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Rebuilding a planarian: from early signaling to final shape

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ABSTRACT **Why some animals can regenerate and others not has fascinated biologists since the first examples of regeneration were reported. Although many animal phyla include species with some regenerative ability, mainly restricted to particular cell types or tissues, there are some other species capable of regenerating complex structures, such as the vertebrate limb and heart. More remarkably, there are some examples of animals that can regenerate the whole body from a tiny piece of them. Understanding how regeneration is triggered and achieved in these animals is fundamental not only to understand this fascinating primary biological question, but also because of its implications for the field of regenerative medicine. Here, we discuss one of the models with higher regenerative capabilities: the freshwater planarians. Two key features make planarians an attractive model to study regeneration: the presence of adult pluripotent stem cells and the permanent activation of the morphogenetic mechanisms that instruct cell fate. Here, we revise our current knowledge of key events that lead to successful regeneration including: how heterogeneous is the stem cell population; what are the immediate changes at the gene level after amputation and what triggers the regenerative response; how is axial polarity re-established; how do the different cell types differentiate from lineage-committed progenitors and how is size and organ proportionality controlled. Finally, we point out some open questions that the field needs to address in the near future.**

KEY WORDS: *planarian, regeneration, neoblast, signaling, morphogenesis, cell differentiation*

Introduction

The majority of model organisms with regenerative capabilities can only regenerate certain organs or extremities. However, a select few, including *Hydra*, planarians, and starfish can regenerate the entire organism from small portions thereof. Planarians can regenerate from a wide array of body fragments, and can arguably regenerate faster and more comprehensively than any other bilaterian. Freshwater planarians are dorsoventrally flattened, soft-bodied, free-living members of the phylum Platyhelminthes. They are acoelomate and triploblastic, and lack circulatory, respiratory, or skeletal structures. Their striking regenerative ability has attracted the interest of scientists for centuries, and has led to their use as a classical model system of regeneration (Saló and Baguñà, 2002; Reddien and Sánchez-Alvarado; 2004, Saló, 2006). In other models such as amphibians and zebrafish regeneration is partially driven by cell dedifferentiation (Tanaka, 2016). By contrast, regeneration in planarians is based upon two key biological properties: 1) the presence in adult organisms of an abundant population of pluripotent stem cells; and 2) the continuous activity and coordination of cell-to-cell communication signaling pathways. Moreover, intact planarians display a high level of plasticity; their morphogenetic pathways are always "on", enabling continuous growing and de-growing depending on environmental conditions, while always retaining proper body proportions (Saló, 2006). With advances in the development of molecular and genomic methodologies, planarians have become the reference organisms for the study of regeneration and homeostatic cell turnover. RNA interference (RNAi), administered by injection, feeding or soaking, shows high penetrance in planarians, enabling effectively interference with gene function. This methodology can be used to alter the expression of several genes simultaneously. Indeed, RNAi loss-of-function studies have led to the discovery of many cellular and molecular processes involved in regulating planarian regeneration.
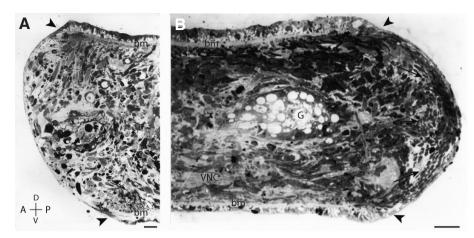
What drives the regenerative response after sectioning?

After injury in planarians, wound healing is mediated by local muscle contraction, which put into contact the existing dorsal and

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Abbreviations used in this paper: ROS, reactive oxygen species.

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Fig. 1. Light micrographs of planarian sagittal sections stained with borax-azur II and methylene blue. (A) *Tail fragment 30 minutes after cutting. A thinly stretched wound epithelium covers the entire wound area. The end of the basal membrane of the existing epidermis indicates the cut level (arrowheads).* **(B)** *Head fragment 24 hours after cutting. Arrowheads indicates the cut level. The wound epithelium is maintained and a dense aggregation of neoblasts, initiating blastema formation (arrows), is visible beneath the wound. bm, basal membrane; A, anterior; P, posterior; D, dorsal; V, ventral; G, gut; VNC, ventral nerve cord. Scale bars: 50 μm.*

encoding extracellular remodeling matrix proteins and signaling components is expressed ubiquitously in the existing epidermis (blue line, Fig. 2). The role of ECM during regeneration in cell migration and anoikis was described (Isolani *et al.*, 2013). In addition, recent studies have demonstrated that changes in the extracellular matrix (ECM) during regeneration are involved in both the assembly of tissues from differentiating progenitor cells (Bonar and Petersen, 2017; Seebeck *et al.*, 2017) and in neoblast migration (Abnave *et al.*, 2017). In the mesenchyme close to the wound site, a second wave of translation-dependent gene expression is initiated during the first 6 hours post-injury. The genes involved, which include *wnt-1, nlg1, inhibin-1,* and *follistatin,* belong to different pathways required for patterning (Sandman *et al.*, 2011, Wenemoser *et al.*, 2012, Wurtzel *et al.*, 2015). This second wave occurs in cases involving tissue loss, and specific gene expression depends on the missing region (Injury specific response, green line, Fig. 2).

A parallel wave of wound-induced gene expression in neoblasts includes transcription factors, chromatin remodeling proteins, histone methyltransferases, and cell cycle factors (dashed green line, Fig. 2). Finally, differentiated tissue markers are expressed 72 hours after injury (red line, Fig. 2) (Wenemoser *et al.*, 2012; Wurtzel *et al.*, 2015). In summary, injury triggers the activation of general injury-response genes, followed by injury type-dependent gene expression, which enables acquisition of the appropriate tissue identity and pattern, depending on the missing tissue. Finally, the expression of cell-differentiation genes promotes tissue and organ differentiation, restoring functionality (Fig. 2). A recent study has shown the importance of ERK signaling in initiating regeneration in planarians (Owlarn *et al.*, 2018). Importantly, in this study it has been shown that the generic wound response to any kind of injury is necessary to induce regeneration in a missing-tissue context.

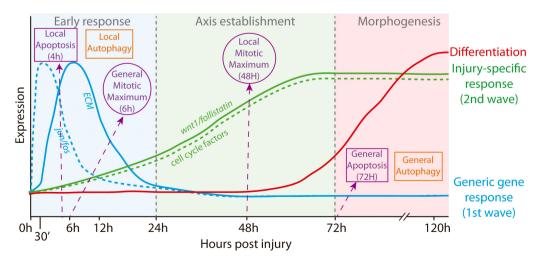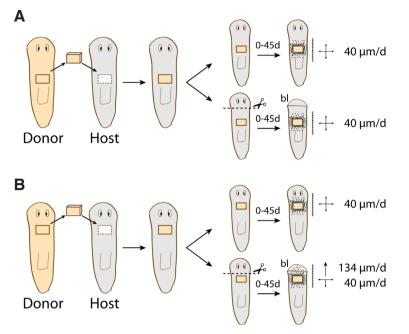Planarian regeneration is dependent on the presence of pluripo-

ventral epidermis. Shortly after muscle contraction, muscle relaxation induces stretching of the existing epidermis, which closes the wound with a thin epidermal film (Fig. 1) (Chandebois, 1980). Direct contact between the healing epidermis and the mesenchyme facilitates epithelial-mesenchymal interactions, which trigger the early gene response. Once the epithelium is regenerated a new basal membrane is produced. All injuries (with or without tissue loss) trigger an early, generic gene response, affecting mainly the epidermis, muscle, and neoblast population (Wenemoser *et al.*, 2012). A first wave of gene upregulation occurs in differentiated cells, and includes two subgroups of early genes that are transiently activated within 30 minutes of injury until 6 or 12 hours after respectively. These genes are activated in the vicinity of the wound in a translation-independent manner, and are predominantly transcription and signaling factors (*e.g., jun-1, fos-1, egrl-1, egr-2,-3,-4, MAP3K-1)* (dashed blue line, Fig. 2). In addition, a group of genes

