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## Types or States? Cellular Dynamics and Regenerative Potential

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### Summary

Many of our organs can maintain and repair themselves during homeostasis and injury, due to the action of tissue-specific, multipotent stem cells. However, recent evidence from mammalian systems suggests that injury stimulates dramatic plasticity, or transient changes in cell potential, in both stem cells and more differentiated cells. Planarian flatworms possess abundant stem cells, making them an exceptional model for understanding the cellular behavior underlying homeostasis and regeneration. Recent discoveries of cell lineages and regeneration-specific events provide an initial framework for unraveling the complex cellular contributions to regeneration. In this review we discuss the concept of cellular plasticity in the context of planarian regeneration, and consider the possibility that pluripotency may be a transient, probabilistic state exhibited by stem cells.

### Keywords

Stem cells; regeneration; homeostasis; planaria; plasticity

### Cell dynamics during regeneration

Stem cells are cells that divide continuously, both to self-renew and to produce various cell types in our bodies. During embryonic development, stem cells are multipotent, capable of producing all the cell types in the animal. As development proceeds, stem cell potential gradually diminishes, eventually becoming lineage-restricted and producing only a subset of cell types matched to the organ [1]. Present throughout our lives, these tissue-specific stem cells replenish dying cells and maintain the physiological function of our organs, in a process called homeostasis.

Based on their low-level activity in adult animals, tissue-resident stem cells participate in tissue repair by producing the particular cell types present in a given organ. However, emerging evidence suggests that while these stem cells can occasionally participate in tissue repair, the changing environment induced by wounding can also stimulate other, differentiated cells to contribute to regeneration [2,3]. This expansion of lineage potential –

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termed *plasticity* – has been described in several mammalian organs, including mammary glands, prostate glands, lung, the small intestine, and hair follicles [4–9]. For example, in the murine small intestine, homeostasis is largely driven by fast-cycling cells located at the crypt base [10]. Wounding or genetic ablation of these rapidly dividing cells causes the typically unipotent quiescent stem cells at the +4 position to become multipotent, now producing all of the cell types comprising the crypt [11]. Similarly, lineage-tracing of cells expressing the differentiated marker *Dll-1* normally produce Paneth, enteroendocrine and secretory goblet cells, but after ablation of stem cells following irradiation, they now give rise to long-lived, multi-lineage clones [12]. Severe injuries in the lung also cause differentiated cells to adopt proliferative behavior and restore damaged tissues [13,14]. Therefore, injury induces environmental stimuli that elicit distinct cellular behaviors, facilitating organ repair.

In general, the dynamic behavior stimulated by injury calls into question our formal definitions of “stem cells” and “differentiated cells”, and suggests that the differentiated state, at least for certain tissues, may not be terminal. Instead, within mature organs, cells may adopt what could be considered a *stable differentiation state*, permitting plasticity to occur upon injury. Recent advances in the transcriptional analyses of single cells underscore this possibility, as these data have now revealed a striking degree of single-cell, non-genetic heterogeneity in otherwise apparently homogeneous cell populations [15–19]. Such heterogeneity likely reflects changes in the dynamics of expression of key regulatory genes. In light of this growing evidence, the traditional definition of “cell type” may be in need of reevaluation.

## The flatworm *Schmidtea mediterranea* as a system for studying *in vivo* stem cell behavior

Freshwater planarians are an exceptional model organism for studying the *in vivo* regulation of stem cells and how they contribute to regeneration [20]. Planarians can regenerate virtually any body part after amputation, due to the involvement of pluripotent stem cells (neoblasts) that are dispersed throughout the body. In this review we argue that the greater regenerative capacity of planarians offers a tremendous opportunity to understand the cellular mechanisms underlying regeneration, including the interplay between differentiated tissues and stem cells, and transitions between homeostatic and regenerative states.

Despite their relatively simple outward appearance, planarian anatomy is quite elaborate (Figure 1), consisting of derivatives of all three germ layers. Planarian organ systems include a complex central nervous system [21,22], photoreceptors [23], a digestive system consisting of a branched gastrovascular system [24], a pharynx, and a primitive excretory system called protonephridia [25,26], all enveloped by body wall muscle and epithelial cells. All of these organs regenerate readily after amputation.

