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PRIMER

Model systems for regeneration: planarians

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ABSTRACT

Planarians are a group of flatworms. Some planarian species have remarkable regenerative abilities, which involve abundant pluripotent adult stem cells. This makes these worms a powerful model system for understanding the molecular and evolutionary underpinnings of regeneration. By providing a succinct overview of planarian taxonomy, anatomy, available tools and the molecular orchestration of regeneration, this Primer aims to showcase both the unique assets and the questions that can be addressed with this model system.

KEY WORDS: Flatworms, Planarians, Tricladida, Triploblastic animals

Introduction

Planarians have long been known to possess astonishing regenerative capabilities. As succinctly stated by John Graham Dalyell in 1814, planarians '…may almost be called immortal under the edge of the knife' (Dalyell, 1814). For example, if a planarian worm is chopped into three pieces (Fig. 1A), each of the pieces regenerates back into a complete and perfectly proportioned animal within ∼2 weeks. In case of the tail (bottom) piece, this entails *de novo* formation of a head complete with brain, eyes and functional neuronal connections to the pre-existing tissue. Likewise, regeneration of the head (top) piece necessitates the *de novo* specification and formation of the trunk and tail. The central trunk (middle) piece needs to regenerate both a head and a tail; the fact that these always form at the front and rear of the piece, respectively, indicates that the regeneration process is primed by the polarity of pre-existing tissues.

Planarians are similarly capable of regenerating tissue along their medio-lateral (M-L) axis. Worms chopped along the midline regenerate the missing half of all paired organs (Fig. 1B) and even thin, lateral slices that have to form the midline *de novo* are capable of restoring bilateral symmetry (Fig. 1C). Furthermore, planarians can restore perfectly proportioned animals from challenges such as oblique cuts (Fig. 1D), triangular deletions (Fig. 1E) or cut-out 'windows' (Fig. 1F). Regeneration also works over a wide range of sizes. Over 100 years ago, T. H. Morgan reported the regeneration of a piece that he estimated to correspond to 1/279th of the donor animal (Morgan, 1898) and later studies placed the lower size limit at <10,000 cells (Montgomery and Coward, 1974). However, even though the planarian regeneration response is extremely robust, the underlying control mechanisms can be tricked into making 'mistakes', as illustrated for example by the double-headed or double-tailed 'monsters' that often result from anteriorly or posteriorly split animals (Randolph, 1897). Furthermore, even the 'almost-immortal' planarians have regenerative 'weak spots': in the model species chosen precisely for their regenerative powers, the tip of the head in front of the eyes and the pharynx are incapable of regeneration (Reddien and Sánchez Alvarado, 2004) and these tissue pieces consequently die if severed from the rest of the animal. Others amongst the many hundreds of planarian species worldwide (see below) have anatomically restricted regenerative abilities (e.g. no head regeneration in the posterior body half) or seemingly no regeneration at all (Brøndsted, 1969; Vila-Farré and Rink, 2018).

Overall, planarians therefore offer unusually broad experimental access to the many unknowns of regeneration. Species with robust and rapid whole-body regeneration provide a model system for studying universal aspects of the regeneration response, for example the mechanisms that signal injury and how they lead to the re-formation of specific organs or body parts. In addition, the comparative analyses of species with poor or absent regeneration provide an opportunity to understand the mechanistic causes of regeneration defects and likely also the evolutionary dimension of regeneration, i.e. why some worms regenerate whereas others cannot. This Primer aims to provide an overview of planarians as a model system for studying regeneration. We start with a brief overview of planarian phylogeny, biodiversity and anatomy and of the currently available tools and techniques. We then discuss the current knowledge regarding planarian regeneration and its relation to steady-state tissue dynamics. The Primer ends with a subjective outlook on how the study of planarians could help address broader questions about regenerative mechanisms and associated problems.

Planarians as model systems for studying regeneration

Planarian phylogeny and biodiversity

Planarians are a group of worms with a flattened body architecture that belongs to the aptly named phylum Platyhelminthes (*platy*=flat;

Model systems for regeneration

This article is part of a series entitled 'Model systems for regeneration'. This series of articles aims to highlight key model systems and species that are currently being used to study tissue and organ regeneration. Each article provides background information about the phylogenetic position of the species, its life-cycle and habitat, the different organs and tissues that regenerate, and the experimental tools and techniques that are available for studying these organisms in a regenerative context. Importantly, these articles also give examples of how the study of these models has increased our understanding of regenerative mechanisms more broadly, and how some of the open questions in the field of regeneration may be answered using these organisms. To see the full collection as it grows, please visit: https://dev.biologists.org/collection/regeneration_models

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Development (2019) 146, dev167684. doi:10.1242/dev.167684