Fig. 2. Summary of the main events that regulate planarian regeneration at the genetic and cellular levels. *Any type of injury, regardless of whether tissue is lost or not, triggers a generic rapid and transient gene response (blue box and continuous and dashed blue lines). During this early response, local increases in apoptosis and autophagy in the regions close to the wound and a general activation of mitosis are observed. In cases involving tissue loss, the decline of the early response is followed by the activation of a new set of genes involved in injury-specific responses (green box and continuous and dashed green lines). The genes activated are related to axis respecification and patterning. During this period mitotic activity in regions close to the wound peaks, resulting in a significant increase in the amount of new blastemal tissue. In the final stage, which is characterized by differentiation and morphogenesis (red box and red line), existing tissue is remodeled in accordance with the new body proportions via general apoptosis and autophagy.*

Fig. 3. Grafting method used to analyze cell migration. *When a fragment of* S. mediterranea *sexual strain is grafted to a* S. mediterranea *asexual strain, the origin of cells can be traced using the chromosomal heteromorphic marker of the asexual strain cells. These experiments revealed that in intact animals (A-B) and amputated far from the transplant no oriented migration of cells is observed (dots)* **(A)**, *showing an non-oriented migration/spreading of 40 µm/day. Interestingly, this migration is preferentially oriented towards the wound when the graft is close to the wound, showing an estimated speed of 134 µm/day* **(B)**. *bl, blastema.*

tent adult stem cells, a unique cell type whose proliferative ability is activated in response to injury. Neoblasts exhibit a coordinated bimodal mitotic response, with a body-wide peak observed 6 hours post-injury, consisting mainly of G2 neoblasts, followed by a second peak around the wound region (Fig. 2) (Saló and Baguñà, 1984). This second local peak occurs only when the injury results in tissue loss; simple poking or small incisions induce only the first general wave of early mitotic activation (Wenemoser and Reddien, 2010). The second local wave of neoblast mitosis occurs in the existing tissue and produces new undifferentiated tissue, known as blastema. Although the blastema continues to grow, the mitotic activity remains mainly localized within the pre-existing tissue close to the wound (an area of ~500 µm in diameter), an area known as the postblastema (Saló and Baguñà, 1989). The blastema therefore grows from its base through the entry and accumulation of non-dividing neoblasts produced in the postblastema (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). Some studies have proposed that the accumulation of mitotic neoblasts in the postblastema is a local process. Grafting experiments demonstrate that an amputation triggers the accumulation of mitotic cells at the wound region and that the origin of those cells is not farther than 500 µm from the wound approximately (Fig. 3) (Saló and Baguñà, 1985, 1989). Long distance migration has only been observed in experiments of partial irradiation (Dubois, 1949; Guedelhoefer and Sánchez-Alvarado, 2012). Thus, if a planarian is partially irradiated in the anterior region and then the head is amputated, neoblasts from the non-irradiated tail region migrate towards the anterior wound in few days. Interestingly, neoblasts only invade the

irradiated tissue after amputation, but never in homeostatic conditions, indicating that wounding triggers the migratory response (Guedelhoefer and Sánchez-Alvarado, 2012). More recently, it has been suggested that the cellular migration would include not only stem cells but also committed cells (Abnave *et al.*, 2017).

Planarian regeneration is a global process, involving the production of new tissue (the blastema) at the amputation planes, as well as the remodeling of existing fragments to adjust to the organism's new proportions. The latter process is dependent on cell death mediated by apoptosis and autophagy. During regeneration, apoptosis occurs in two waves. The first peaks a maximum at 4 hours post-injury and is local, affecting the region close to the wound. The second peaks a maximum at 3 days post-injury and is general in nature (affecting the entire body) (Fig. 2) (Pelleteri *et al.*, 2010). Autophagy is also activated very early during regeneration, with activity observed 1 day post-amputation localized mainly in the postblastema region, spreading gradually to all existing tissues as remodeling processes occur (Fig. 2). However, additional activation of autophagy occurs in the reproductive system, testis, and ovaries at 1 day post-amputation (González-Estévez *et al.*, 2007).

Recent studies in animal models indicate that cell death is a pre-requisite to trigger the regenerative response (Bergantiños *et al.*, 2010; Vriz *et al.*, 2014). However, in planarians it remains unclear whether apoptosis is required for regeneration; no studies have examined the effects of directly inhibiting apoptosis during regeneration. What it is known is that in planarians conserved JNK expression is required to induce the early apoptotic response 4 hours after injury (Almuedo-Castillo *et al.*, 2014). Importantly, inhibition of JNK in planarians not only inhibits the early apoptotic response but also unblocks the G2-M checkpoint of neoblasts entering mitosis, and prevents the activation of early-response genes. JNK therefore serves as a hub, translating wound signals into apoptotic cell death, controlled stem cell proliferation, and differentiation during regeneration (Almuedo-Castillo *et al.*, 2014). Also, there is evidences that Akt signaling mediates the spatial distribution of cell death during tissue repair (Peiris *et al.,* 2016). Although the molecular and cellular mechanisms upstream remain unclear, cell death is commonly triggered by the activation of reactive oxygen species (ROS), mainly through MAPK or JNK signaling (Vriz *et al.*, 2014).

Reactive oxygen species and planarian regeneration

In recent years, ROS have emerged as key players in the initiation of the regenerative program in a variety of models. ROS include molecules such as O_2^-, peroxide (H_2O_2), and hydroxyl radicals (OH^-), which are produced from oxygen as by-products of aerobic metabolism or enzymatic production and are implicated in multiple pathologies including cancer, aging, and neurological dysfunction. However, ROS also participate in cell regulation and signaling in development, immunology, wound healing, and angiogenesis (Alfadda and Sallam, 2012; Hernández-Garcia *et al.*, 2010). Remarkably, ROS signaling is required for regeneration in a variety of models from *Drosophila* to newts, in which its blockade results in defective impaired regeneration. During *Xenopus* tadpole tail regeneration, sustained ROS production is necessary for cell

proliferation and activation of the Wnt/β-catenin pathway (Love *et al.*, 2013). Fin regeneration in zebrafish also requires the production of ROS, which then activate apoptosis and the JNK pathway to trigger the cell proliferation necessary to form the blastema (Gauron *et al.*, 2013). ROS production also stimulates the proliferation of ependymiglia cells during regeneration of midbrain dopamine neurons in newts (Hameed *et al.*, 2015). Finally, in *Drosophila* imaginal discs, ROS produced by dying cells activate JNK and p38 in the surrounding living cells, triggering a regenerative response (Santabárbara-Ruiz *et al.*, 2015).