Distributed throughout the animals are small, dividing cells called neoblasts. Thought to be the only dividing cells in the animal, neoblasts produce various cell types based on lineage tracing [24,27–29] and uniformly express many markers including the Argonaute protein *piwi-1* and histone *H2B* [30,31]. Aspects of the molecular regulation of stem cells have been

extensively reviewed elsewhere [32,33]. Recently, transplantation assays were developed to determine the differentiation potential of single stem cells in irradiated hosts (which lack stem cells). With time, single cells produced all of the animal's tissues, formally demonstrating that one neoblast (termed "cNeoblast", for clonogenic Neoblast) can be truly pluripotent [29]. However, rescue occurs at a low frequency (7/120 transplanted cells), and it is unclear whether this reflects a relatively low, natural occurrence of cNeoblasts, or is a result of technical limitations. Furthermore, molecular markers for these cells have not yet been identified, leaving questions about their physical location and behavior unresolved.

The abundance of stem cells, their broad distribution, and their ability to produce so many different tissue types suggests that this is a heterogeneous cell population. Measurements of gene expression in isolated stem cells [34–36] and *in vivo* [37] have confirmed the heterogeneity of neoblasts, and some heterogeneously expressed genes are transcription factors essential for organ regeneration. These have been identified by transcriptional profiling of mature tissues [38,39], testing candidates [23,28], RNAi screening for organ regeneration defects [25,40], gene expression analysis of neoblasts [36] or RNA-sequencing purified neoblasts [41] (Figure 2A). Knockdown of these organ-specific transcription factors causes a failure to regenerate the cognate organ, indicating that these genes may function to mark the future fate of stem cells. Coexpression of these lineage markers in subsets of neoblasts (broadly defined as *piwi-1*<sup>+</sup>) raises the possibility that lineage specification occurs at the level of the stem cell. Therefore, the stem cell population may be fragmented into lineage-restricted subsets [42], but the significance of heterogeneity, and how it may contribute to regeneration, is not yet clear.

### Stem cell lineages during homeostasis

During homeostasis, neoblasts undergo steady rates of mitosis, replacing cells lost to the normal wear and tear of adult life. Unlike other invertebrate model systems, planarians can grow (or shrink) to any size depending on feeding status [20,43,44]. Growth is fueled by stimulating stem cells to proliferate [45], and is counterbalanced by a constant rate of cell death, maintaining body size [46]. As they experience cycles of growth and degrowth, overall scaling of organs is preserved [47–49], suggesting that all cell types are produced (or lost) in a proportional manner [50–54].

Lineage tracing experiments show that during homeostasis, stem cell progeny are steadily incorporated into existing organs including epithelial cells, intestine and neurons [24,27,29,55]. As these cells are produced, they stably express tissue-specific transcripts. Therefore, a general assumption is that during normal steady-state conditions, pluripotent cells produce various specialized progenitors at a constant rate, perhaps stochastically. Now that several progenitor markers are known, a quantitative analysis of the lineages generated by stem cells during homeostasis will resolve this issue. An intriguing possibility is that during homeostasis, the extensive diversity of transcription factors expressed within neoblasts may be explained by dynamic and possibly transient expression of determinants across the stem cell pool over time. It is not yet known, however, whether the presence of stable progenitor cells influences the behavior of individual stem cells during homeostasis.

## Organ regeneration highlights dynamic stem cell responses

The presence of progenitor markers within the stem cells creates an opportunity to understand how regeneration alters the dynamics of these cell populations. Because most organs in planarians are broadly distributed throughout the body (Figure 1), amputation typically removes only part of the organ, leaving the remainder damaged. This incomplete organ loss confounds analysis of progenitor behavior. Two examples of progenitor markers for anatomically restricted organs offer an opportunity to observe how stem cells contribute to *de novo* organ regeneration.