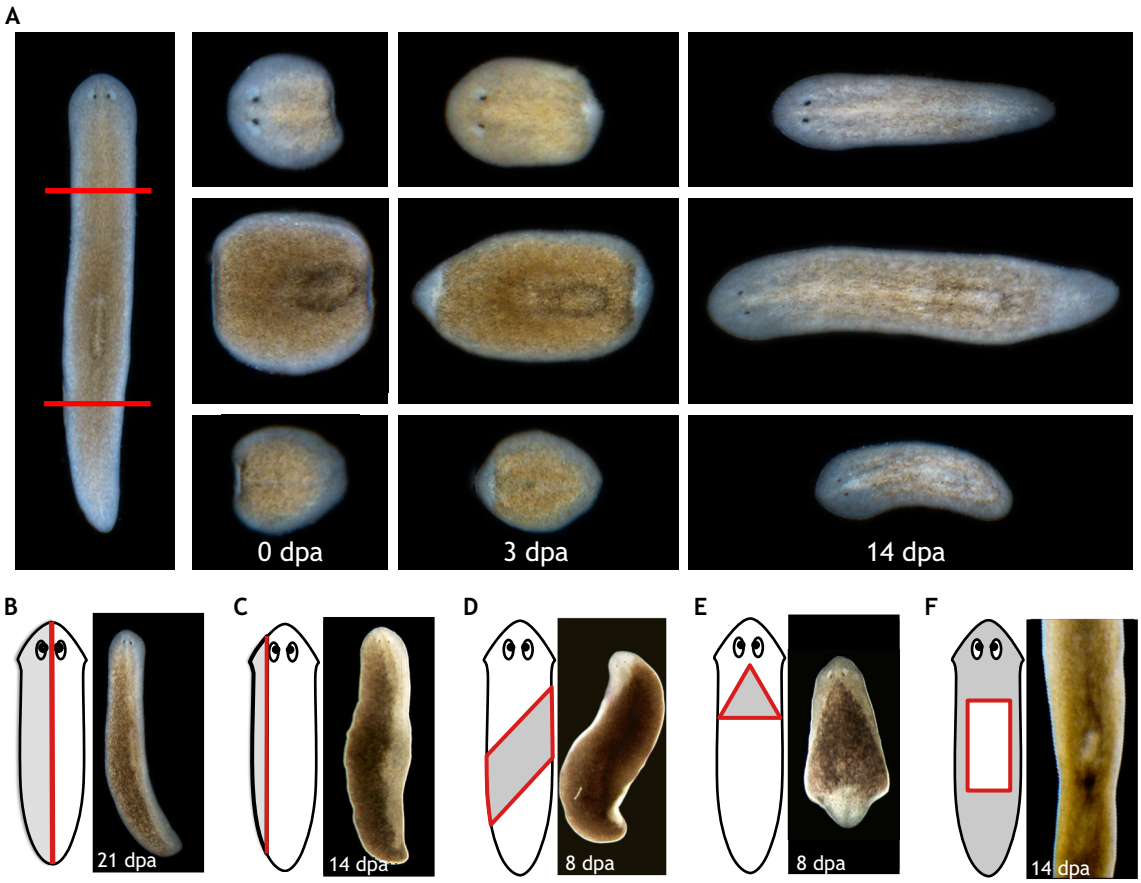

Fig. 1. Planarian regeneration. (A) Images depicting the regeneration of head (top), trunk (middle) and tail (bottom) fragments obtained via amputation of an intact *Schmidtea mediterranea* specimen (left; red lines indicate approximate cutting planes). Time points in days post-amputation (dpa) are indicated. (B-F) Regeneration of tissue fragments following amputation/injury as depicted in the cartoons (grey indicates regenerating piece; red lines indicate the cutting plane) at the indicated dpa. Although intermediate time points are shown for D and E, all fragments eventually regenerate normal body plan proportions.

helminthes=worms), within which they form a distinct evolutionary clade, the order Tricladida (Fig. 2) (Sluys and Riutort, 2018). The Platyhelminthes in turn group within the superphylum Lophotrochozoa/Spiralia, along with, for example, leeches, earthworms and snails. The roundworm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* are only distantly related as they both group within the Ecdysozoa.

Hundreds (if not thousands) of planarian species exist worldwide in marine, freshwater or terrestrial habitats and are spread across the three taxonomic suborders: Maricola, Cavernicola and Continenticola (Sluys and Riutort, 2018; Vila-Farré and Rink, 2018). The regenerative abilities of planarian species vary greatly, from robust whole-body regeneration, as seen in *Schmidtea mediterranea* or *Dugesia japonica*, to anatomically limited regenerative abilities (e.g. no head regeneration in the posterior body half), as seen in *Dendrocoelum lacteum*, or even the reported near-absence of regeneration in *Bdelloura candida* and other marine planarians (Brøndsted, 1969; Vila-Farré and Rink, 2018). *S. mediterranea* and *D. japonica* have been developed into model species precisely for their robust and rapid whole-body regeneration (Newmark and Sánchez Alvarado, 2002; Rink, 2018; Saló and Agata, 2012) and the two species have consequently enjoyed most of the scientific limelight so far.