In planarians, however, although ROS inhibition also blocks regeneration (Pirotte *et al.*, 2015) it is unknown whether it is required to initiate it. After amputation, a rapid burst of ROS production occurs in the wound region. The use of chemical inhibitors of ROS production such as DPI (diphenyleneiodonium chloride) and APO (apocynin) results in a marked reduction in blastema growth during regeneration. Interestingly, in contrast to the reported pro-proliferative role of ROS in other models, these defects in planarians do not appear to be due to a reduction in either cell proliferation or neoblast number. However, ROS inhibition clearly affects cell differentiation, inhibiting CNS regeneration and significantly reducing numbers of different neuronal cell types and epidermal progenitors (Pirotte *et al.*, 2015). Future studies in planarians will need to identify the sources of ROS after amputation, as well as the downstream signaling pathways they affect. In other models, ROS action has been linked to MAKP signaling. The EGFR/ERK pathway, which plays an important role in cell differentiation during planarian regeneration (Fraguas *et al.*, 2011, 2017; Tasaki *et al.*, 2011), may also constitute a necessary target of ROS to ensure successful regeneration
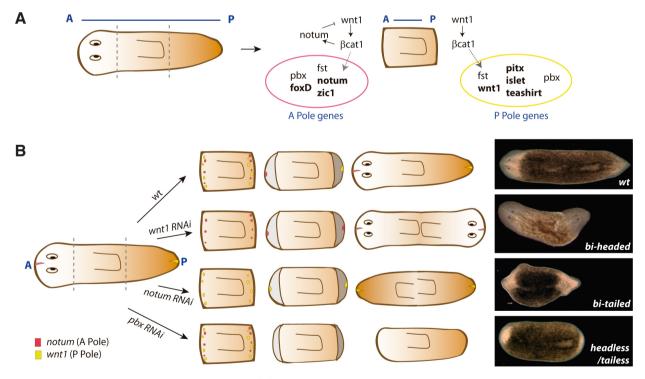
Axial establishment

Successful and functional regeneration requires triggering of a wound response, to stimulate the generation of new cells, and activation of the signals that assign the correct fate to newly generated tissue. Thus, if a tail is amputated, the blastema cells must acquire posterior, and not anterior, identity. This process is particularly complex in planarians, which are capable of regenerating any missing region, regardless of the position or the size of the amputated portion. The molecular mechanisms that confer positional identity to the new blastema cells in planarians have been extensively studied over the last 10 years (Owlarn and Bartscherer, 2016; Almuedo-Castillo *et al.*, 2012; Reddien, 2011; Molina *et al.*, 2011). To stimulate regeneration, a signaling center is formed in the blastema very soon after amputation (Vogg *et al.*, 2016). Depending on the position of the wound, the signaling center acquires an anterior (A) or a posterior (P) instructive activity. Thus, when the missing portion is anterior, the signaling center provides the signals required to properly regenerate a brain and all missing structures with the correct orientation and position with respect to the existing tissue. Similarly specialized signals are generated when the missing portion is posterior. These signaling centers, also known in planarians as poles (A or P), consist of a cluster of muscle cells located at the tip of the blastema from early regenerative stages (12-24 h), which appearance requires proliferation and differentiation of pre-existent stem cells (Petersen and Reddien 2009). The molecular signatures of the A and P poles have been partially deciphered. The A pole expresses *notum* (a Wnt inhibitor) and *follistatin*, as well as

the transcription factors *foxD* and *zic-1*, which are required for the specialization of neoblasts into A pole progenitors (Petersen and Reddien, 2009; Vasquez-Doorman and Petersen, 2014; Scimone *et al.*, 2014a; Roberts-Galbraith and Newmark, 2013; Gavino *et al.*, 2013; Vogg *et al.*, 2014; Oderberg *et al.*, 2017; Owlarn and Bartscherer, 2016). The P pole expresses *wnt1* and *follistatin* (Adell *et al.*, 2009; Gavino *et al.*, 2013; Petersen and Reddien, 2009). Several studies have shown that the key elements required for the formation of the A versus the P pole are *notum* and *wnt-1*, since RNAi of *notum* induces regeneration of a tail instead of a head, and RNAi of *wnt1* induces regeneration of a head instead of a tail (Adell *et al.*, 2009; Petersen and Reddien, 2009; Petersen and Reddien, 2011) (Fig. 4). However, the results of a recent transcriptomic study suggest that the decisive determinant of AP fate is *notum*, which is the only element differentially regulated in A and P early blastemas (Wurtzel *et al.*, 2015). This finding is of particular significance, given that in other organisms the main role of *notum* is to inhibit Wnt function. Interestingly, the expression of both secreted elements, *notum* and *wnt1*, begins as early as 6 hours post-amputation as a dotted pattern in muscle cells of any wound, and is not restricted to their ultimate location at the A and P poles, respectively, until 24 to 48 hours later (Petersen and Reddien, 2009; Petersen and Reddien, 2011; Sureda-Gómez *et al.*, 2015). This dotted early expression pattern of both genes appears to be independent of proliferation, whereas their expression at the poles requires the proliferation of neoblasts. At which specific stages do *notum* and *wnt1* specify the A versus the P fate? It is assumed that this decision is made very early during regeneration. However, the lack of suitable tools to study gene function at specific times and locations has hindered researcher's efforts to answer to this question. The available data suggest that the A and P poles, once formed, have a role in the patterning and growth of the regenerating tissues, but that the decision to assign an A or P identity is taken earlier. For instance, inhibition of *pbx*, which blocks the formation of both A and P poles but does not affect the early expression of *notum* or *wnt1*, gives rise to planarians that display correct polarity but regenerate small blastemas (Chen *et al.*, 2013; Blassberg *et al.*, 2013) (Fig. 4). In fact, the A and P signaling centers of planarians share several properties with embryonic signaling centers such as the Spemann-Mangold organizer of amphibians and the zone of polarizing activity (ZPA) of vertebrate limbs (Reversade and De Robertis, 2005; Harfe, 2011). These centers may therefore be essential for proper tissue growth, in addition to providing patterning cues. In support of this view, silencing of *pbx*, as well as other elements involved in the formation of the A or P planarian poles, results in a no-tail or no-head phenotype instead of a head-to-tail or a tail-to-head transformation, illustrating the requirement of the poles for tissue growth (Petersen and Reddien, 2009; Rink *et al.*, 2009; Petersen and Reddien, 2011; Sureda-Gómez *et al.*, 2015; Adell *et al.*, 2009; Hayashi *et al.*, 2011; Vasquez-Doorman and Petersen, 2014; Scimone *et al.*, 2014; Vogg *et al.*, 2014).

Interestingly, in the no-tail and no-head phenotypes generated when the poles cannot be organized, the mediolateral (ML) organization of A or P structures is lost. Thus, if a small brain is formed, it will lack the anterior commissure, while in posterior regeneration the two digestive branches and ventral nerve cords (VNCs) fuse and close to form a circle. These phenotypes suggest that the formation of the poles is required to pattern the new regenerating tissue relative to all body axes (AP, dorsoventral/DV and ML. In

agreement, a recent study by the Reddien group proposes that the location at which the A pole forms is dependent upon the axial coordinates of the pre-existing tissue (*i.e.,* the pre-existing AP axis, pre-existing DV plane, and the pre-existing midline (Oderberg *et al.*, 2017). Thus, the A signaling center is formed at the intersection between the pre-existing DV plane and the midline. Using the positional information of the pre-existing tissue, any kind of wound can be correctly patterned and integrated into the pre-existing tissue. Another study by the same group demonstrated that axial coordinates do exist in adult intact planarians, and provide positional identity to adult planarian cells, including neoblasts (Witchley *et al.*, 2013). These axial coordinates are provided by the so-called positional control genes (PCGs). PCGs are secreted elements (typically morphogens) expressed in muscle cells that surround the planarian body. The PCGs that have been functionally studied specify the 3 planarian body axes: the "posterior Wnts", which are expressed in a nested pattern along the AP axis and are responsible for its specification; BMP and its inhibitors, which are differentially expressed along the DV axis and are responsible for its specification; and Slit and Wnt5, which are responsible for the positioning of tissues with respect to the midline.