Photoreceptors are located in the anterior of the animal, and the transcription factor *ovo* is required for their regeneration [38]. During homeostasis, these *ovo*<sup>+</sup>/*piwi-1*<sup>+</sup> progenitors are produced in a steady stream and are expressed in vanishingly few neoblasts directly posterior to the photoreceptors. Head amputation stimulates the production of new photoreceptors, initiated by activation of *ovo* within stem cells (Figure 2B). Knockdown of *ovo* causes a highly specific inability to regenerate all cell types within the photoreceptors, while other tissues regenerate normally, suggesting that *ovo* functions as a selector gene [56] to drive photoreceptor formation.

The pharynx can be completely removed without overt damage to other tissues, facilitating the study of the stem cell response to selective organ loss. The Forkhead transcription factor *FoxA* is essential for regeneration of the pharynx but not other organs [40,41]. *FoxA* is expressed in a subset of stem cells, but only those in the immediate vicinity of the pharynx (Figure 2B). After chemical amputation, stem cells activate expression of *FoxA* specifically within stem cells. Although the mechanism of *FoxA* upregulation is not known, the increase in the proportion of stem cells expressing *FoxA* suggests that a dynamic shift in gene expression in progenitor cells is induced by organ loss.

Amputation of heads or tails (as in Figure 3) generates fragments lacking pharynges. In contexts where *de novo* regeneration of the pharynx occurs in fragments, stem cells in the anterior or posterior (not normally expressing *FoxA*) activate its expression [57] (and Adler, unpublished), indicating plasticity in the stem cells during regeneration. For both photoreceptors and the pharynx, pre-existing stem cells in the body must recognize the absence of these organs, and subsequently initiate expression of organ progenitors. This highlights key questions in planarian regeneration: are stem cells constantly monitoring the presence and absence of every organ? If so, how are the stem cells instructed to produce specific organs after amputation? Answering these questions are important future goals of planarian research.

## Injury-induced Responses in Planarians

After injuries, planarians rapidly heal wounds and quickly re-establish proper axial polarity. Tissues are regenerated within days, almost without error, after almost any type of amputation [24,58,59]. Organs, transcripts, and antigens that are normally restricted to specific regions now redistribute to establish proper proportioning of the animal [57,60,61]. Wounding induces potent changes throughout the animal, in both differentiated tissues and in stem cells, significantly altering the organismal context in which differentiated and stem

cells reside [62,63]. Understanding how injury induces this transition from a homeostatic state into a regenerative state, and how modified stem cell behavior contributes to regeneration are key issues to resolve.

### Immediate transcriptional changes in differentiated tissues

Although the identity of a wound signal is unknown, it likely diffuses quickly because within minutes of injury, dramatic transcriptional changes ensue throughout the animal [64,65], in both stem cells and differentiated tissues. Analysis of these early-response genes, mostly present in differentiated cells, showed distinct classes of wound-response genes. Probably because of functional redundancy, knockdown of many of these genes do not cause detectable phenotypes [64], leaving some uncertainty as to their function in regeneration. However, these early transcriptional changes in the pre-existing tissue are essential for initiating regeneration.

Many patterning genes are expressed in distinct domains during adult homeostasis. Removal of heads or tails disrupts this normal pattern, inducing disproportional expression patterns (Figure 3) [66]. After amputation, these markers reappear in a stereotypical manner at anterior-facing (*sFRP-1*, *wnt2* and *sFRP-2*) or posterior-facing (*wntP-2*) wounds, even in the absence of stem cells [63,67,68], suggesting that these potential morphogens could provide axial information during regeneration. In fact, grafting of stem-cell-depleted tissue influences the fate of regenerated tissues [69,70]. Thorough analysis of several potential positional markers shows that they are expressed subepidermally, in subsets of muscle cells [71], and may create a coordinate system for regeneration, providing positional information by transcriptional activation of patterning genes [72]. Therefore, differentiated cells actively participate in regeneration by expressing patterning genes that directly influence cell fate decisions.

