated animals. n = 152 (unc-22), n = 14 (follistatin), n = 125 (glypican-1), n = 57 (evi/wls) and n = 133 (junctionophilin-1) notum\* cells. i. Selected images and raw x-y position of wnt1\* posterior pole cells visualized using whole-mount in situ hybridization. Scale bars, 150\(\mu\)m. j. The number of wnt1\* cells in RNAi-treated animals. n = 3 (unc-22), n = 6 (follistatin), n = 4 (notum), n = 5 (glypican-1), n = 5 (evi/wls) and n = 5 (junctionophilin-1) biologically independent animals. k. The distribution of wnt1\* cells in RNAi-treated animals. n = 15 (unc-22), n = 71 (follistatin), n = 28 (notum), n = 20 (glypican-1), n = 36 (evi/wls) and n = 27 (junctionophilin-1) wnt1\* cells. P values were determined using two-sided Mann–Whitney U-tests with no adjustments for multiple comparisons compared with the unc-22 control (c) and two-sided unpaired t-tests (g, h, j and k), with no adjustments for multiple comparisons. Data are mean ± s.d. compared with the unc-22 control.
To test the function of epidermal TRACS during regeneration, genes enriched in EC2 and EC20 were depleted using RNAi and the phenotypes were observed at homeostasis and after amputation. Surprisingly, a range of homeostatic and regeneration defects were observed after depletion of genes enriched in agat-3 epidermal progenitors (E20) (Fig. 7a–e and Extended Data Fig. 9e,f).

agat-1–RNAi-treated animals had reduced photoreceptor pigmentation in regenerates at 14 d.p.a. (Fig. 7b,d). Animals treated with RNAi targeting agat-3 or zinc finger homeobox protein 3 (zfhx3) had no blastema growth or reduced blastema growth (Fig. 7b,d) and RNAi against cyp3142a1 or agat-2 produced lesions at homeostasis and either no blastema or lesions after amputation.
(Fig. 7b,d). Finally, RNAI against a gene enriched in the mature epidermis, sodium/potassium-transporting ATPase subunit α (atp1a1) (Fig. 7b,d), resulted in lesions or a small blastema after amputation. Visualization of the central nervous system and intestine at 7 d.p.a. confirmed a reduction in the size of regenerating cephalic ganglia, but minimal defects in anterior fusion of the left and right gut branches (Fig. 7c,e). EC20-enriched genes (for example, actin-3, agat-1, agat-2, agat-3, cyp3142a1) were expressed in dispersed cells in the mesenchymal space at homeostasis, but more highly expressed in more densely packed cells around the regenerating blastema, while atp1a1 was expressed throughout the mature epidermis in both intact and amputated animals (Extended Data Fig. 9g).

Given the increased incidence of lesions in RNAI-treated animals, we visualized piwi-1+ stem cells and quantified H3P+ mitotic cells during the early (2 d.p.a.) and late (7 d.p.a.) stages of regeneration (Fig. 7f,g and Extended Data Fig. 9h–k). Surprisingly, zfkhx3-, agat-2-, cyp3142a1- and atp1a1-RNAI-treated animals had reduced proliferation at 2 d.p.a. and piwi-1+ stem cells were less plentiful in agat-2-, cyp3142a1- and atp1a1-RNAI-treated animals (Fig. 7f,g). The reduction in the proliferation in RNAI-treated animals was less significant after the wound-induced mitotic burst, but the continued loss of piwi-1+ stem cells could be observed in agat-2-, cyp3142a1- and atp1a1-RNAI-treated animals (Extended Data Fig. 9j,k). We also assessed stem cell proliferation in intact animals for genes that produced the most severe phenotypes. Both agat-1- and agat-3-RNAI-treated animals had increased mitotic divisions at 7 d after RNAI feeding, whereas agat-2- and atp1a1-RNAI-treated animals had reduced H3P+ mitotic cells (Extended Data Fig. 9h,i).

Previous studies identified regulators of epidermal differentiation expressed in agat+ progenitor cells5,6,9. Our results indicate that agat+ late epidermal progenitors respond to injury and express many genes required for regulation of stem cell proliferation. We also identified a Na/K ATPase subunit expressed in mature epidermal cells that is required for regulation of stem cell proliferation. Thus, our results indicate that both mature epidermal cells and agat+ late progenitors are transcriptionally responsive to amputation and are required for the regulation of stem cell proliferation in intact and regenerating tissues.

Intestinal genes regulate stem cells and tissue remodelling

The endoderm is a well-established model for stem cell regulation and are required for the regulation of stem cell proliferation at 2 d.p.a. and piwi-1+ stem cells were less plentiful in agat-2-, cyp3142a1- and atp1a1-RNAI-treated animals (Extended Data Fig. 9j,k). We also assessed stem cell proliferation in intact animals for genes that produced the most severe phenotypes. Both agat-1- and agat-3-RNAI-treated animals had increased mitotic divisions at 7 d after RNAI feeding, whereas agat-2- and atp1a1-RNAI-treated animals had reduced H3P+ mitotic cells (Extended Data Fig. 9h,i).

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Discussion

To test whether genes upregulated in intestinal TRACS after amputation are required for planar regeneration, IC9- and IC12-enriched genes were depleted using RNAI. Seven genes (Fig. 8a) produced reproducible defects in either tissue homeostasis or tissue regeneration. RNAI depletion of atp12a and spectrinβ which are both enriched in outer/basal cells; Extended Data Fig. 10g) resulted in lesions or inch-worming at 3 d.p.f. (Fig. 8b) and lysis before 21 d after feeding (Fig. 8c). Animals treated with atp12a RNAI had a substantial reduction in stem cell proliferation 7 d after RNAI feeding, whereas spectrinβ-RNAI-treated animals had reduced piwi-1 expression in posterior tissues (Fig. 8d,e). atp1a1 and spectrinβ were upregulated in wound-adjacent intestinal tissues, but were also expressed in intestinal, phagocytic and protonephridial tissues at homeostasis (Extended Data Fig. 10g). Thus, while we conclude that basal/outer intestinal cells alter transcription after amputation (Figs. 3–5) and express genes that are important for stem cell maintenance (Fig. 8b–e), we have not yet determined whether they have a regeneration-specific function.