Thus, correct patterning and growth of regenerating blastemas depends on the organization of signaling centers in the A or P poles of the wound, formed at the intersection between the pre-existing

DV plane and the midline. In this sense, the requirements of adult planarians, which use pre-existing tissue as a reference point, differ to those of an embryo, which must specify all structures *de novo*. Planarians use this mechanism to ensure correct tissue specification and integration after any kind of injury. But how are the axial coordinates provided by the PCGs maintained in an organism that continuously adjusts its size, reproduces by fission, and can regenerate from any tiny fragment? Answering this question has been the recent focus of several research groups. It is now known that a gradient of β-catenin activity, which is regulated by a morphogenetic gradient of "posterior Wnts", patterns gene expression along the planarian AP axis (Iglesias *et al.*, 2008; Petersen and Reddien, 2008; Gurley *et al.*, 2008; Adell *et al.*, 2010; Sureda-Gómez *et al.*, 2016). The findings of a recent mathematical modeling study suggest that this gradient occurs autonomously in the tail and that it takes part of a self-organizing patterning system that patterns the AP axis during homeostasis and regeneration (Stückemann *et al.*, 2017). This self-organizing system would require functional antagonism between the tail Wnt gradient and an as-yet-unidentified head-patterning system.

It was proposed that a FGF-like signal might function as a brain activator in planarians since inhibition of *noudarake* (*ndk*, a FGF receptor-like) produces an expansion of the neural tissues (Cebria *et al.*, 2002a). However, functional studies demonstrate that *ndk*,

Fig. 4. Axial specification during planarian regeneration. (A) *Schematic representation of the genes required for the specification of the A and the P poles. In the A facing wound, the early expression of* notum *inhibits the early activity of* wnt1 *and* βcatenin1, *allowing the expression of the A Pole specific genes (*foxD, zic1, notum*). In the P facing wound,* notum *is not expressed and the early* wnt1 *can activate the expression of the P Pole genes (*wnt1, pitx, islet, teashirt*), through* βcatenin1. *The hierarquichal relationship between the A and the P pole genes is still not well resolved.* βcatenin1 *is also expressed in the A pole, where it could activate the expression of its own inhibitor,* notum *(Petersen and Reddien 2011; Sureda-Gómez* et al., *2016).* pbx *and* follistatin *are required for the specification of both poles.* **(B)** *Schematic representation of the impact of* wnt1, notum *and* pbx *inhibition during the regeneration of the A and P poles. The final phenotype is shown. Inhibition of* wnt1 *produces the regeneration of an A pole in P (leading to bi-headed planarians), inhibition of* notum *produces the regeneration of a P pole in A (leading to bi-tailed planarians), and inhibition of* pbx *impairs the regeneration of both poles (leading to animals that are not able to regenerate the missing tissues). Some images courtesy by Miquel Sureda-Gómez and Eudald Pascual.*

wntA and *fz5/8-4* are required to restrict the expansion of the head domain towards P, but they would not specify A identity (Adell *et al.*, 2009; Hill and Petersen, 2015; Scimone *et al.*, 2016). Recently, two groups of researchers identified new genes that are expressed in an overlapping manner along the body of planarians and provide positional information to cells (Lander *et al.*, 2016; Scimone *et al.*, 2016). Both studies corroborate the role of Wnt and FGF signals in the specification axial identities. They demonstrate that *ndl-3* (a second FGF-like receptor), expressed in the prepharyngeal region, is required to restrict trunk identity, since its inhibition generates ectopic pharynges and mouths. The same phenotype is observed after inhibition of *wnt11-2* (also called *wntP-2* or *wnt4-b*) (Sureda-Gómez *et al.*, 2015). Although the signals that specify the A signaling center need further clarification, together these findings suggest that in planarians the Wnt and FGF pathways coordinate positional identity along the planarian body axis. The restoration of regional identity during regeneration would depend upon the interpretation of those signals.

Interestingly, several studies demonstrate that the formation of the correct signalling center in regenerating blastemas also depends on a pre-existent polarity of the nervous system and on membrane voltage signalling pathways. Thus, H,K-ATPase-mediated membrane voltage and gap junction-dependent signals control head-versus-tail identity during planarian regeneration (Beane *et al.*, 2011, Oviedo *et al.*, 2010). It could be that bioelectric signals underlie the long-range information that specifies the activity of morphogens in the correct location (Lobo *et al.*, 2012).

Stem cell differentiation and morphogenesis

Neoblasts are a population of heterogeneous cells that includes truly pluripotent stem cells as well as lineage-committed progenitors that give rise to the different specific mature tissues (Scimone *et al.*, 2014b; van Wolfswinkel *et al.*, 2014; Wagner *et al.*, 2011; Zhu and Pearson, 2016). These progenitor populations are characterized by the co-expression of *Smedwi-1*, a neoblast marker, and transcription factors specific for the different cell lineages whose RNAi silencing inhibits the differentiation of specific cell types, suggesting that their co-expression with *Smedwi-1* is characteristic of committed cell progenitors still capable of proliferating. How these committed progenitors are specified from pluripotent neoblasts remains to be elucidated. A homologue of the MEX3 RNA-binding protein is proposed to play a general role in cell differentiation, since silencing of its expression results in expansion of the stem cell compartment in parallel with a decrease in the number of lineage-restricted progenitors (Zhu *et al.*, 2015). On the other hand the CCR4-NOT complex, which is involved in the regulation of mRNA degradation through deadenylation, promotes neoblast differentiation through the regulation of stem cell mRNA (Solana *et al.*, 2013). Silencing of *Smed-not1*, a central scaffolding protein of the complex, inhibits regeneration, probably by blocking cell differentiation, as suggested by the specific accumulation of key neoblast markers exclusively in neoblasts, which would account for their failure to differentiate into different cell lineages (Solana *et al.*, 2013). Finally, it has also been reported that a PIWI homologue inherited from pluripotent neoblasts may be necessary for transposon silencing and normal differentiation of those descendent cells (Shibata *et al.*, 2016). However, the exact cues that cause pluripotent neoblasts to activate the genetic programs that convert them into epidermal, gut, or neural progenitors, among other cell types, remain poorly understood. These cues may be both intrinsic and extrinsic. A recent study proposed that the complex body-wall musculature may serve as the source of positional information (Witchley *et al.*, 2013). In the case of planarian eyes, in intact planarians the progenitors of the new photoreceptor and pigment cells are found exclusively in the head region posterior to the eyes (Lapan and Reddien, 2011, 2012). Those progenitors are thought to be responsible for the normal turnover of eye cells. The fact that those progenitors are only found around the eyes suggests that extrinsic cues may regulate their specification from pluripotent neoblasts that are uniformly distributed throughout the body.

Once the individual progenitor populations have been specified, each is characterized by the expression of specific transcription factors required for their final differentiation into distinct cell fates. For example, *ap-2* is required for the differentiation of TrpA+ neurons (Wenemoser *et al.*, 2012), *klf* for mechanoreceptor sensory neurons (Scimone *et al.*, 2014b), *pax3/7* for DBH+ (dopamine-β-hydroxylase) neurons (Scimone *et al.*, 2014b), *lhx1/5-1* and *pitx* for serotonergic neurons (Currie *et al.*, 2013; März *et al.*, 2013), *foxA* for the pharynx (Adler *et al.*, 2014), and *foxD* and *prep* for cells of the anterior pole (Scimone *et al.*, 2014a). However, researchers have only recently begun to identify the genes and signaling pathways that are activated downstream of these transcription factors during the final stages of differentiation of these progenitors. Below, we review current knowledge of the differentiation of several cell types.