### Stem cell response to wounding

Stem cells also respond rapidly to injuries. After wounding, two bursts of cell proliferation occur: six hours after injury, a wave of proliferation spreads throughout the entire body, and then two days after amputation, there is a localized peak of proliferation near the wound site [73–75]. Transplantation assays show that wounding mobilizes stem cell migration toward the amputation plane [76]. Transcriptional profiling with microarrays [64] and RNA-sequencing of purified stem cells [41] also identify several transcription factors that are upregulated in the first two days of regeneration.

Transcriptional plasticity of stem cells is best understood in the formation of a new anterior pole during regeneration. After removal of anterior tissues, neoblasts near the wound site initiate a cascade of several transcription factors, beginning with the Forkhead transcription factor *FoxD* [77,78] and then *Zic-1*, which further specifies subpopulations of stem cells to generate an anterior pole [79]. Anterior pole cells (which are differentiated cells) express several genes required for normal head formation: *notum*, *folliculin*, *prep*, and *pbx* [78–81]. To promote head formation, Wnt signaling must be suppressed, which is achieved by expression of the Wnt inhibitor notum. The rapid response of pre-existing tissue to activate this cascade of transcription factors demonstrates the plasticity of stem cells to wounding.

The signaling machinery responsible for altering stem cell behavior is poorly understood. Some potential receptors have been identified by transcriptional profiling or candidate approaches, such as the FGF receptor *fgfr-1* [31], the EGF receptor *efgr-3* [82] and the G-protein-coupled receptor *P2X-A* [83]. Future efforts will extend our knowledge of the signaling machinery regulating stem cell behavior, as well as whether they instruct specific responses (e.g. inducing proliferation or differentiation).

## The end of regeneration and the return of homeostasis

Regeneration is expected to invoke distinct biological responses as compared to the homeostatic state. This is supported by evidence that knockdown of some genes impairs regeneration and not homeostasis. For example, the Bmp inhibitor follistatin [80,84] and the transcription factor *runt-1* [64] exhibit regeneration-specific defects, while homeostasis is unaffected. Other genes, including the growth-regulatory kinase Tor and JNK, decouple rates of proliferation and apoptosis normally observed during regeneration [85,86].

Therefore, stem cells may adopt two distinct states – homeostasis and regeneration – and each state may exhibit different transcriptional and cellular characteristics. In adult animals, most of our organs reach steady state after development completes [87]. However, for organs that regenerate readily (such as blood, liver, and intestine), a feedback mechanism may exist to limit organ size and prevent excessive overgrowth [88]. Without this inhibition, these organs may undergo unlimited growth, potentially resulting in a cancerous state. This feedback control could be either intrinsically or extrinsically regulated [89], and the mechanism may differ among organs. Because planarians' bodies continually expand and shrink during their lives, these types of organism-wide signals may exhibit heightened activity throughout a planarian's lifetime. It will be interesting to determine which cell types may be involved in sensing tissue loss and alters its behavior after amputation.

One appealing hypothesis is that mature organs produce specific factors that inhibit their own growth [90]. The most well-characterized example of these types of signals is the TGF $\beta$  family member myostatin, which is expressed in mammalian muscle; mutations in myostatin cause vast muscle overgrowth [91,92]. In planarians, distinct, localized organs like the pharynx and photoreceptors could produce this kind of signal. In fact, some evidence exists that the pharynx can inhibit its own regeneration [93–95]. How might this signaling be regulated? For example, are stem cells constantly monitoring the presence of every differentiated cell type in the animal? Because cNeoblasts do not increase their proliferative rates after amputation [29], these cells are unlikely to sense the absence of organs. Instead, this responsibility may fall on the organ-specific progenitors embedded in the stem cell population.