Anatomy and physiology

In contrast to other flatworm clades, such as tapeworms or flukes, planarians are non-parasitic. They are triploblastic animals with a complex internal anatomy (Fig. 3). Organ systems include a true brain connected to ventral nerve cords and simple eye cups, which give planarians their characteristic cross-eyed appearance (Cebrià, 2007; Umesono and Agata, 2009). Planarians also possess a highly branched intestinal system comprising three major branches (Forsthoefel et al., 2011), which gave rise to the clade designation Tricladida (*tri*=three; *cladida*=branches) (Sluys and Riutort, 2018), and a protonephridial excretory system with interesting homologies to the vertebrate kidney (Thi-Kim Vu et al., 2015). Planarians feed via a muscular pharynx that they extrude through a ventral mouth opening. The pharynx is the only body opening and also functions as the anus of the animal. Circulatory and respiratory systems are absent (Sluys and Riutort, 2018). Neoblasts – the adult stem cells of planarians (Baguñà, 2012) – reside in the mesenchyme that surrounds all internal organs. The three-layered body wall musculature in turn surrounds the mesenchyme like a shell and provides both mechanical stability and patterning information to the cells below (Scimone et al., 2017; Witchley et al., 2013).

Planarians generally harbour a hermaphroditic reproductive system, comprising a pair of ovaries located behind the brain, testes and yolk glands along the entire anterior-posterior (A-P) axis, and the copulatory organs in the tail (Newmark et al., 2008; Sluys and Riutort, 2018). However, asexual reproduction by parthenogenesis or fission/regeneration is also common amongst planarians (Vila-Farré and Rink, 2018). Strains that rely on asexual reproduction by fission often have poorly developed reproductive

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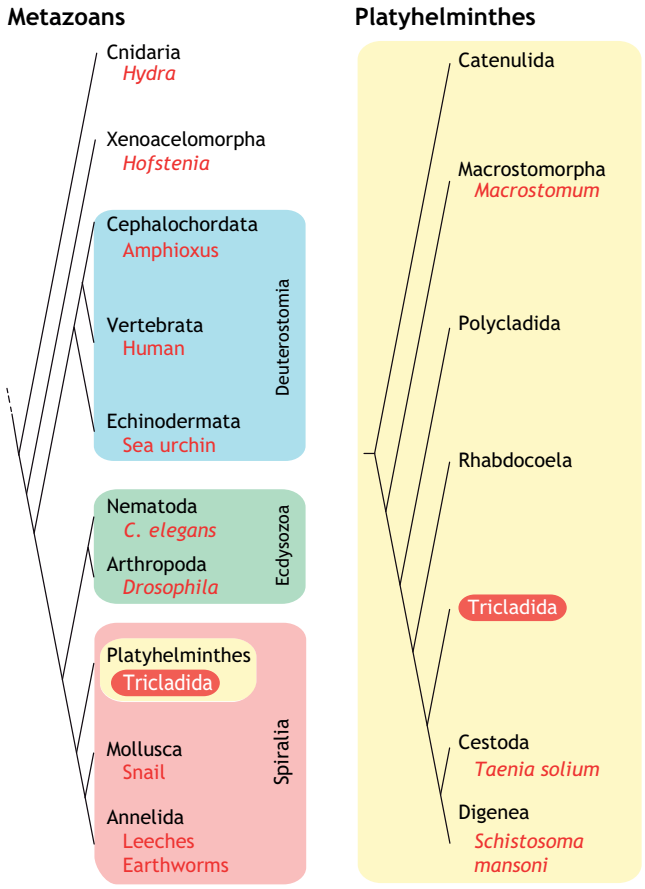

Fig. 2. Planarian phylogeny. Left: Simplified phylogenetic relationship between flatworms (phylum Platyhelminthes) and major taxonomic groups within the Metazoa. Boxes highlight major clades. Red text denotes well-known (model) species representatives of specific groups. Right: Simplified phylogenetic relationship between planarians (order Tricladida) and other major taxonomic groups within the flatworms. Figure based on Martín-Durán et al. (2018) and Egger et al. (2015).

organs, as is the case in the clonal laboratory strains of the model species *S. mediterranea* and *D. japonica* (Pongratz et al., 2003; Vila-Farré and Rink, 2018). Planarian body sizes vary from less than a millimetre in length to more than one metre in the case of *Bipalium nobile* (Kawakatsu et al., 1982). In addition, body shape and coloration display strong inter-species variation, as do the number and anatomical placement of eyes or other organ systems (Sluys and Riutort, 2018).