Gut cells

The planarian digestive system consists of highly ramified branches that expand to occupy almost the entire body (except in the anterior-most region), thereby ensuring that nutrients are distributed to all cells and thus compensating for the absence of a circulatory system. At the cellular level, the digestive system is a monostratified epithelium composed of two cell types, phagocytes and secretory goblet cells, surrounded by enteric musculature. Regeneration of the gut is accomplished by the differentiation of stem cells and remodeling of the pre-existing gastrodermis (Forsthoefel *et al.*, 2011). In a remarkable RNAi screening study Forsthoefel and colleagues (2012) identified a set of genes expressed in phagocytes and required for several processes, including branching morphogenesis, phagocyte differentiation, and neoblast proliferation. One of those genes, a homologue of ceramide synthase, is required for the differentiation of functional phagocytes (Forsthoefel *et al.*, 2012). Gut progenitors have been defined by the expression of two conserved transcription factors, *hnf-4* and *gata4/5/6*, in *Smedwi-1* positive cells (van Wolfswinkel *et al.*, 2014; Scimone *et al.*, 2014b; Wagner *et al.*, 2011). In other animals, *hnf-4* and *gata4/5/6* are important for endodermal development (Ang *et al.*, 1993; Murakami *et al.*, 2005). Although no functional evidence supporting a role of *hnf-4* in planarian regeneration has been published to date, the silencing of *gata4/5/6* gives rise to severe defects in gut regeneration and maintenance that ultimately result in death, suggesting an important role of this gene in gut cell differentiation and survival (Flores *et al.*, 2016; González-Sastre *et al.*, 2017). A recent study demonstrated a key role of the epidermal growth factor receptor (EGFR) signaling pathway in the differentiation of gut progenitors during regeneration and homeostasis (Barberán *et al.*, 2016a). *Smed-egfr-1* is one of several planarian homologues of the EGFR family of tyrosine kinase receptors (Barberán *et al.*, 2016b) and is

expressed in differentiated gut cells and in gut progenitors in the mesenchyme around the gut branches (Barberán *et al.*, 2016a). Whereas *Smed-egfr-1* silencing inhibits the differentiation of new gut cells, as demonstrated in EdU incorporation experiments, the number of gut progenitors defined by co-expression of *hnf-4*/SMEDWI-1 and *gata4/5/6*/SMEDWI-1 increases significantly. These results strongly suggest that *Smed-egfr-1* is not required for the specification of gut progenitors but is required for their differentiation into mature phagocytes and goblet cells (Barberán *et al.*, 2016a). In this same study, the authors identified a putative ligand of *Smed-egfr-1* named *Smed-nrg-1,* which shares homology with neuregulin-type EGF ligands (Barberán *et al.*, 2016b). The silencing of *Smed-nrg-1* phenocopies the defects observed after knockdown of *Smed-egfr-1*, supporting the hypothesis that *Smed-nrg-1* may act as a ligand of *Smed-egfr-1 in vivo,* thereby regulating the differentiation of gut progenitors. Further experiments will need to determine whether *Smed-egfr-1* expression is regulated by *hnf-4* and/or *gata4/5/6,* and characterize the cell type(s) in which *Smed-nrg-1* is expressed.

Epidermis

The planarian epidermis is a monostratified layer consisting of a number of non-ciliated and ciliated cell types. Intercalated between the epidermal cells are specialized mucus-secreting rhabditic cells, as well as the ducts of mesenchymal secretory cells that open to the outside. The secreted mucus is thought to play a protective role and to facilitate gliding locomotion. The epidermal cells are strongly polarized, with cilia and microvilli at the apical end, lateral septate junctions, and hemidesmosomes at the basal end. Of all planarian cell types the epidermal lineage is among the best characterized. A subclass of neoblasts, named zeta-neoblasts, appear to be involved in epidermal regeneration and homeostatic cell turnover (van Wolfswinkel *et al.*, 2014). Transcription factor *zfp-1* is specific to zeta neoblasts and its silencing results in a significant decrease in the number of newly differentiated epidermal cells (van Wolfswinkel *et al.*, 2014). More recently, several intermediate cellular stages between undifferentiated zeta-neoblasts and mature epidermal cell types have been identified (Tu *et al.*, 2015). In 2008 Eisenhoffer and collaborators identified the genes *NB21.11e* (currently renamed *prog-1*) and *AGAT-1* as markers of early and late neoblast progeny, respectively (Eisenhoffer *et al.*, 2008). That conclusion was based on the time required for the depletion of those populations after the elimination of all neoblasts by lethal irradiation. NB21.11e-expressing cells disappear earlier than AGAT-1-expressing cells. Moreover, these early and late neoblast progeny are spatially separated, with the early progeny located deeper in the mesenchyme and the late progeny located closer to the outside. *prog-1-* and *AGAT-1*-positive cells are derived from zeta-neoblasts, placing them in the epidermal lineage (van Wolfswinkel *et al.*, 2014). A current model of epidermal lineage differentiation proposes that zeta neoblasts gives rise to *prog-1* cells through the activity of *zfp-1*, and that *prog-1* cells in turn give rise to *AGAT-1* cells from which *zpuf-6*-expressing descendants emerge. Finally, *zpuf-6* cells differentiate into different mature epidermal cell types including multi-ciliated cells and marginal adhesive cells (Tu *et al.*, 2015). In agreement with previous reports describing spatial separation of *prog-1* and *AGAT-1*, *zpuf-6* cells are mainly found within or just below the epidermis, whereas *AGAT-1* cells are located deeper within the mesenchyme. The different stages

of the epidermal lineage are thus spatially segregated, with the zeta neoblasts located deepest in the mesenchyme and *zpuf-6* cells closer to the outside. More recently, other transcription factors such as soxP-3, pax-5 and p53 have been shown to participate in the differentiation of the epidermal lineage (Cheng *et al.*, 2018).

A key element in the differentiation of the epidermal lineage is *egr-5*, a member of the "early growth response" family of zinc-finger transcription factors. Planarian *egr-5* is mainly co-expressed with *AGAT-1* in epidermal progenitors, and is barely expressed in mature epidermal cells. *egr-5* silencing leads to a significant decrease in the number of mature epidermal cells and the numbers of *AGAT-1-* and *zpuf-6*-expressing progenitors, ultimately resulting in the loss of epidermal integrity, suggesting a key role of *egr-5* in the differentiation of epidermal progeny cells (Tu *et al.*, 2015). Remarkably, although *egr-5* silencing does not affect the differentiation of cell lineages other than the epidermal lineage, it results in the expansion of neoblast populations and increases numbers of multiple lineage progenitors, including zeta-neoblasts. How the loss of epidermal integrity is sensed by neoblasts and why neoblast expansion occurs after *egr-5* RNAi silencing remain to be elucidated. It has been proposed that this expansion may be part of a stress response to cope with the loss of epidermal integrity (Tu *et al.*, 2015). Interestingly, a similar scenario has been described for the gut lineage: silencing of *Smed-egfr-1* leads to the gradual loss of the digestive system, since gut progenitors are unable to fully differentiate. The increase in the neoblast population and in proliferation observed in these same animals may constitute one element of a general stress response (Barberán *et al.*, 2016a), a view supported by the similar effects observed after *egr-5* silencing.