## Redefining regenerative potential as a cellular state

Whether during homeostasis or regeneration, communication between cells in changing environments directly impacts their behavior and output. Considering the large number of neoblasts in planarians, the extensive heterogeneity in expression of cell fate determinants in these cells [36,40,41] and the relative scarcity of cNeoblasts as assayed by single cell transplantation [29], an important question arises: how are stem cells maintained in these



changing conditions? Two distinct and testable models become apparent. First, a standard model, in which cNeoblasts represent a core of naïve, pluripotent stem cells constantly undergoing self-renewal (Figure 4, Deterministic Model). However, given that in serial transplantations an estimated 168 neoblasts [76] and only 7/120 single cell injections [29] are capable of rescuing lethally irradiated animals, combined with the uncertainty about the abundance and distribution of cNeoblasts within the animal, it is difficult to explain how small fragments can regenerate complete animals. A second, non-standard model in which cNeoblasts arise stochastically, or on demand, from a larger population of fate-restricted or primed neoblasts (Figure 4, Probabilistic Model). In this latter model, self-renewal becomes a conceptual property not possessed by a discrete population, but transiently held by a small number of cells and arising probabilistically depending on the demands of the animal. If these stem cells stochastically express progenitor markers for specific organs, perhaps injury induces changes in the frequency or periodicity of expression, resulting in altered differentiation of stem cell progeny. Such a model allows us to frame the remarkable plasticity of planarian in terms of dynamic cell states rather than statically defined cell types.

Our ability to identify these dynamic states of a cell's life history may have been limited by the methods we have used to probe the system – fixed analysis of tissues and single-timepoint analysis of transcriptomes. With technologies that permit the observation of cell behavior and transcriptional output over time, we are beginning to realize that cell types are not static: even though anatomical position and cell function may be fixed, stochastic and transient changes may occur at the cellular level. As technologies advance, our understanding of the responses of cells to various environmental stimuli will only improve. Future efforts aimed at understanding the dynamics of these cellular states – both in planarians and in other animals – may allow us to redefine cells as having dynamic “states” instead of just fates.

### Concluding Remarks: Sic Transit Gloria Cella

Understanding how stem cell behavior is influenced by environmental signals may be the key to deciphering regenerative capacity. Planarians provide intriguing examples of the dynamism of transcriptional activity in differentiated and undifferentiated cells during both tissue homeostasis and injury. However, evidence for the ability of cells to modulate their states under homeostasis and tissue repair is also provided by mammals, particularly by committed intestinal cells that revert to stem cells upon crypt damage. Thus, even though these cells express fate determinants like *Dll-1* [12], they can nonetheless be recalled to the stem cell compartment when needed. Another example of plasticity comes from committed enteroendocrine cells expressing fate determinants such as *neurogenin 3*, which can produce both non-endocrine and non-secretory cells [96,97].

The planarian stem cell population exhibits three key features: 1) the ability to self-renew; 2) the presence of truly pluripotent cells (cNeoblasts); and 3) a high degree of heterogeneity. Although the transcriptional heterogeneity observed appears to be static, current, static experimental methods prohibit the possibility of observing more dynamic transcriptional events. Stochastic and transient transcriptional changes may allow for rapid responses to injury and regeneration demands. Understanding how this may be accomplished will require

molecular identification of the cNeoblasts, further characterization of the lineage produced by them during organ regeneration, and additional knowledge about the molecules responsible for communication between stem cells and their environment (Outstanding Questions Box).

Given the desire to harness and manipulate our own cells for the benefit of regenerative medicine, it is essential to understand the complex processes of stem cell maintenance and fate dynamics in different contexts. Studying how planarian stem cells and differentiated cells respond to injury may unlock the key to controlling cell states, allowing us not only to shed light on the mechanisms controlling developmental timing but also on viable approaches leading to the production of particular organs on demand.

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### Outstanding Questions Box

**What is the wound signal that stimulates distinct stem cell behaviors?**

Injury triggers rapid transcriptional changes, cell cycle entry, and repatterning. The signals responsible for initiating these responses are presently unknown.

**What distinguishes cNeoblasts from the rest of the stem cell population?**

cNeoblast is an operational definition for planarian pluripotent stem cells, but specific molecular markers for these cells have not yet been identified. Defining specific molecular attributes for these cells will facilitate the identification of their anatomical location and allow analysis of their behavior during regeneration.

**How do stem cells sense missing tissues?**

Regeneration proceeds without error after any type of amputation, suggesting that stem cells rapidly alter their homeostatic states in response to missing tissue. With their complex anatomy and numerous cell types, how this response is coordinated and executed at the molecular and cellular levels is unclear.