In contrast to genes in IC9, RNAI depletion of IC12-enriched genes resulted in regeneration defects, but minimal defects in intact animals. RNAI knockdown of myosin1E, polybromo-1 and talin-1 resulted in small blastemas and limited or no pharynx regeneration, whereas RNAI knockdown of cathepsinB and lrrk2 resulted in an increased incidence of cyclopia or unpigmented photoreceptors (Fig. 8f,g). Visualization of the central nervous system confirmed a reduction in the size of regenerated cephalic ganglia in myosin1E- and polybromo-1-RNAI-treated animals (Fig. 8h,i). Furthermore, visualization and quantification of notum+ cells revealed a reduction in either the number or distribution of notum+ cells in RNAI-treated animals (Extended Data Fig. 10i,j,k). A reduction in or loss of posterior intestinal branches was also observed in myosin1E and talin-1 RNAI-treated animals (Fig. 8h,i). Thus, inhibition of 112-enriched genes (for example, myosin1E) resulted in defects in the regeneration of anterior structures and in gut remodelling. These defects were not due to a reduction in piwi-1+ stem cells or the number of proliferating stem cells at 2 d.p.a. (Extended Data Fig. 10h,i), but there was a slight reduction in proliferation observed 14 d.p.a. in myosin1E- and polybromo-1-RNAI-treated animals (Fig. 8j,k).

Together, our data indicate that a rare subset of intestinal enterocytes upregulates phagocytic gene sets in response to amputation, and a screen of enterocyte enriched genes identified several important for new tissue growth during regeneration.

Fig. 7 | Epidermal TRACS express genes that are required for the regulation of stem cell proliferation. a, UMAP feature plots showing epidermal expression of genes that produced penetrant RNAI phenotypes. Scale bar shows scaled gene expression. b, Representative images of homeostatic (21 d.p.f.) and regeneration phenotypes (14 d.p.a.) in RNAI-treated animals. Insets, 2.8X magnification. c, Selected images of whole-mount dfISH visualization of CNS (PC2) and intestine (gata4/5/6) in RNAI-treated animals. d, Scoring of regeneration phenotypes. n = 20 animals. e, Quantification of cephalic ganglia length normalized to total body length in RNAI-treated animals. n = 5 (unc-22), n = 3 (agat-1), n = 3 (zfkhx3), n = 4 (agat-2), n=1 (cyp3142a1) and n = 3 (atp1a1) biologically independent animals. f, Selected images of whole-mount in situ hybridization of piwi-1+ stem cells and immunohistochemistry of H3P+ mitotic cells in RNAI-treated animals 2 d.p.a. g, H3P+ cell density in RNAI-treated animals at 2 d.p.a. n = 8 (unc-22), n = 7 (agat-1), n = 8 (zfkhx3), n = 11 (agat-3), n = 9 (agat-2), n = 9 (cyp3142a1) and n = 7 (atp1a1) biologically independent animals. For the box plots in e and g, the centre line shows the median, the bounds of the box show the first and third quartile, and the whiskers extend from the quartile to the minimum and maximum values. P values were determined using two-sided Mann-Whitney U-tests compared with the unc-22 control, with no adjustments for multiple comparisons (d) and two-sided unpaired t-tests compared with the unc-22 control, with no corrections for multiple comparisons (e and g). For b, c and f, scale bars, 500 μm.

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were taken from unirradiated, sublethally irradiated and lethally irradiated planarians, enabling us to define the cellular dynamics of successful and unsuccessful regeneration across an entire animal and to identify post-mitotic TRACS. We next determined which TRACS were dependent on stem cell proliferation and identified TRACS-enriched genes that are important for tissue regeneration and remodelling of pre-existing tissues.

We identified TRACS in the mesoderm (collagen°notum°, MC16), ectoderm (agat-1°actin-3°, EC20; hadrian°, EC2) and endoderm (prcn°lectin2b°, IC9; and prcn°ira-1°, IC12). A subset of anterior-facing muscle cells induces genes (notum, follistatin, evi/wls, glypican-1, junctophilin-1) that are required for the re-establishment of the anterior and posterior poles and subsequent tissue regeneration (Fig. 6). agat-1°actin-3° cells are induced by injury and are
required for stem cell proliferation (Fig. 7). Finally, basal/outer intestinal cells respond to amputation and regulate stem cell proliferation, and a rare subset of enterocytes transiently express genes that are required for tissue remodelling (Fig. 8). Importantly, TRACS were independent of cell divisions (Fig. 5), consistent with observations that some wound-induced genes do not require new proliferation\(^3\)\(^8\)\(^,\)\(^4\).
An increase in transcriptional plasticity in rare cell types is often required for tissue repair, particularly in systems in which stem cells have been ablated\(^{5,6}\). Our results build on these observations and indicate that regenerative capacity may arise from an organism’s ability to produce rare, transient and functionally distinct cell states within a subset of differentiated tissues after injury. We refer to these as TRACS. The observation that TRACS are both transient and rare may have important implications for the regulation of growth ability. Transience of regenerative signalling may act as a guard against unchekced growth. Indeed, genes expressed in intestinal TRACS (polybromo-1 and TDO) have been implicated in cancer proliferation and tumour immune evasion\(^{7,8}\). The rarity of TRACS may also account for why regenerative ability is so unevenly distributed across animals. A rare cell state regulating a subset of regenerative signalling is more easily lost during evolution and is perhaps more easily reactivated\(^{9}\). The regenerative ability of planarian species that are incapable of head regeneration has been restored through inhibition of Wnt signalling\(^{10}\) and we can now postulate that this may have restored rare muscle TRACS (notum, MC16) and the ability to repattern the body plan. If generally applicable, reactivation of TRACS could unleash regenerative capacity in systems in which such abilities are more limited.