Central nervous system

One of the most astonishing regenerative abilities of freshwater planarians is their ability to regrow a complete functional central nervous system (CNS) *de novo* from a tiny portion of the organism (Ross *et al.*, 2017). The planarian CNS consists of an anterior bilobed brain or cephalic ganglia connected by a transverse commissure, lying on top of two ventral nerve cords that extend throughout the length of the animal (Agata *et al.*, 1998; Cebrià *et al.*, 2002b). In contrast to its apparent morphological simplicity, the planarian CNS displays a high degree of molecular sub-compartmentalization and complexity (Umesono *et al.*, 1999; Cebrià *et al.*, 2002c), and a wide range of neuronal types (reviewed in Cebrià, 2007; Ross *et al.*, 2017). These include cholinergic (Nishimura *et al.*, 2010), GABAergic (Nishimura *et al.*, 2008a), dopaminergic (Nishimura *et al.*, 2007a), octopaminergic (Nishimura *et al.*, 2008b), serotonergic (Nishimura *et al.*, 2007b; Cebrià, 2008), and neuropeptide F- and GYRFamide-positive (Cebrià, 2008) neurons, as well as several other subpopulations characterized by the expression of specific neuropeptides (Collins *et al.*, 2010).

Recent studies have begun to elucidate how neoblasts are specified into the different neuronal lineages. Molinaro and coworkers, using a combination of single-cell sequencing and *in silico* lineage tracing methodologies, identified a novel neoblast population that may act as neural stem cells (Molinaro and Pearson, 2016). According to the model proposed by those authors, this subpopulation of neoblasts, termed νNeoblasts (nu-Neoblasts), is defined by the expression of *Smedwi-2* mRNA, SMEDWI-1 protein, and a set of neural-specific transcription factors. These νNeoblasts are typically found in the stem cell compartment adjacent to the

brain, and are derived from the apparently pluripotent population of sigma-neoblasts (van Wolfswinkel *et al.*, 2014). They migrate to the cephalic ganglia, where they are integrated as mature differentiated neurons (Molinaro and Pearson, 2016). If we assume that νNeoblasts give rise to the large diversity of planarian neuronal populations, the next challenge is to identify the factors responsible for their specification from νNeoblasts. To this end, several populations of progenitor cells specific for different neuronal types have been characterized in recent years based on their expression of distinct transcription factors. For example, *pitx* and *lhx1/5-1* are required for the differentiation of serotonergic neurons; *soxB2-2* for dopaminergic neurons (Roberts-Galbraith *et al.*, 2016); pax3/7 for DBH (dopa-β-hydroxylase) neurons (Scimone *et al.*, 2014b); *runt* for the expression of markers such as *ovo* and *sp6-9,* which specify photoreceptor and pigment cell progenitors, and *klf* and *ap2,* which are required for the differentiation of mechanosensory and TrpA neurons, respectively (Roberts-Galbraith *et al.*, 2016; Lapan and Reddien, 2011, 2012; Currie *et al.*, 2013; März *et al.*, 2013; Wenemoser *et al.*, 2012; Scimone *et al.*, 2014b). *runt* appears to be required for the specification of several distinct neuronal lineages. Transcription factors belonging to the bHLH family are also expressed in neural progenitors capable of giving rise to several mature neuronal types (Cowles *et al.*, 2013, 2014). These include planarian homologues of *sim*, *coe,* and *hesl-3,* which are expressed in mature neurons and in progenitor cells that continue to express the neoblast marker *Smedwi-1* and are located in the mesenchyme in close proximity to the CNS (Cowles *et al.*, 2013, 2014). *coe* expression has been demonstrated in mature cholinergic, GABAergic, octopaminergic, dopaminergic, and serotonergic neurons, while *sim* is expressed in octopaminergic and dopaminergic neurons (Cowles *et al.*, 2013). The silencing of these two genes results in the regeneration of smaller brains in which several neuronal types are markedly under-represented (Cowles *et al.*, 2013). Given the large number of transcription factors that have been associated with planarian neural progenitors, and the large diversity of neuronal types found in these animals, further research will be required to better understand how these different cell types are specified and differentiate from putative vNeoblasts. Similarly, more studies are required to characterize the mechanisms by which these transcription factors regulate the terminal differentiation of planarian neurons (*i.e.,* to identify downstream elements and/or determine how transcription factors interact with different signaling pathways). Two recent studies have described the role of the Hedgehog and Wnt/β-catenin pathways in neural cell differentiation (Currie *et al.*, 2016; Sureda-Gómez *et al.*, 2016). Silencing of *β-catenin-1* results in the regeneration of smaller brains containing reduced numbers of octopaminergic and dopaminergic neurons (Sureda-Gómez *et al.*, 2016). The transcription factors *nkx2.1* and *arx* are expressed in some ventral-medial (VM) neuronal types, including cholinergic, GABAergic, and octopaminergic neurons, and in *Smedwi-1*-expressing progenitors. The silencing of these transcription factors results in a decrease in the size of these populations, indicating a role in their maintenance (Currie *et al.*, 2016). Moreover, many of these VM neurons also express *hedgehog* (*hh*), and the number of *hh*-positive cells decreases after *arx* RNAi. Remarkably, some stem cells located in the mesenchyme between the two cephalic lobes express the *patched* (*ptc*) receptor, suggesting that they may respond to the Hh signal originating in VM neurons. Interestingly, *hh* silencing

results in significant reductions in several populations of neural progenitors, including those defined by the expression of *lhx1/5-1, coe, nk2-1,* and *arx*. Based on these observations, a model has been proposed in which *hh* expression by VM neurons in the brain signals to stem cells in the mesenchyme, thereby regulating their differentiation into neurons to maintain homeostatic neurogenesis (Currie *et al.*, 2016).

Many transcription factors and signaling pathway have been implicated in the specification and differentiation of the wide range of neuronal types found in planarians. Future studies incorporating the use of lineage-tracing tools should help improve our understanding of the mechanisms involved in their differentiation.

Eye cells

Planarians have simple eyes consisting of 2 cell types: pigment cells and photoreceptors. Pigment cells form a pigmented optic cup surrounding the cell bodies of clustered photoreceptors, which are bipolar neurons with rhabdomeric dendrites facing the pigment cup and visual axons that project to a specific region of the cephalic ganglia (Okamoto *et al.*, 2005). While some visual axons project ipsilaterally to the brain, others do so contralaterally, giving rise to the optic chiasm. The overall projection pattern of visual axons is quite stereotypical, and several studies have uncovered the key functions of genes involved in the guidance of these axons (Cebrià and Newmark, 2005; Cebrià *et al.*, 2007; Almuedo-Castillo *et al.*, 2011). Specific transcription factors regulate the differentiation of pigment cells and photoreceptors. In the case of pigment cells, *sp6-9* and *dlx* are expressed in mature cells and in their specialized progenitors. Silencing of those genes results in animals that are unable to regenerate eye pigment cells, but does not affect photoreceptor cell differentiation (Lapan and Reddien, 2011). Remarkably, during regeneration, eye pigment progenitors expressing *sp6-9* and *dlx* form a trail of cells behind to the newly differentiating eyes. In fact, a gradient of differentiation is observed, such that the cells farthest from the new eyecups are committed undifferentiated progenitors co-expressing *sp6-9* and the proliferation marker *histone h2b,* while those closer to the eyecups continue to express *sp6-9,* together with markers of mature differentiated cells (*e.g.,* tyrosinase) (Lapan and Reddien, 2011). Silencing of either *dlx* or *sp6-9* prevents the regeneration of pigment cell progenitors, indicating a key role of these transcription factors in the specialization of neoblasts towards the pigment cell lineage (Lapan and Reddien, 2011). In fact, specification of the eye lineage implies several additional transcription factors, many of which interact with one another to specify progenitors of both photoreceptors and pigment cells. The earliest stage of neoblast specification towards the eye lineage appears to be determined by co-expression of the transcription factors *ovo*, *sine oculis,* and *eya* (Pineda *et al.*, 2000; Mannini *et al.*, 2004; Lapan and Reddien, 2012). The descendants of those progenitors then split into the lineages that give rise to each mature eye cell type (Lapan and Reddien, 2011, 2012). Photoreceptor cells do not constitute a homogeneous cell population, as they can be divided into anterior, posterior dorsal, and posterior ventral subpopulations based on the expression of different neuropeptide-encoding genes. Anterior cells are positive for *eye53-1* and *npp-12*, posterior dorsal cells co-express *eye53-2* and *mpl-2,* and posterior ventral photoreceptors express *mpl-2* (Collins *et al.*, 2010). While it remains unclear how posterior photoreceptors are specified from intermediate

progenitors expressing *otxA*, anterior photoreceptors appear to be characterized by the expression of the transcription factor *soxB* (Lapan and Reddien, 2012).