**What cellular signaling machinery is present on stem cells?**

Extracellular signaling likely instructs changes in cellular dynamics. Our knowledge of the molecules responsible for changes in stem cell behavior is limited, but will continue to expand with high-resolution sequencing and improved molecular techniques.

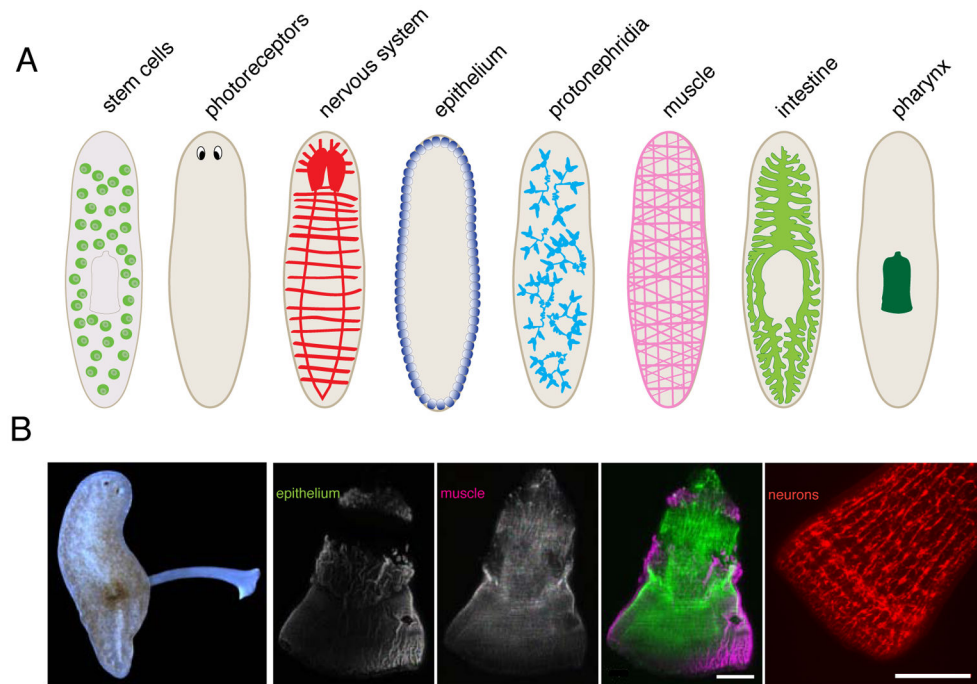
**When does regeneration stop?**

Once organs reach their final size and are properly integrated, the accelerated activity of stem cells should subside and return to a homeostatic state. Future work may identify how this is coordinated in the animal.