The results presented here characterize the cellular components that are required for regeneration at a high molecular resolution, facilitating the discovery of rare cell states (TRACS) and identification of genes that are required for patterning, stem cell proliferation and tissue remodelling. These data also support a model in which regenerative ability may be linked to coordinated plasticity in differentiated cells across all three germ layers.

Online content
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**Methods**

**Animal husbandry.** *S. mediterranea* animals from the asceonal strain C1W-4 (C4) were maintained in 1x Montjuic salts in a planarian recirculation culture system as previously described. Tissue biopsies were taken from large animals that were fed chunk liver for three times per week as a recirculation system. Whole animals that were used for in situ hybridization analyses and RNAi screens were fed chunk liver once a week in a recirculation system and starved in static culture at least 4 weeks before use. Worms were dosed with gamma irradiation in the MDS Nordom Gammacell 40 Exact Low-Dose Rate Irradiator. All animal husbandry was performed in accordance with the ethical guidelines of the Stowers Institute for Medical Research.

**Tissue biopsies.** Animals were anesthetized using cold chloroform solution (0.1–0.2% w/v) chloroform in 1x Montjuic salts for 4–5 min or until immobile, then rinsed with cold Holfreter’s solution (3.5 g/l NaCl, 0.2 g/l NaHCO₃, 0.05 g/l KCl, 0.01 g/l CaCl₂, 0.05 g/l MgCl₂, pH 7.5) and placed on Whatman filters to collect tissue biopsies. Tissue biopsies were maintained in 1x Montjuic salts for 10 min and stored on ice before fluorescence-activated cell sorting analysis. Cells were centrifuged for 10 min at 1,000 × g, fixed as previously described for SPLiT-seq analysis, and resuspended in 1 ml PBS+RNase inhibitors (Invitrogen, AM2694; Qiagen, Y9240L) and incubated on ice for 20 min, then centrifuged for 5 min at 300 × g and resuspended in 1 ml PBS+RNase inhibitors. Fluorescence-activated cell sorting analysis was performed using a FACSAria III sorter (BD Biosciences) with CellProfiler (v.2.2.0.4) to classify cells in our dataset by tissue using one of the reference datasets used for tissue annotation and LabelTransfer were downloaded from GEO accession GSE11764 and processed using the same functions and parameters used for the SPLiT-seq datasets as described above.

**Library preparation and sequencing.** Cell lysates were thawed and sequencing libraries were generated according to the SPLiT-seq protocol with some modifications. Incubation and step incubation steps were performed at room temperature with 500 rpm agitation using a thermomixer (with the final 42 °C, template switching incubation agitation at 300 rpm). After template switch clean-up, all of the steps were conducted in the 96-well plate format. Monitored cDNA amplification was stopped once the signal left exponential phase (10 cycles), and a SPRI bead clean-up was performed using two additional (200 µl) ethanol washes. Amplified cDNA was spiked for quality control and quantitation with the Qubit 2100 Bioanalyzer and Invitrogen Qubit Fluorometer and normalized to 600 pg per sample for sequencing library preparation using the Illumina Nextera XT library preparation kit. The PCR amplification program was modified to incorporate an initial 3 min 72 °C hold for increased yield. The resulting short fragment libraries were assessed for quality and quantity, pooled equal molar in batches of eight, and sequenced as a total of nine Hiseq runs at the Duke Flow Cytometry Core Facility on an Illumina Next Seq 500 instrument using the NextSeq Control Software (v.2.2.0.4) with the following pairs read lengths: 66 bp (Read1), 6 bp (Index) and 94 bp (Read2). After sequencing, the Illumina primary analysis tools NextSeq RTA v.2.4.11 and bcl2fastq v.2.20 were run to demultiplex reads for all libraries and generate FASTQ files.

**Data processing and alignment of SPLiT-seq dataset and public Drop-seq dataset.** Pooled SPLiT-seq libraries were demultiplexed into individual time point samples using a custom Python script (available at GitHub). In brief, Read2 contains three rounds of cell barcodes that were designed during the library preparation; and, pooling step. Cells were counted using a baccalaureate cell counter (C4) and flow cytometry. The barcodes were extracted from raw reads and cell barcode 1 was used to assign each read to the well/sample line. Reads were assigned a sample, UMI and 24 bp cell barcode allowing for up to one mismatch per cell barcode round, with a quality score more than 10. Samples of each time point were then processed using Drop-seq v2.4.0 (http://mccarrellab.org/dropseq/) for barcode extraction, UMI collapse, gene annotation and cell expression matrix generation. All samples were aligned using STAR aligner with the following parameters: --outFilterMatchNmin 0 --outFilterMismatchNmax 10 --outFilterMismatchNoverLmax 0.3 --outFilterMultimapNmax 100 --outFilterMultimapNoverNoReads 1. Unique aligned reads were annotated using the Drop-seq tools function TagReadWithGeneExon on the basis of the planarian gene interval refflat file. The final UMI expression matrix was collapsed using the Drop-seq tools function DigitalExpression with the parameter DETDISTANCE = 1. Raw and processed data files are available at the Gene Expression Omnibus (GEO) under accession number GSE46685. The public Drop-seq reference datasets used for tissue annotation and LabelTransfer were downloaded from GEO accession GSE11764 and processed using the same functions and parameters used for the SPLiT-seq datasets as described above.