Experimental accessibility and tools

Planarians are generally cheap and easy to maintain in the laboratory (Merryman et al., 2018) and a broad and rapidly expanding cell and molecular biology tool kit is now available. The organism-wide knockdown of gene function by RNA interference (RNAi) (Rouhana et al., 2013; Sánchez Alvarado and Newmark, 1999) remains the workhorse in the field. This technique is often used in combination with robust *in situ* hybridization and (immuno)histological staining protocols (Adell et al., 2018; Forsthoefel et al., 2018; King and Newmark, 2018; Solana, 2018; Umesono et al., 1997; Winsor and Sluys, 2018). Simple fluorescence-activated cell sorting protocols and the identification of specific surface labels allow the isolation and characterization of neoblasts and other cell types (Hayashi and Agata, 2018; Hayashi et al., 2006; Moritz et al., 2012; Zeng et al., 2018). Whole-body or regionalized irradiation protocols provide a convenient means of stem cell ablation (Abnave et al., 2017; Bardeen and Baetjer, 1904; Guedelhoefer and Sánchez Alvarado, 2012), and single-cell and tissue transplantation protocols permit the investigation of differentiation potential (Rojo-Laguna and Saló, 2018; Wagner et al., 2011; Wang et al., 2018).

The recent embracement of next-generation sequencing by the planarian research community has generated transcriptome assemblies of model and ‘wild’ planarian species (Adamidi et al., 2011; Blythe et al., 2010; Cantarel et al., 2008; Liu et al., 2013; Nishimura et al., 2012; Sandmann et al., 2011; Sikes and Newmark, 2013). This has been complemented by rich documentation of the effects of individual gene knockdowns (Lin and Pearson, 2014; Reuter et al., 2015; Tu et al., 2015; van Wolfswinkel et al., 2014), and by gene expression time series during regeneration (Kao et al., 2013; Stückemann et al., 2017; Wurtzel et al., 2015). More recently, organism-wide single-cell sequencing atlases have been developed (Fincher et al., 2018; Plass et al., 2018). Likewise, the very recent completion of a high-quality *S. mediterranea* genome assembly (Grohme et al., 2018) and a *D. japonica* genome assembly (An et al., 2018) have made the genome sequences of the two model species accessible, for instance, to chromatin immunoprecipitation (ChIP) protocols that can provide insights into gene regulatory mechanisms (Dattani et al., 2018; Duncan et al., 2015; Zeng et al., 2013). A number of community resources provide access to these data and allow the online querying of planarian biology (see Box 1).

Remaining community challenges include the establishment of robust transgenesis protocols, further improvement of live-imaging strategies (Boothe et al., 2017; Shen et al., 2018), and the broad general adaptation of the tool kit to the non-model species. Nevertheless, planarians now provide a bona fide model system for understanding the mechanistic basis of regeneration.

Mechanisms of regeneration in planarians

Neoblasts: the lynchpin of planarian biology

The regenerative powers of planarians derive largely from an abundant population of unusual adult stem cells, the neoblasts. Neoblasts are relatively small, round cells (7-12 µm in diameter) with a high nuclear-cytoplasmic volume ratio that are distributed throughout the planarian mesenchyme (Fig. 4A). They often possess filopodia-like extensions and, interestingly, harbour prominent RNA/protein granules (chromatoid bodies) with morphological and molecular similarities to the RNA/protein granules found in the germ cells of many animals (Agata et al., 2006; Baguñà, 2012; Reddien and Sánchez Alvarado, 2004; Rink, 2018; Tanaka and Reddien, 2011). Neoblast-specific genes include those encoding conserved chromatoid body components (Rouhana et al., 2010, 2012, 2014), but also other conserved germ line genes including homologues of *piwi. piwi-1* expression further continues to be used as generic neoblast marker (Guo et al., 2006; Reddien et al., 2005b; Shibata et al., 2016).

Neoblasts are unusually abundant in comparison with adult stem cells in other animals and have been estimated to account for as much as 20-30% of all cells (Baguñà and Romero, 1981). Remarkably, the transplantation of a single neoblast into a stem cell-depleted host is sufficient to restore a complete animal via the gradual replacement of all host cells by descendants of the transplanted neoblast (Wagner et al., 2011). This experiment demonstrates conclusively that the neoblast fraction contains stem cells (termed clonogenic or cNeoblasts) capable of giving rise to all adult cell types, which is the functional definition of pluripotency. Although this abundance of pluripotent stem cells in adult tissues