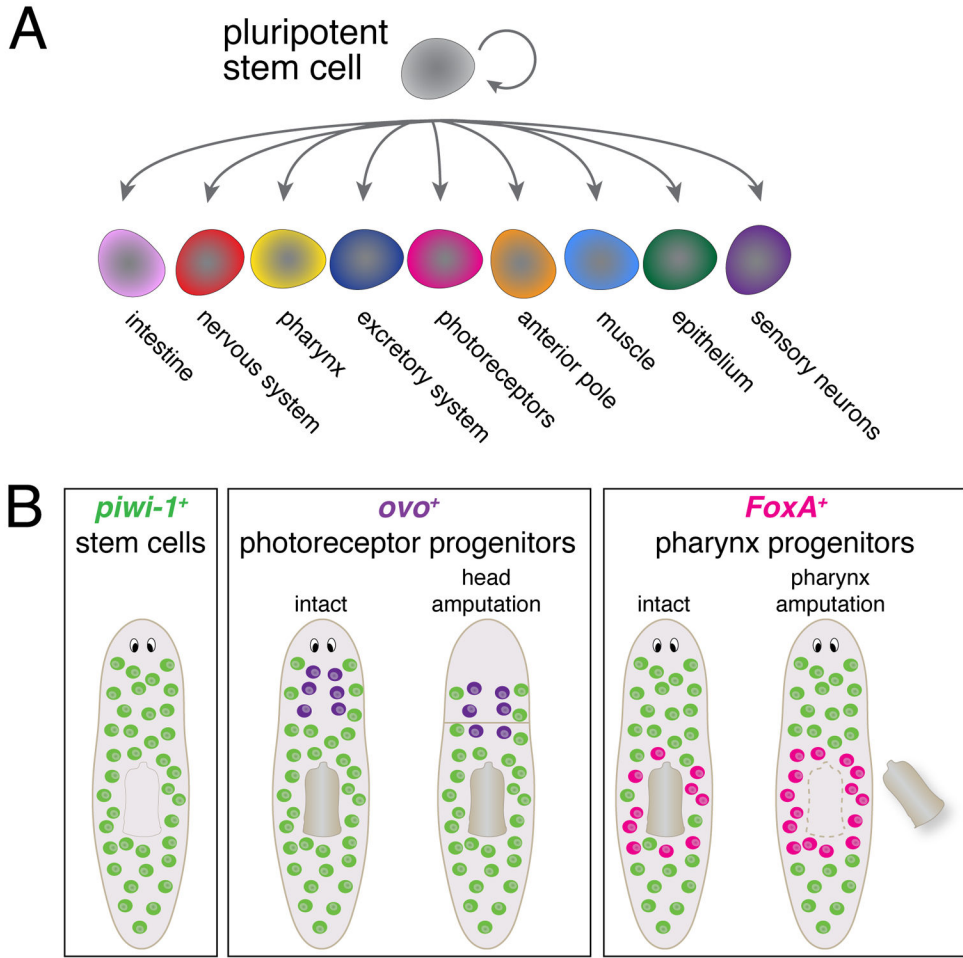
### Trends Box

- Injury induces dramatic changes in tissues. Emerging data from mammalian systems suggest that injury triggers transcriptional and cellular changes in tissues, demonstrating that plasticity of cellular states may be a unique state that facilitates regeneration.
- Planarian flatworms readily regenerate after any amputation, demonstrating a rapid response to tissue loss. Because regeneration depends on pluripotent stem cells, swift homeostatic state changes of these cells must occur.
- The planarian stem cell population exhibits transcriptional heterogeneity. Many transcription factors required for organ-specific regeneration are expressed in distinct segments of the stem cell population.
- After injury, differentiated cells and stem cells alter their transcriptional profiles and cellular behavior. These dynamic changes may describe unique cellular states that drive regeneration.

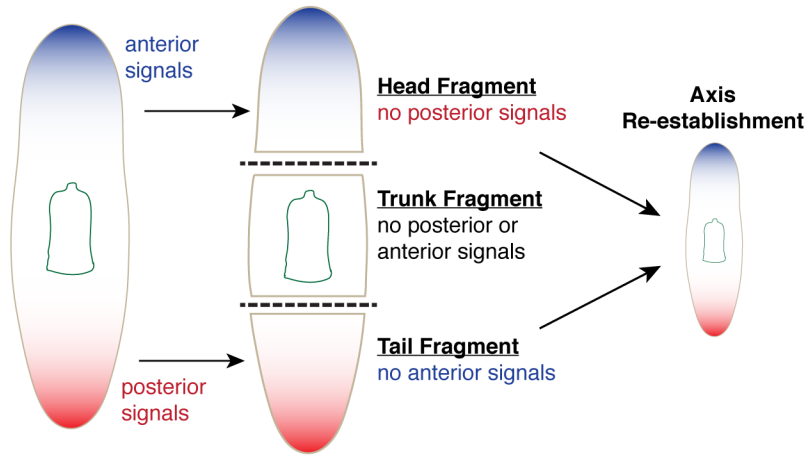




**Figure 1.** Planarian anatomy. (A) Various organs in asexual flatworms. Each organ illustrated here consists of several cell types. (B) Left, live animal extending its pharynx. Right, pharynx anatomy in isolated pharynges with stained epithelial cells, muscle, neurons ( $\alpha$ -FMRF-amide). Scale bars, 100 $\mu$ m.