**Quality filtering, data normalization and clustering of cells.** Low-quality cells from the raw sequencing (at UMI < 500 and/or nGene < 75) were excluded from subsequent analysis. Using the R package Seurat v3.1.0 and the R packages FindCellMarkers v3.1, 100 random cells were ordered from GEO accession GSE11764 and processed using the same functions and parameters used for the SPLiT-seq datasets as described above.

**Identification of enriched transcripts and tissue annotation.** We used Seurat’s FindAllMarkers using the default parameters to identify highly variable transcripts that serve as markers for each of the 89 global clusters, resulting in a total of 3,558 markers comprising 1,015 unique transcripts (some transcripts were markers for more than one cluster). Gene annotations were sourced from S. Alvarado laboratory’s Rosetta Stone Transcript Mapper. The most significant 20–40 transcripts per cluster were mapped to dd_Smed_v4 using the same mapper and their expression and tissue specificity was characterized in published planarian single-cell datasets. Most global clusters were easily assigned to a previously described tissue lineage due to a shared gene expression profile and, in many cases, a cluster could be matched one-to-one to a cluster from a previously published dataset or to a planarian ontology term (Supplementary Table 15). In addition to manually annotating each global cluster, we used Seurat’s TransferData function to classify cells in our dataset by tissue using one of the previous planarian single-cell studies as a reference for tissue annotation. Raw data (GSE117764) were aligned using STAR aligner using the same parameters outlined above, with 50 µl of expression matrix generated using Drop-seq tools v1.13. Only cells that could be unambiguously identified in the published Fincher metadata using a combination of cell barcode and the type of tissue sample were retained. Tissue annotations from the published metadata were added to each cell barcode. FindIntegrationAnchors (dmins = 1:30) was used to identify anchor
cells, then IntegrateData (dims = 1:30) was used to integrate the published dataset with our full SPLiT-seq dataset. PCA and UMAP embedding were performed with 30 PCs. FindTransfer Anchors (dims = 1:30) found transfer anchors using the published “data” as the reference and our dataset as the query. TransferDataset used the identified transfer anchors and the published metadata’s tissue annotations to predict tissue annotations for all cells in our SPLiT-seq dataset. The manual and TransferData tissue annotations were combined to produce a predicted tissue type for each cluster, which was then added to the gene expression matrix metadata for each cell in that cluster.

Interestingly, global cluster 1 contained very few significantly enriched genes and most of the enriched genes were also highly expressed in cells from the epidermis, muscle or phagocytic cell lineages. In previous research, clusters lacking the identified transfer anchors and the published metadata’s tissue annotations to ends of each primer. PCR homologous to the pPR-T4P vector were added to 5 S. mediterranea transcripts.

connectedness of subclusters/partitions, we used PAGA’s native plotting (Fig. 5a–c). We chose to use PAGA, which uses fewer assumptions and determines connectivity between clusters of cells, making it more robust to

Subclustering of tissue subsets. The full dataset was broken into subsets by tissue type for tissue-specific subclustering. For each tissue subset, the raw RNA counts were normalized using SCTransform before performing PCA. After normalization, PCA was performed on a number of PCs set on a tissue basis. A summary of the global clusters combined into each tissue subset and the PC parameters for tissue level subclustering is provided in Supplementary Table 3. As described for the full dataset, we generated both two-dimensional and three-dimensional UMAP embeddings and a shared nearest neighbour graph using the same number of PCs as in the principal component analysis, then clustered using the default settings. We then used FindAllMarkers using the default settings to find markers for each of the tissue-level clusters (subclusters). This resulted in a total of 211 subclusters (18 phagocytic/epidermis and intestine, stem cells-2 for muscle). Tissue subset PCA embeddings similarity to any tissue subcluster was chosen as the progenitor cluster for that and all of the stem cell subclusters. The stem cell cluster that showed the highest performed a pairwise comparison of expression between all of the tissue subclusters and all of the stem cell subclusters. This resulted in 211 subclusters (18 phagocytic/epidermis and intestine, stem cells-2 for muscle). Tissue subset PCA embeddings similarity to any tissue subcluster was chosen as the progenitor cluster for that and all of the stem cell subclusters. The stem cell cluster that showed the highest

Proportional downsampling for global data visualization. The number of cells in each sample in the dataset varies from 3,461 to 36,344. While the abundance of cells in each cluster could be normalized by sample quite easily in tissue proportion heat plots, this was more difficult in UMAP plots. To mitigate this difficulty, we created a proportionately downsampled dataset with a target maximum of 6,000 cells per sample. Both the unirradiated and sublethally irradiated day-0 samples had fewer than 6,000 cells, so the entire sample was included. For each of the other samples, we calculated the relative proportion of each global cluster and multiplied by 6,000 to determine the number of cells to retain from each cluster. The desired number of cells was then randomly selected from among the cells in that cluster in the given sample. When needed, we rounded all cell counts up to the next equal or greater integer. This subsampled dataset was used as the reference object for most global UMAP plots (Fig. 5a–c) (tissue highlight plots) and Extended Data Fig. 6a). All of the other plots (cluster

Laemmli buffer (250 mM Tris, 2% SDS, 10% glycerol, 1% bromophenol blue) and 10 µl of sample were loaded into each well and the gels were run at 80–100 mA for 1.5 h. Images were obtained with a Gel Doc 2000 imaging system (Bio-Rad) and analyzed with Quantity One software (Bio-Rad). Western blots were scanned, quantified and analyzed as described.