In addition, other genes and pathways have also been implicated in eye cell differentiation. Silencing of *Smed-smad6/7-1*, a homologue of the inhibitory SMAD proteins that regulate the BMP pathway, inhibits differentiation of the anterior photoreceptor cells without affecting posterior photoreceptor cells (González-Sastre *et al.*, 2012). Following *Smed-smad6/7-1* knockdown, the number of eye pigment cells during the initial stages of regeneration is similar to that of controls (González-Sastre *et al.*, 2012). However, this number decreases as regeneration, suggesting that *Smed-smad6/7-1* is also directly involved in the differentiation of eye pigment cells, or that this decrease is indirectly caused by the decrease in photoreceptor cell number. Indeed, a relationship between the differentiation of both eye cell types has been proposed. For example, when eye pigment cells fail to differentiate after silencing of *dlx* or *sp6-9,* the visual axons of photoreceptors are often mistargeted (Lapan and Reddien, 2011). Moreover, silencing of *Smed-egfr-1* results in a decrease in the number of regenerated eye pigment cells, in addition to the aforementioned defects observed in the gut (Fraguas *et al.*, 2011). Although the number of photoreceptor cells is unaffected by *Smed-egfr-1* silencing, the clustering of their cell bodies is abnormal (Fraguas *et al.*, 2011). Studies of the BMP pathways have found that low-dose inhibition of *Smed-bmp* results in the regeneration of abnormally patterned eyes with an increased number of anterior photoreceptor cells (González-Sastre *et al.*, 2012). These results suggest that BMP signaling is involved in the differentiation of the anterior photoreceptor population (González-Sastre *et al.*, 2012). Future experiments will need to determine whether *bmp*, *smad6/7-1,* and *egfr-1* are expressed exclusively in mature eye cells or also in eye progenitor cells, and to identify any transcription factors that regulate their expression.

Finally, a recent study has shown that a novel β-catenin homologue named *Smed-β-cat4* appears to act as a dominant-negative regulator of *Smed-β-cat1* to control eye regeneration (Su *et al.*, 2017). Thus, planarians in which *Smed-β-cat4* is silenced regenerate smaller eyes containing fewer photoreceptors and pigment cells. Remarkably, *Smed-β-cat4* is expressed in *ovo*-positive progenitor cells but is not co-expressed with *sp6-9* (Su *et al.*, 2017). This suggests that either (i) *Smed-β-cat4* is involved in the specification of the earliest neoblast descendants that give rise to the eye cell lineage, and from which both photoreceptor and intermediate pigment cell progenitors originate; or ii) *Smed-β-cat4* specifies the photoreceptor lineage, and the defects observed in pigment cell differentiation are an indirect consequence of the decrease in photoreceptor cell number caused by *Smed-β-cat4* silencing, similar to the situation observed after of *Smed-smad6/7* silencing.

Excretory system

The planarian excretory system consists of epithelial tubules known as protonephridia, which are distributed throughout the entire body. This system includes a series of tubules formed by terminal flame cells, the cilia of which drive filtration. The recent identification of a variety of molecular markers has enabled better characterization and visualization of these complex structures (Rink *et al.*, 2011; Scimone *et al.*, 2011). The *Smed-DNAH-β3* (dynein heavy chain) homologue is expressed in terminal flame cells; *Smed-innexin-10* and *Smed-rootletin* are expressed in intermediate ciliated tubules
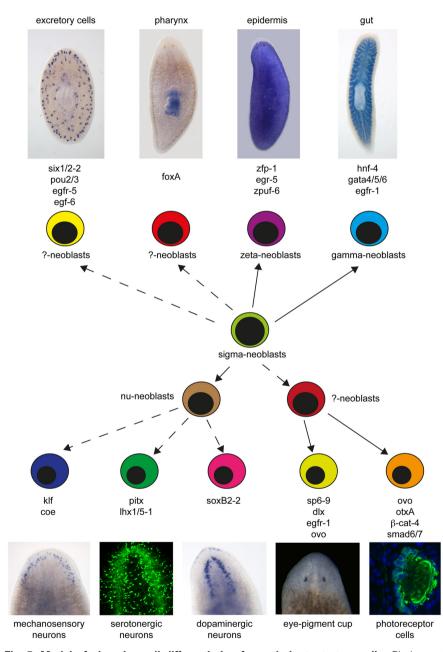
connected to flame cells; and *Smed-CAVII-1* (a carbonic anhydrase homologue) is expressed in non-ciliated distal tubules (Rink *et al.*, 2011; Scimone *et al.*, 2011). Protonephridia progenitors express some conserved transcription factors, such as *Six1/2-2*, *pou2/3,* and *Sall*, the silencing of which impairs protonephridia regeneration and maintenance (Scimone *et al.*, 2011). In fact, the silencing of *Six1/2-2* or *pou2/3* inhibits the generation of progenitor cells, which may explain the subsequent failure to regenerate and maintain the excretory system (Scimone *et al.*, 2011). A current model thus proposes that progenitor cells co-express *Six1/2-2* and *pou2/3* and that descendants from this population terminally differentiate into at least 2 populations: intermediate ciliated tubules, which express *Six1/2-2* and *rootletin;* and distal non-ciliated tubules, which express *pou2/3* and CAVII-1 (Scimone *et al.*, 2011). It remains to be determined whether terminal flame cells are also derived from these progenitors.
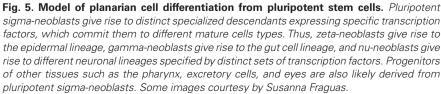
The EGFR signaling pathway has been also implicated in the regeneration and maintenance of the excretory system (Rink *et al.*, 2011; Barberán *et al.*, 2016b). *Smed-egfr-5* is expressed in flame cells and in adjacent ciliated tubules and its silencing results in edema formation, aberrant cell morphology and overall branching pattern as well as in the reduction of flame cells (Rink *et al.*, 2011). The silencing of *Smed-egf-6* induces edema and defects in the expression of Smed-CAVII-1, suggesting that it may function as a ligand of *Smed-egfr-5* to regulate regeneration and maintenance of the excretory system (Barberán *et al.*, 2016b).

In summary, planarian pluripotent stem cells give rise to different populations of descendant progenitors that become specified to differentiate into discrete lineages based on the expression of distinct sets of transcription factors. Once specified, additional transcription factors and/or different signaling pathways can regulate their final differentiation (Fig. 5). Moreover, it appears that specialized progenitors are usually found around the region in which the tissues derived from them will be required during regeneration, suggesting that local signals may control the specification of pluripotent neoblasts towards their final fates. Similarly, during homeostatic cell turnover, progenitors are found in close proximity to the tissues that they will renew, further supporting the view that local signals regulate neoblast differentiation. In addition to the cell-cell communication signals (Wnt, BMP, FGF, and EGF) already mentioned in the preceding sections, recent studies have highlighted the importance of cell-extracellular matrix signaling interactions in the correct positioning of progenitor cells and the regeneration of patterned tissues (Bonar and Petersen, 2017; Seebeck *et al.*, 2017).