Development (2019) 146, dev167684. doi:10.1242/dev.167684

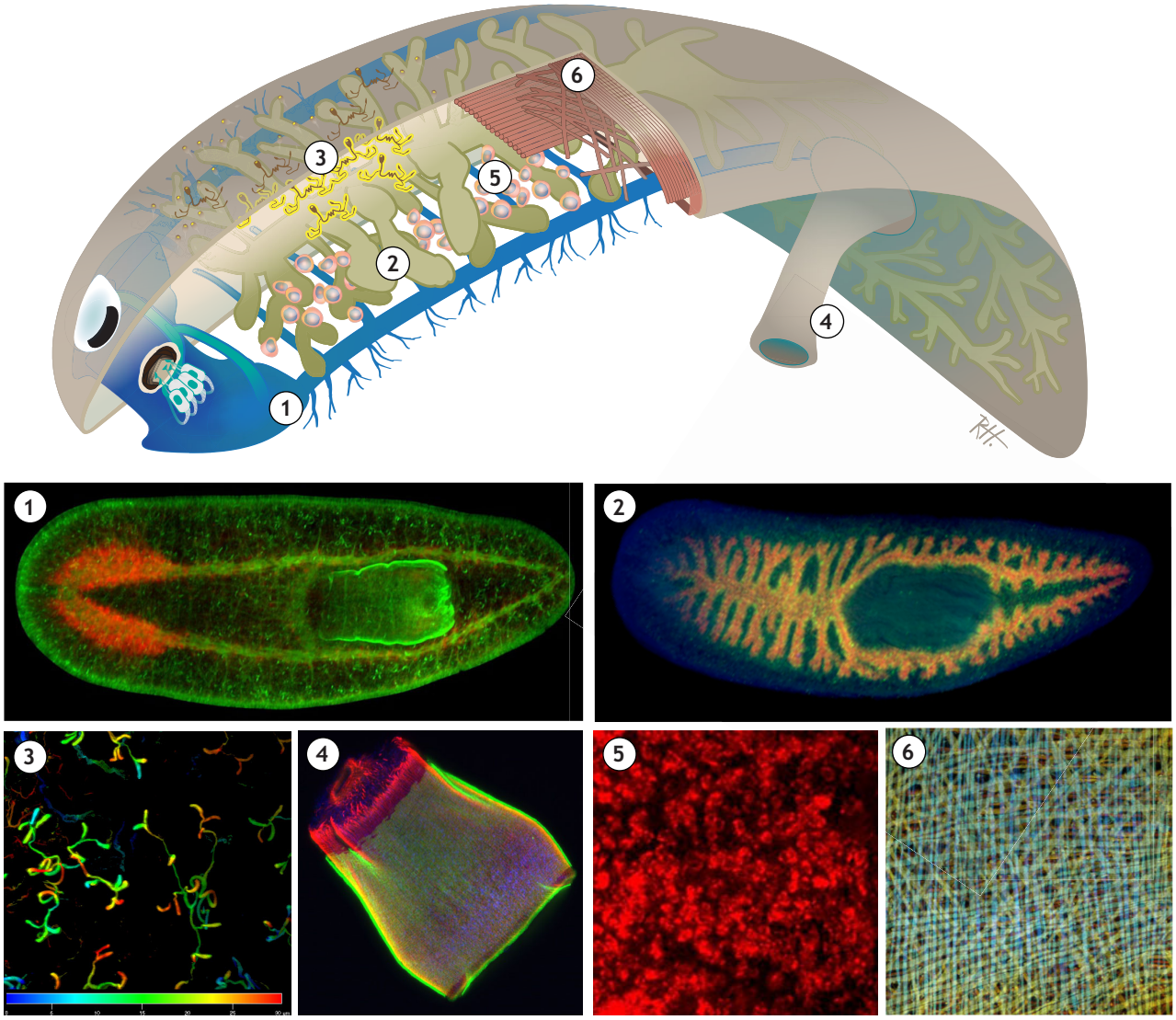

Fig. 3. Planarian anatomy. Schematic (top) and microscopic images (bottom) of the major planarian organ systems. (1) Brain (red, *Smed-pc2 in situ* hybridization), CNS and pharynx (both green, α-tubulin immunostaining). (2) Intestine (red, *Smed-porcupine-A*; green, *Smed-sufu in situ* hybridization). Nuclear counterstaining (blue, DAPI) reveals the silhouette of the specimen. (3) Protonephridia. Depth-coded confocal maximum projection showing individual protonephridial units (acetylated-tubulin immunostaining). (4) Pharynx (red, phalloidin staining of muscle actin; green, acetylated-tubulin immunostaining of cilia; blue, nuclear counterstaining). (5) Neoblasts (red, confocal maximum projection of *piwi*-1 *in situ* hybridization in the tail area). (6) Body wall musculature (depth-coded confocal maximum projection of 6G10 immunostaining; for antibody details, see Ross et al., 2015).

may seem highly exotic by comparison with more familiar stem cell systems, the comparatively recent discovery of *piwi*-expressing (likely) somatic stem cells in a broad range of animals (Lai and Aboobaker, 2018) indicates that planarian neoblasts may not be so exotic after all, and that they may represent one end of a continuum of stem cell architectures in animal phylogeny (Rink, 2018). The mechanistic basis of neoblast pluripotency and of its indefinite maintenance in adult planarians therefore poses a fascinating question for future research, also from a comparative point of view.

Neoblasts are not only a source of all adult cell types, but they are in fact the only source of new cells in planarians (Baguñà, 2012; Rink, 2018; Tanaka and Reddien, 2011). This stems from the fact that neoblasts are the only somatic cells that are division-competent (Forsthoefel et al., 2011; Newmark and Sánchez Alvarado, 2000; Reddien et al., 2005b). Not surprisingly, therefore, neoblasts are essential for regeneration, and the depletion of neoblasts by irradiation completely blocks regeneration. Moreover, the tip of the head and pharynx, which are the only 'naturally' neoblast-devoid tissues, are the only body parts incapable of regeneration (reviewed by Baguñà, 2012).