Quantification of FISH images. TRACS quantification. Confocal z stacks of wound-site-adjacent tissues (anterior tissues in regenerating tail fragments or

RNAi food preparation and feeding. Cloned gene vectors transformed into E. coli strain HT115 were cultured in 24-well, round-bottom culture plates in 2x YT bacterial growth medium with 50 µg ml⁻¹ kanamycin and 10 µg ml⁻¹ tetracycline for 16–18 h at 37 °C. Production of double-stranded RNA was induced by adding 6 ml 2x YT bacterial growth medium with 50 µg ml⁻¹ kanamycin, 10 µg ml⁻¹ tetracycline and 25 µM L–isopropyl–β-D–thiogalactoside (IPTG) at 2 h intervals. Bacteria were cultured for an additional 4 h at 37 °C after induction. Beef liver was homogenized by adding 800 µl 0.2X food colouring in Montjuic salts to 2 g beef liver puree, followed by pipetting until homogenized. Cultures were centrifuged for 10 min at 1,500g, the supernatant was removed and bacterial pellets were resuspended in 60 µl homogenized beef liver. Planarians were fed RNAi food four times with 2d between each feeding. Water exchanges were performed after each feeding and dish exchanges along with full water exchanges were performed 24 h before each new feeding.

Phenotype quantification. After RNAi feeding, the animals were moved to clean dishes with fresh Montjuic solution plus 50 µg ml⁻¹ gentamycin. In the primary RNAi screen, animals were imaged 7 d.p.f. and all of the surviving animals were bisection through the pharyngeal region to produce anterior and posterior fragments. Regeneration phenotypes were visually inspected and scored at 7 d.p.a. and animals were imaged and scored at 14 d.p.a. for the secondary RNAi screen, the number of animals was doubled (n = 20). RNAi depletions that had produced lesions by 3 d.p.f. in the primary screen were assigned to survival curve assays, in which animals were monitored daily after the fourth feeding and lysis events were noted until all of the animals were dead. A log-rank (Mantel–Cox) test was used to compare the survival of all RNAI conditions with the unc-22–RNAi-treated control. Genes that produced regeneration phenotypes or homeostatic defects only after amputation were assigned to regeneration assays. In regeneration assays, animals were imaged 7 d.p.f. and 10 animals were bisected to produce anterior and posterior fragments, while 10 were left intact. At 14 d.p.a. or 21 d.p.f., homeostatic and regeneration

Microscopy. Colorimetric whole-mount in situ hybridization samples and live-worm or fragment images were acquired using a Leica M205 microscope using the Leica Application Suite (LASX). After image acquisition, non-tissue background was subtracted, contract and image intensity adjusted, and the edited image saved as a raw image data file. No quantifications were performed on contrast- or intensity-adjusted images, and all raw, original data are available in the original data database (http://www.stowers.org/research/publications/libp-1513). Confocal images of FISH samples were acquired using an LSM-700 inverted confocal microscope with Zeiss Xenon Black Software (v8.1) or using high-throughput imaging on a Nikon Eclipse Ti with a Yokogawa W1 spinning disk and robotic plate loader. Using automated image capture, whole slides were imaged at x4 and objects of interest were automatically detected, reimaged at x10, and then batch stitched, quantified and aligned using Fiji macros (https://github.com/jovyan/smc-macros). Stitching of tiles for whole-worm images was performed using Fiji plugins (grid/collecction stitching) with custom macros for Fiji. Z-projections of all images were performed. Images were generated to visualize gene expression across the entire animal, then rotated and cropped for data presentation.

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Cephalic ganglia size. The Fiji straight line tool was used to measure anterior to posterior length of the cephalic ganglia and total body length of each animal and the ratio of cephalic ganglia length/body length was reported. Unpaired two-sided t-tests were used to compare the number of notom* or wnt11* cells per animal in RNAi-treated animals compared with the unc-22 RNAi-treated animals.

H3P quantification. Imaging and quantification were performed as described previously44. Unpaired two-sided t-tests were used to calculate the statistical difference between the number of H3P* cells in whole animals or amputated fragment. Colchicine-treated animals were compared to 0% colchicine controls, whereas RNAi-treated animals were always compared with the unc-22 RNAi-treated animals.

Statistics and reproducibility. No statistical method was used to predetermine the sample size in the reported experiments. For RNAi primary and secondary screens (Supplementary Tables 16 and 17), animals were randomly assigned to treatment conditions and investigators were blinded to allocation during experiments and phenotype assessment. For phenotype characterization after initial screening, the investigators were not blinded to allocation during experiments and outcome assessment. No data were excluded from the analyses. Data analysis and statistical tests were performed using Microsoft Excel (v.16.36) and Graph Prism (v.8.3.0).

Cytoplasmic data collection and analysis were performed using BD FACSDiva (v8.0). The single-cell sequencing data presented in the study were obtained in a single experiment that was not independently replicated due to resource constraints. Gene expression patterns of subcluster-enriched genes shown by in situ hybridization (Figs. 4d and 5c and Extended Data 3c–i, 4d–h, 5c–i, 6g, 9g and 10g) are representative of 4–5 animals from a single experiment. Experiments related to the visualization and characterization of TRACS (Figs. 4e and 5e and Extended Data Fig. 7a) were repeated twice independently with similar results. RNAi phenotyping of survival and regeneration (Figs. 6b,c, 7c and 8h) was repeated at least twice independently with similar results. Visualization of notom* or wnt11* cells in RNAi-treated animals (Fig. 5e–k and Extended Data Fig. 10j) is representative of 4–6 animals from a single experiment. Quantification of H3P density (Figs. 7f,g and 8d,e) was repeated at least twice independently with similar results.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All scRNA-seq data supporting the findings of this study have been deposited at the GEO under accession code GSE146685. scRNA-seq data can also be explored in our Shiny app at https://simcompbio.shinyapps.io/bbp_app/. Previously published sequencing data that were reanalysed here are available under accession codes GSE111764 (ref. 45) and GSE107874 (ref. 45). A list of SMEDIDs for all of the cloned genes is provided in Supplementary Table 15 and sequence information are available online (https://planosphere.stowers.org/findgenes). All other data supporting the findings of this study are available from the corresponding author on reasonable request or can be accessed from the Stowers Original Data Repository (http://www.stowers.org/research/publications/lhpbp-1513). Source data are provided with this paper.