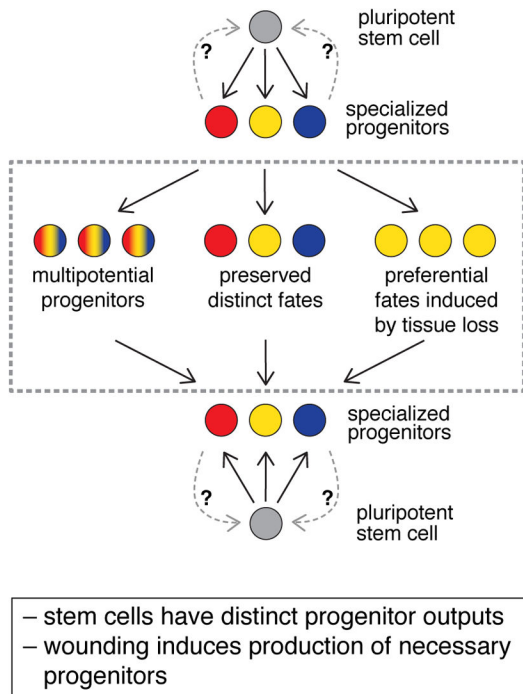
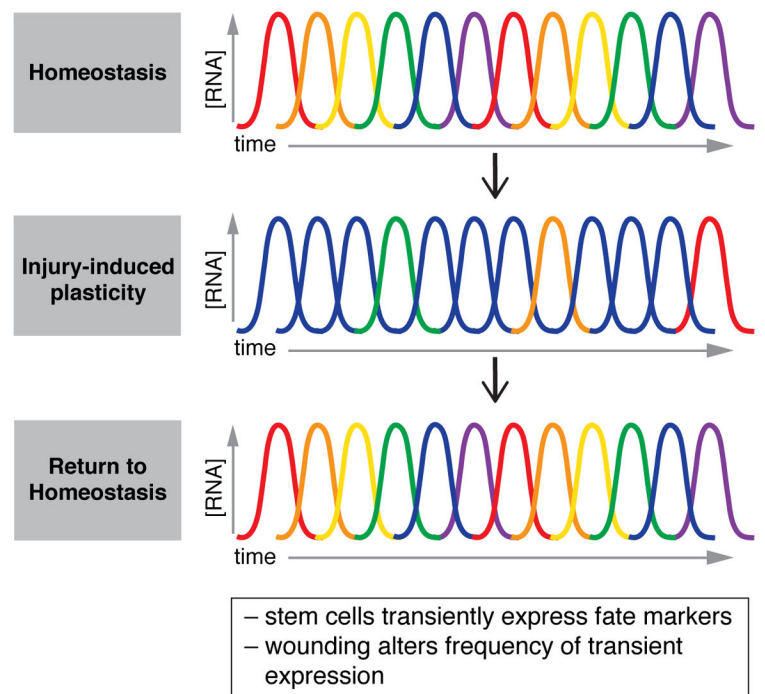


**Figure 2.** Lineage progenitors for organ regeneration. (A) Several specialized progenitors for many tissues are produced during homeostasis, derived from pluripotent stem cells. (B) Changing distribution of organ-specific progenitor markers after amputations (highlighted in pink or purple).



**Figure 3.**

Amputation initiates polarity re-establishment. Removal of anterior and posterior regions induces dramatic changes in the animal. Transcriptional markers of the anterior and posterior (represented by blue and red, respectively) are expressed in differentiated cells, and removed after significant amputations, leaving disproportional axial patterning in fragments. Within 12–24 hours, expression of these markers is reestablished in fragments and instructs fate determination of regenerating tissues.

**Deterministic Model****Probabilistic Model****Figure 4.**

Two models for progenitor dynamics during homeostasis and regeneration. In the deterministic model, stem cells produce specialized progenitors that statically express specific fate markers. Injury stimulates changes in the rates and types of progenitors produced, allowing animals to respond to different wounds. In the probabilistic model, stem cells and their progenitors transiently express fate markers (RNA concentration represented by curves, with colors representing different genes being expressed at different times). Wounding may alter the frequency and/or persistence of expression of fate determinants, thus producing the necessary cell types required for regeneration.