Any wounding event, even the prick of a needle, activates neoblast divisions (Baguñà, 1976a; Wenemoser and Reddien, 2010). Wounds involving tissue removal attract the neoblast progeny by an unknown mechanism and the consequent accumulation of postmitotic neoblast progeny underneath the freshly sealed wound gives rise to a blastema – a mass of differentiating cells in the process of tissue formation. The blastema first becomes apparent as a thin rim of unpigmented tissue at ∼24 h post wounding and continues to grow due to high levels of local neoblast proliferation at its base (Baguñà, 1976b; Newmark and Sánchez Alvarado, 2000; Wenemoser and Reddien, 2010). Bromodeoxyuridine pulse-chase experiments have demonstrated that the blastema is largely composed of the post-mitotic progeny of wound-induced neoblast divisions (Eisenhoffer et al., 2008),

Development (2019) 146, dev167684. doi:10.1242/dev.167684

Box 1. Online communities and further resources

PlanMine (http://planmine.mpi-cbg.de; Brandl et al., 2016; Rozanski et al., 2019). Queryable repository of flatworm sequence data, provides interactive tools for functional and comparative gene/transcript analyses. Has an associated UCSC-based genome browser instance.

SmedGD (http://smedgd.stowers.org; Robb et al., 2015; Robb et al., 2008). Queryable database for *S. mediterranea* sequence data.

Planosphere (https://planosphere.stowers.org; Davies et al., 2017). Interactive access to *S. mediterranea* cell and developmental biology datasets.

Genome browser of *D. japonica* (http://www.planarian.jp/; An et al., 2018).

Planaria SCS 2015 (https://radiant.wi.mit.edu/app/; Wurtzel et al., 2015). Single-cell sequencing data of flow-sorted planarian cells.

Planarian digiworm (https://digiworm.wi.mit.edu/; Fincher et al., 2018). Large-scale single-cell transcriptomic resource for *S. mediterranea*.

Planaria SC Atlas (https://shiny.mdc-berlin.de/psca/; Plass et al., 2018). Large-scale single-cell transcriptomic resource for *S. mediterranea*, including cellular lineage trees.

Developmental Studies Hybridoma Bank monoclonal antibodies (http://dshb.biology.uiowa.edu/; Forsthoefel et al., 2014; Ross et al., 2015). Collection of monoclonal antibodies with validated reactivity towards planarian epitopes.

suggesting that the trans-differentiation of existing cells contributes little, if any, to planarian regeneration (reviewed by Baguñà, 2012). As regenerating pieces often cannot eat until the completion of regeneration, the blastema cannot possibly rebuild all missing tissues in their original size. Rather, the blastema appears to restore the ends of the cardinal body axes (e.g. the 'head' or 'tail' and 'body edge'), and subsequent restoration of the adjacent anatomy is accomplished via the dynamic re-modelling of existing tissues (Agata et al., 2007). In terms of Morgan's classic terminology (Morgan, 1901), planarian regeneration therefore combines epimorphic aspects in the form of *de novo* tissue formation until ∼day 5 of regeneration, with the morphallactic remodelling of existing tissues occurring during the subsequent ∼9 days. However, neoblast divisions are likely instrumental in both, which limits the utility of Morgan's terminology with respect to planarian biology.

Neoblasts are also crucial for the maintenance of planarian anatomy in the absence of wounding. Continual neoblast divisions and their resulting progeny continuously replace all differentiated cell types. The importance of continuous cell turnover is underscored by the stereotypic deterioration of irradiated (i.e. neoblast-depleted) animals, which first lose their head, followed by ventral curling and eventual lysis of the remaining tissues∼30 days after irradiation (Bardeen and Baetjer, 1904; Reddien et al., 2005a; Wolff and Dubois, 1948). Moreover, the basal division rate of neoblasts increases strongly after every meal (Baguñà, 1976a; Newmark and Sánchez Alvarado, 2000) and the resulting burst of postmitotic progenitors translates into a growth burst at the organismal level. Neoblast divisions continue under starvation, but the basal division rate is insufficient to replace all cells and the animals consequently shrink owing to a net loss of cells (Baguñà, 1976a; Baguñà and Romero, 1981; González-Estévez et al., 2012; Thommen et al., 2019).