Code availability
Original scripts used for the analysis and visualization of single-cell sequencing data are available at GitHub (https://github.com/0x644BE25/smedSPLiT-seq).

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Author contributions
Conceptualization and data interpretation: B.W.B.-P. and A.S.A. Data analysis: B.W.B.-P., C.E.B. and S.C. Acquisition of data: B.W.B.-P., A.M.K., A.R.S. and A.C.B. Cloning of planarian gene transcripts: F.G.M. Writing the original manuscript: B.W.B.-P. Supervision and funding acquisition: A.S.A. All of the authors revised and edited the manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Identification of optimal biopsy sizes and treatments for single cell reconstruction. (a) Schematic of experimental design. (b) Piwi-1 expression of parent animals at time biopsy was taken after irradiation treatment (right), as well as representative images 14 days post amputation (dpa), survival curves, and scoring of regeneration of photoreceptor pigmentation of biopsies 0.75mm–1.50mm taken from comparable parent animals following irradiation treatment. Notation on representative images indicates number of fragments that regenerated photoreceptors by 14dpa out of total surviving at 14dpa (exact n is provided in source data file). (c) Representative images of biopsies from un-irradiated, sub-lethally irradiated, and lethally irradiated animals imaged 1, 2, 4, 7, 10, and 14 days post amputation. Scale = 500µm.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Optimization and acquisition of a single cell reconstruction of planarian regeneration. (a) Schematic of experimental design using Atto-conjugated linker molecules to visualize SPLiT-seq reagents after second [2] and third [3] round barcoding, and to detect biotin tagged molecules [4]. (b) Representative images of cells/objects detected in the Hoechst + compartment at all steps of the barcoding process and area vs. Hoechst intensity plots with Hoechst + cell compartment highlighted from each stage of barcoding. Note the accumulation of non-nucleated debris that occurs during the barcoding process that needed to be removed prior to sequencing. As a result, Hoechst + intact cells were sorted following barcoding using the plot depicted in B [4] as a guide. Gating strategy utilized pre-barcoding (c) and post-barcoding (d). Abundance of sorted population pre-barcoding (e) and post-barcoding (f). (g) Number of cells captured, mean nUMI/cell, and mean nGene/cell for each of the 21 conditions. (h) UMAP embeddings cells sampled from each of the 21 conditions (see materials and methods for sub-sampling methodology) illustrating the change in tissue composition and captured transcriptional states across the dataset.
Extended Data Fig. 3 | Annotation of muscle, epidermal, and intestinal tissue subclusters. UMAP embedding of global dataset with tissue highlighted and UMAP embedding of tissue cells colored by tissue subcluster ID for muscle (a), epidermis (b), or intestine (c). Scaled mean expression of cluster enriched genes by tissue and tissue subcluster for muscle (d), epidermis (e), and intestine (f) enriched genes. Whole mount in situ hybridization of tissue markers analyzed in D (g), E (h), and F (i). Scale = 500µm.
Extended Data Fig. 4 | Annotation of neural and parenchymal tissue subclusters. UMAP embedding of global dataset with nervous system (a) or parenchyma (e) highlighted. UMAP embedding of neural (b) or parenchymal (f) cells colored by tissue subcluster ID. Scaled mean expression of cluster neural-enriched (c) or parenchymal-enriched (g) genes by tissue and tissue subcluster. Whole mount in situ hybridization of tissue markers analyzed in C (d) or G (h). Scale = 500µm.
Extended Data Fig. 5 | Annotation of phagocytic, protonephridial, and pharyngeal tissue subclusters. UMAP embedding of global dataset with tissue highlighted and UMAP embedding of tissue cells colored by tissue subcluster ID for phagocytic (a) and protonephridial (d) cells. Scaled mean expression of cluster enriched genes by tissue and tissue subcluster for phagocytic (b) and protonephridia (e) enriched genes. Whole mount in situ hybridization of tissue markers analyzed in B (c) and E (f). (g) UMAP embedding of global dataset with pharyngeal clusters highlighted. (h) UMAP embedding of pharyngeal cells. (i) Whole mount in situ hybridization of pharynx-enriched genes. Scale = 500µm.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Additional Data supporting tissue annotations and identification of TRACS. (a) Tissue annotation prediction made using Fincher et al. tissue annotations transferred to SPLiT-seq dataset using Seurat’s TransferData function. (b) Proportion of cells from each global tissue cluster assigned to tissue lineages by TransferData. UMAP embedding of all neural (a), parenchymal (c), phagocytic (e), protonephridial (g), and pharyngeal (i) cells, colored by time. Scaled proportion of cells from each neural (b), parenchymal (d), phagocytic (f), protonephridial (h), or pharyngeal (j) subcluster across sampled conditions, normalized to the sample in which the subcluster had maximum representation.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Data supported the visualization and quantitation of TRACS. (a) Selected images of whole mount double-fluorescent in situ hybridizations of wound-induced genes (yellow) and tissue-specific markers (magenta). (b) Schematic representation of TRACS quantitation strategy. (c) Selected images of whole mount in situ hybridization of piwi-1* stem cells and immunohistochemistry of H3p* mitotic cells. (d) Quantitation of density of H3p* cells in control and colchicine-treated animals. n = 9 (0%), 7 (0.10%), 10 (0.15%), 9 (0.20%), and 9 (0.25%) biologically independent animals. Data are presented in box plots of min, max, and median, with the bounds of the box at the first and third quartile and whiskers extending from quartile to the minimum or maximum. P values are two-sided unpaired t-tests compared to 0%, with no corrections for multiple comparison. Scale = 500 μm.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Additional data supporting wound-induced muscle cluster and M16-enriched genes requires for tissue maintenance and regeneration. UMAP embedding of all muscle cells, colored by time after amputation and split by cells from biopsies taken from un-irradiated (a), sub-lethally irradiated (b), or lethally irradiated (c) animals. (d) Scaled proportion of cells from each muscle subcluster across sampled conditions, normalized to sample in which subcluster had maximum representation. (e) Scaled mean expression of muscle cluster 16 enriched genes (black arrow) by tissue, muscle subcluster, and sample. (f) Gene expression of screened muscle genes in bulk RNA-seq dataset of planarian regeneration. (g) UMAP feature plots and gene expression patterns of grp78, CaATPase, and tubulin-β visualized by fluorescent in situ hybridization in intact animals and regenerating fragments 2 days post amputation. (h) Representative images RNAi-treated animals 3 days post feeding. (i) Survival of RNAi-treated animals shown in H (n = 20 for each condition). (j) Representative images of homeostatic (21 days post feeding, 10 animals) and regeneration phenotypes (14dpa, 20 animals) in unc-22 and tubulin-β RNAi-treated animals. (k) Gene expression patterns of M16-enriched genes visualized by fluorescent in situ hybridization in intact animals and regenerating fragments 2 days post amputation. P values are log-rank test (i) compared to unc-22 control. Scale = 500µm.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Additional data supporting amputation-specific epidermal clusters and epidermal genes requires for stem cell proliferation.