Size control and organ proportionality

The majority of animal species have a defined body shape and size in adulthood, and their organs attain a specific size proportional to total body size. The ability of planarians to regenerate from any fragment is dependent upon a robust mechanism that allows the adjustment of organ size as appropriate. During regeneration planarians adjust their final size to the size of the original fragment, thus resulting in well-proportioned body parts, in which new and pre-existing tissues are integrated. While the blastema regenerates some of the missing organs, in some fragments organs such as the pharynx or the reproductive system are regenerated in the pre-existing tissue. Thus, during regeneration extensive and tightly

controlled remodeling of the existing tissue takes place, mediated by cellular death via apoptosis and autophagy (Pelleteri *et al.*, 2010; González- Estévez *et al.*, 2007). The molecular signals that control blastema size in planarians are those involved in regulating cell death and proliferation, and include the JNK signaling pathway, the mTOR pathway and the Hippo pathway. As described above, in planarians JNK translates the wound signals into apoptotic cell death and controlled stem cell proliferation (Almuedo-Castillo *et al.*, 2014). Consequently, JNK RNAi results in the regeneration of tiny blastemas, and prevents the remodeling of existing tissue. Interestingly, the inhibition of genes that impede proper blastema regeneration prevents remodeling and re-patterning of existing tissue to match the new body size. For instance, inhibition of *pbx*, which is required to establish organizing centers in blastemas, not only inhibits differentiation of the missing tissues but also impairs re-scaling of the "posterior Wnts" expression gradient (Blassberg *et al.*, 2013; Chen *et al.*, 2013).

Inhibition of the Insulin receptor in planarians prevents their growth after feeding (Miller and Newmark 2012). The TOR signal is an evolutionary conserved pathway that integrates nutrient status with cell growth and division (Loewith *et al.*, 2011). Although its role in relation to the nutritional status of planarians has not been uncovered, its inhibition in regenerating planarians leads to the inability to form blastemas and to remodel the pre-existing tissues (Peiris *et al.*, 2012, Tu *et al.*, 2012). The inhibition of *PTEN* and *smg-1* in planarians produces overproliferation and outgrowths that are rescued by Rapamycin, a TOR pathway inhibitor (Oviedo *et al.*, 2008; González-Estévez *et al.*, 2012). Those results show the relevance of the insulin-TOR pathway in the control of growth in planarians, although further studies are required for a deeper understanding. The Hippo pathway is a conserved signaling pathway that integrates signals controlling proliferation, cell death, and differentiation during development (Pfleger, 2017). While inhibition of *yki* results in blotted planarians due to altered homeostasis of the excretory system (Lin and Pearson 2014), in-depth molecular analyses have shown that *yki* RNAi animals display a hyper-activated wound response (Lin and Pearson 2017). Thus, inhibition of *yki* induces blastema regeneration, with increases both in cell number and in the number of differentiated cells. This finding appears to contradict the reported role of *yki* in other regenerative contexts; in both zebrafish and mice *hippo* inhibition (which induces Yki phosphorylation and nuclearization) activates the regenerative response (Zhou *et al.*, 2015; Loforese *et al.*, 2016). However, we recently found that *hippo* inhibition (*i.e.,* Yki activation) in planarians promotes cell plasticity and acquisition of stemness (de Sousa *et al.*, 2018). While this effect does not promote a regenerative response, it is in agreement with the reported role of Yki as a stemness promoter in other models (Pfleger, 2017). It is possible that hippo inhibition (*i.e.,* yki activation) in planarians fails to facilitate regeneration because cell dedifferentiation fuels the regenerative response in vertebrate regenerative systems (Yanger *et al.*, 2013; Kikuchi, 2015), whereas expansion of the stem cell population is the only source of new cells in planarians.

Fig. 5. Model of planarian cell differentiation from pluripotent stem cells. *Pluripotent sigma-neoblasts give rise to distinct specialized descendants expressing specific transcription factors, which commit them to different mature cells types. Thus, zeta-neoblasts give rise to the epidermal lineage, gamma-neoblasts give rise to the gut cell lineage, and nu-neoblasts give rise to different neuronal lineages specified by distinct sets of transcription factors. Progenitors of other tissues such as the pharynx, excretory cells, and eyes are also likely derived from pluripotent sigma-neoblasts. Some images courtesy by Susanna Fraguas.*

In planarians the Hippo signaling cascade controls the wound response and plays a crucial role during cell differentiation. However, it does not directly regulate organ size, as described in other systems (Pfleger, 2017). In one of the few studies to shed light on the molecular mechanisms that regulate organ size in planarians, Petersen and coworkers examined the maintenance of the brain proportion with respect to body size (Hill and Petersen 2015). They found that *notum*, which is expressed in the anterior part of the brain, and *wnt11-6*, expressed in the posterior region of the brain, form an inhibitory spatial feedback loop that regulates planarian brain size through reversible regenerative growth. Using RNAi inhibition of *notum* and *Wnt11-6*, and analysis of *cintillo* (a marker of head chemoreceptors, numbers of which are constantly correlated with body size (Oviedo *et al.*, 2003), they found that *wnt11-6* inhibits the division of brain progenitors. Based on their findings, they proposed that *wnt11-6,* via the canonical Wnt pathway, activates the expression of its own inhibitor, *notum*, in the anterior brain, which in turn promotes brain growth by inhibiting *wnt11-6.* Importantly, Wnt signaling appears to be required to control brain size, but not brain patterning.

Conclusions

Regeneration is a fascinating biological process that occurs naturally in a large variety of animals. Among all of them, freshwater planarians are one of the champions, as they can regrow, for example, a complex CNS *de novo* from almost any piece of their bodies. The active research in the planarian field in the last years has provided essential data to understand the main events that leads to a successful regeneration. According to the genetic and cellular response, three different phases can be distinguished. Following, we highlight the main discoveries as well as the main gaps still present in each one:

The early response is characterized by the triggering of a generic regenerative program, likely induced by ERK signaling, in which genes related with cell death (apoptosis and autophagy), and stress (JNK and ROS) are activated. In contrast to other models, it is currently unknown whether the apoptotic cells are the source of proliferative or survival signals, neither the relationship between apoptosis and the activation of ROS.

The injury-specific response is characterized by the activation of the specific program to regenerate the missing tissues. The molecular nature of the A and the P organizing centers has been broadly described. And the discovery of the PCGs has been one of the main recent advances in the field. However, the precise mechanism that triggers the regeneration of the appropriate A versus P organizer in each wound after any type of amputation remains a mystery.

The late phase corresponds to the differentiation and morphogenesis of the new organs. A great advance has been seen in the discovery of the specific transcription factors that give rise to specific lineages arising from neoblasts, which are nowadays clearly seen as a heterogeneous population of stem cells, including true pluripotent stem cells and subpopulations of lineage-committed progenitors. However, many cell types as well as the origin of several lineages are still waiting to be molecularly uncovered.

In contrast to other models, especially vertebrates, planarian regeneration relies on the proliferative potential of their adult pluripotent stem cells, the neoblasts, and not in a process of dedifferentiation. The potency of planarian adult stem cells, the evolutionary conservation of the main molecular signals, and the malleability of the model, makes planarians very attractive not only to understand the basic mechanisms of regeneration, but also for the implications it may have in the field of regenerative medicine.

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