The pivotal importance of neoblasts as the sole source of new cells for regeneration and homeostatic tissue dynamics raises the problem of how to orchestrate the orderly differentiation of all adult cell types from a single pluripotent stem cell population (Rink, 2018). In contrast to vertebrate adult stem cells, which supply progeny to comparatively few tissue-specific cell lineages, every single cNeoblast can give rise to potentially hundreds of adult cell types that its division progeny must consequently 'choose' amongst (Fig. 4B,C). Several recent studies detailing gene expression in individual neoblasts have provided some glimpses into how this might occur. Collectively, they demonstrate the onset of lineage specification within *piwi-1*-positive and irradiation-sensitive 'neoblasts' (Hayashi et al., 2010; Molinaro and Pearson, 2016; van Wolfswinkel et al., 2014; Wurtzel et al., 2015), which therefore constitute a heterogeneous cell population. The selective expression of a number of genes, often transcription factors with evolutionarily conserved roles in lineage specification, define a number of *piwi-1*-expressing neoblast subclasses (Fincher et al., 2018; Molinaro and Pearson, 2016; Plass et al., 2018; van Wolfswinkel et al., 2014; Wurtzel et al., 2015; Zeng et al., 2018). Moreover, recent results have suggested that the operationally defined pluripotent cNeoblasts (Wagner et al., 2011) – the nexus of all planarian cell lineages – fall within a subclass marked by the cell surface protein Tetraspanin (Zeng et al., 2018). Planarian cell fate specification is likely, therefore, a hierarchical process that involves the initial 'differentiation' of cNeoblasts into a comparatively small number of lineage-restricted neoblast subclasses (Fincher et al., 2018; Molinaro and Pearson, 2016; Plass et al., 2018; van Wolfswinkel et al., 2014), and terminal cell fates emerge during post-mitotic progenitor differentiation in concert with the migration of these progenitors to their target organs (Eisenhoffer et al., 2008; Lapan and Reddien, 2011; Tu et al., 2015; Wurtzel et al., 2017). Important open questions include the precise differentiation potential of specific neoblast subclasses, and the extent to which they function analogously to the transit-amplifying stages observed in vertebrate stem cell lineages. But above all, there is the question of how to generate the right cells at the right time and place, or, more specifically, how to guide differentiating progenitors through the maze of the planarian cell lineage tree.

Maintenance of the planarian body plan

Neoblasts situated in the tail or in a tail blastema do not make eye progenitors (LoCascio et al., 2017), even though they are equally pluripotent as elsewhere in the animal. This suggests the existence of patterning processes that instruct location-specific cell fate choices, analogous to positional information during development. Indeed, the experimental perturbation of conserved signalling pathways results in dramatic body plan transformations. Inhibition of canonical Wnt signalling, for example, causes the appearance of eyes in the tail by transforming the existing tail into a head, or by re-programming tail blastemas into head development in regenerating animals (Fig. 5A, top) (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Activation of Wnt signalling causes the opposite phenotype, namely loss of the head and all anterior structures and transformation of the entire animal into a mass of tail tissue, or the re-programming of head blastemas into tails in regenerating animals (Fig. 5A, bottom) (Gurley et al., 2008; Iglesias et al., 2011; Stückemann et al., 2017). Interference with the BMP signalling pathway has similarly dramatic consequences, causing ventralization of animals inclusive of the duplication of the entire (ventral) nervous system (Gaviño and Reddien, 2011; Molina et al., 2011; Reddien et al., 2007). Collectively, these phenotypes identify BMP signalling and canonical Wnt signalling as determinants of dorsal or posterior tissue identity, respectively.

Consistent with these phenotypes, the respective signals are constitutively expressed in homeostatic animals. For example, *Smed-bmp4* (which encodes a planarian BMP4 homologue), is expressed dorsally in a medio-laterally graded manner (Orii et al.,