UMAP embedding of all epidermal cells, colored by time after amputation and split by cells from biopsies taken from un-irradiated (a), sub-lethally irradiated (b), or lethally irradiated (c) animals. (d) Scaled proportion of cells from each epidermal subcluster across sampled conditions, normalized to sample in which subcluster had maximum representation. (e) Scaled mean expression of E2/E20 enriched genes (black arrows) by tissue, muscle subcluster, and sample. (f) Gene expression of screened epidermal genes in bulk RNA-seq dataset of planarian regeneration\(^5\). (g) Gene expression patterns of epidermal genes visualized by fluorescent in situ hybridization in intact animals and regenerating fragments 2 days post amputation. Selected images of whole mount in situ hybridization of piwi-1\(^+-\) stem cells and immunohistochemistry of H3P\(^+\) mitotic cells in RNAi-treated animals 7 days post feeding (h), or 7 days post amputation (j). H3P\(^+\) cell density in RNAi-treated animals 7 days post feeding (i) or 7 days post amputation (k). n (7dpf) = 9 (unc-22), 8 (agat-1), 9 (agat-3), 10 (agat-2), 6 (cyp3142a1), and 10 (atp1a1) biologically independent animals. n (7dpa) = 7 (unc-22), 11 (agat-1), 7 (zfhx3), 9 (agat-3), 10 (agat-2), 8 (cyp3142a1), and 7 (atp1a1) biologically independent animals. Data are presented in box plots of min, max, and median, with the bounds of the box at the first and third quartile and whiskers extending from quartile to the minimum or maximum. P values are two-sided unpaired t-tests with no corrections for multiple comparison compared to unc-22 control. Scale = 500\(\mu\)m.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Additional data supporting amputation-induced intestinal clusters and intestinal genes required for tissue homeostasis and regeneration. UMAP embedding of all intestinal cells, colored by time after amputation and split by cells from biopsies taken from un-irradiated (a), sub-lethally irradiated (b), or lethally irradiated (c) animals. (d) Scaled proportion of cells from each intestinal subcluster across sampled conditions, normalized to sample in which subcluster had maximum representation. (e) Scaled mean expression of I9/I12-enriched genes (black arrows) by tissue, muscle subcluster, and sample. (f) Gene expression of screened intestinal genes in bulk RNA-seq dataset of planarian regeneration63. (g) Gene expression patterns of intestinal genes visualized by fluorescent in situ hybridization in intact animals and regenerating fragments 2 days post amputation. (H) Selected images of whole mount in situ hybridization of piwi-1+ stem cells and immunohistochemistry of H3P+ mitotic cells in RNAi-treated animals 2 days post amputation. (i) Selected images and raw x,y position of notum+ anterior pole cells visualized by whole mount in situ hybridization. (j) H3P+ cell density in RNAi-treated animals 2 days post amputation. n = 8 (unc-22), 9 (lrrk2), 8 (cathepsinB), 7 (myosin1e), 8 (polybromo-1), and 8 (talin-1) biologically independent animals. (k) Number of notum+ cells in RNAi treated animals. n = 4 (unc-22), 5 (lrrk2), 4 (cathepsinB), 3 (myosin1e), 4 (polybromo-1), and 6 (talin-1) biologically independent animals. (l) Distribution of notum+ cells in RNAi-treated animals, n = 152 (unc-22), 180 (lrrk2), 125 (cathepsinB), 92 (myosin1e); 88 (polybromo-1), and 180 (talin-1) notum+ cells. P values are a two-sided unpaired t-tests, with no adjustments for multiple comparisons. Data are presented in box plots of min, max, and median, with the bounds of the box at the first and third quartile and whiskers extending from quartile to the minimum or maximum (i) or as mean values +/− SD (k,l). Scale = 500µm (G,H) or 150µm (I).
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☑️ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

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☑️ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☑️ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☑️ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

*Our web collection on [statistics for biologists](https://natureresearch.com/statistics) contains articles on many of the points above.*

Software and code

Policy information about [availability of computer code](#)

Data collection: Images were collected using Zeiss Zen Black (v8.1) and the Leica Application Suite (LASX).

Data analysis: Microsoft Excel (Version 16.36), Fiji (Version 2.0.0-rc-68/1.57), IDL (Version 8.3.0) and Graphpad Prism (Version 8.3.0) were used for Data Analysis. In addition, scRNA-seq data was processed and analyzed in R (Version 3.6.1) using Dropseq Tools (v 2.4.0) and the Seurat software package (v3). Original software [scripts](#) utilized for analysis and data visualization are available at https://github.com/Okx0448E25/smedSPLIT-seq. Cytometry data collection and analysis was performed with BD FACSDiva Software (v8.0).

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All scRNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE146685. scRNA-seq data can also be explored in our shiny app at: https://simrcompbio.shinyapps.io/bbp_app/. Previously published sequencing data that were re-analysed here are available under accession codes GSE111764 (24) and GSE107874 (49). SMEDIDs for all cloned genes are listed in Supplementary Table 15 and sequence information can be found at https://planosphere.stowers.org/limd/ggenes. All other data supporting the findings of this study are available from the corresponding author on reasonable request or can be accessed from the Stowers Original Data Repository at [http://www.stowers.org/research/publications/bbpb-1513](http://www.stowers.org/research/publications/bbpb-1513).
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No sample calculations were performed. Sample sizes were chosen to be similar to previously published data (5-10 animals) for highly penetrant phenotypes (Manuscript References 21, 25-27, 30). |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the analyses |
| Replication | The single cell sequencing data presented in the study was a single experiment and was not independently replicated due to resource constraints. Gene expression patterns of subcluster enriched genes shown by in situ hybridization (Figures 1D, 2E, Extended Data 3C, F, I, 4D, H; 5C, F, I, 6G, K, 9G, 10G) are representative of 4-5 animals from a single experiment. Experiments related to the visualization and characterization of TRACS (Figures 4B, 5E, Extended Data 7A) were repeated twice independently with similar results. RNAi phenotyping of survival and regeneration (Figures 6B, C, 7C, D; 8B, C, F, G) were repeated at least three times with similar results. Characterization of the CNS and gut in RNAi treated animals (Figures 6D, 7C, and 8H) was repeated at least twice independently with similar results. Visualization of notum + and Wnt1+ cells in RNAi treated animals (Figure 5E-K, Extended Data 10J) is representative of 2-6 animals from a single experiment. Quantitation of H3P Density (Figures 7F-G, 8D, J, Extended Data 9H-J, 10H) were repeated at least twice independently with similar results. |
| Randomization | Animals were randomly allocated to RNAi treatment conditions and stainings. |
| Blinding | For RNAi primary and secondary screens (Tables S16 and S17), investigators were blinded to allocation during experiments and phenotype assessment. For all H3P imaging and quantitation, investigators were blind to allocation for outcome assessment. For other phenotype characterization after initial screening (PCT, gata456, Wnt, Notum), the investigators were not blinded to allocation during experiments and outcome assessment. For quantitation of TRACS (Figs 4-5), investigators could not be blinded to time point or treatment because animal morphology was obviously impacted by treatment independent of marker imaged. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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| ☒ ☐ Eukaryotic cell lines      | ☒ Flow cytometry |
| ☒ ☐ Palaeontology              | ☐ MRI-based neuroimaging |
| ☐ ☑ Animals and other organisms |         |
| ☒ ☑ Human research participants |         |
| ☒ ☑ Clinical data               |         |

Antibodies

Antibodies used

- Anti-phospho-histone H3 (Ser10)[H3P] antibody (1:500, abcam, ab32107) and Alexa-conjugated goat anti-rabbit secondary antibodies (1:1000, abcam, ab150086) were used to stain mitotic cells.

Validation

- Anti-phospho-histone H3 (Ser10)[H3P] antibody (1:500, abcam, ab32107) was validated by KD my Abcam and was independently validated by absence or reduction of staining in irradiated animals (Reference 67).

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Adult animals of the clonal CW4 strain of Schmidtea mediterranea were used for all experiments. Since animals were asexual clones, age since birth cannot be measured. However, all animals used in the study were allocated 6-8 weeks after the most recent regeneration event. |
|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
Wild animals
This study did not involve wild animals.

Field-collected samples
This study did not involve field-collected samples.

Ethics oversight
Husbandry and experimental protocols for invertebrate planarians are not subject to ethical approval via IACUC.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
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Methodology

Sample preparation
Animals or biopsies were rinsed in calcium-magnesium free buffer with 1% BSA (CMF8) (67), finely chopped with a single edge stainless steel blade, then agitated on a benchtop rocker in CMF8 for 20 – 30 minutes with vigorous pipetting every 3 – 5 minutes. After maceration, cells were filtered through a 30µm Falcon cell-strainer and centrifuged for 5 minutes at 300g. Cells were re-suspended in 1X CMF8 + 1:1000 LIVE/DEAD Dye (Life Technologies L34975) and incubated on ice for 20 minutes, then centrifuged for 5 minutes at 300g and resuspended in 1 ml PBS + RNase Inhibitors (Invitrogen AM769, Qiagen Beverly Inc Y9240L) and fixed as previously described for split-pool ligation based RNA-seq (22). Cells were centrifuged for 10 minutes at 1000g, re-suspended in 1ml 5µg/ml Hoechst 33342 in 0.5x PBS + RI, incubated at RT for 10 minutes, and stored on ice prior to FACS.

Instrument
BD Influx Sorter

Software
BD FACSDiva Software (v8.0)

Cell population abundance
Nucleated cells were 22% of FACS events [Extended Data 2E] pre SPLITseq barcoding and 24% of FACS events after SPLITseq barcoding [Extended Data 2F]

Gating strategy
Singlet cells were identified by combining a SSC-SCC/FSC-FSC gate with a Trigger Pulse Width (Extended Data 2C-D). From this population, nucleated cells were sorted based on low staining with a NearIR Live/Dead Dye (750LP Red APC.Cy7), Extended Data 2C-D) and high intensity staining with Hoechst 33342 (460.50.UV-Hoescht Blue, Extended Data 2C-D).

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