Development (2019) 146, dev167684. doi:10.1242/dev.167684

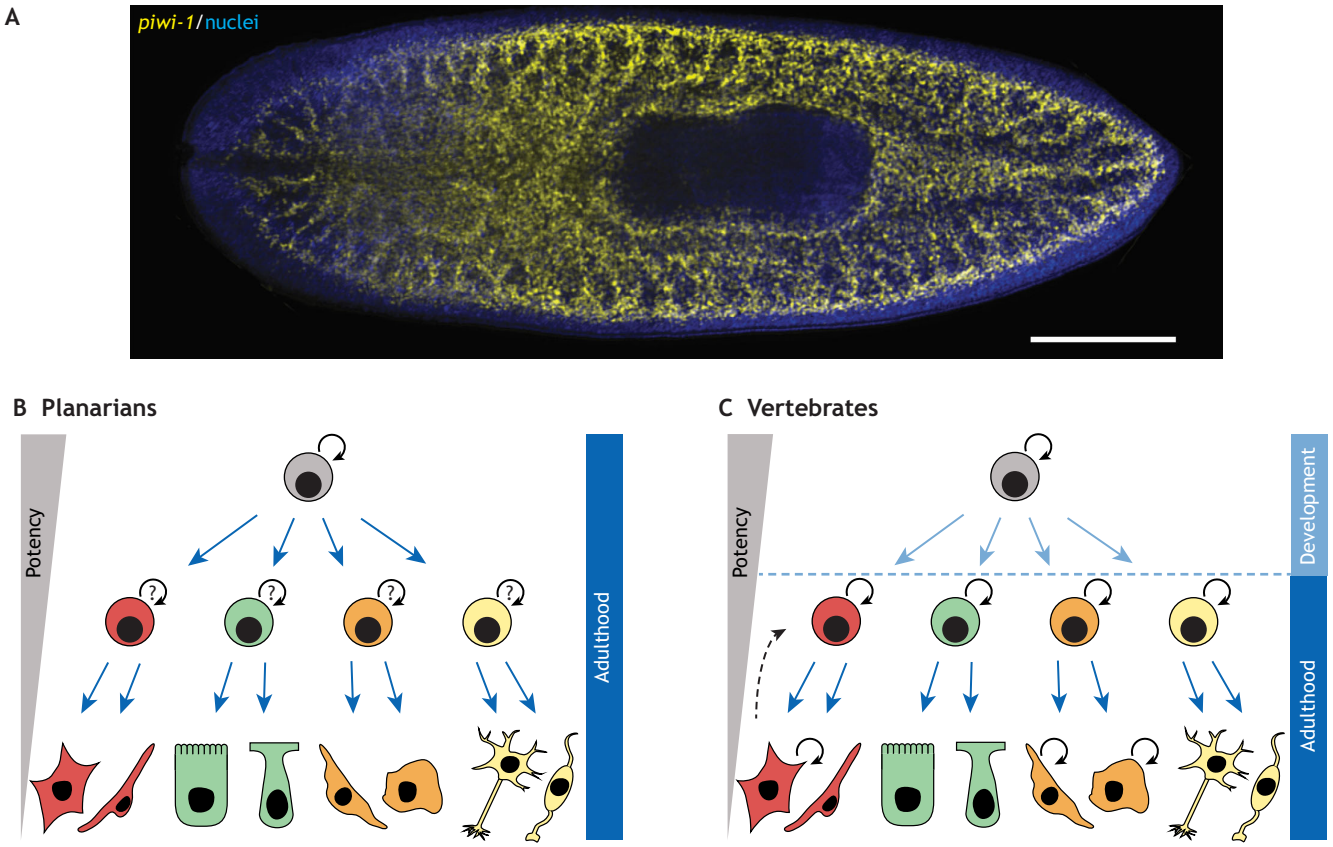

Fig. 4. The planarian stem cell system. (A) Whole-mount *in situ* hybridization highlighting the localization of neoblasts, which are visualized using the generic neoblast marker *piwi*-1 (*smedwi*-1; yellow), counterstained with DAPI (blue) to highlight nuclei. Scale bar: 500 µm. (B,C) Schematics contrasting key organizational features of the planarian and vertebrate stem cell systems. In adult planarians (B), the indefinitely self-renewing and pluripotent cNeoblasts (grey cells, top) likely give rise to lineage-committed progenitors of currently unknown self-renewal potential (middle) that terminally differentiate into various postmitotic cell types (bottom). In vertebrates (C), self-renewing pluripotent stem cells (grey cells, top) only ever occur transiently during early developmental stages; in this context, multiple multipotent stem cells instead persist in adults (middle) and terminally differentiate into various, occasionally mitotically active, cell types (bottom).

1998; Reddien et al., 2007). In addition, many canonical Wnt signalling pathway components are expressed in tail-to-head gradients (Gurley et al., 2010; Petersen and Reddien, 2009; Reuter et al., 2015; Stückemann et al., 2017; Sureda-Gómez et al., 2015), which result in a corresponding gradient of canonical Wnt signalling activity in the tissue (Stückemann et al., 2017). The tail-deployed Wnt gradient further appears to functionally oppose independent signalling gradients emanating from the tip of the head (Stückemann et al., 2017). Although multiple homologues of the vertebrate FGF pseudoreceptor FGFRL1 (termed nou-darake in planarians) are involved (Cebrià et al., 2002; Lander and Petersen, 2016; Scimone et al., 2016), multiple aspects of planarian head identity specification remain to be clarified. Overall, the above phenotypes and expression patterns now amount to a rudimentary coordinate system of the planarian body plan to specify positional information along the A-P, dorso-ventral (D-V) and M-L axes (Fig. 5B). Moreover, the identity of the primary patterning signals, together with the prominent theme of signalling gradients in their deployment, raise many intriguing parallels to embryonic axis establishment.

Practically all of the aforementioned signals are expressed in the multi-layered sheet of muscle fibres that lies beneath the planarian epithelium (see Fig. 3) (Scimone et al., 2017; Witchley et al., 2013). Importantly, existing muscle fibres can rapidly and dynamically change the complement of patterning molecules they express; for example, swapping tail for head gradient genes in the case of tail piece regeneration (Witchley et al., 2013). A further important theme that is currently emerging is the functional specialization of muscle fibre subtypes with regard to the patterning signals they express (Scimone et al., 2017). A subclass at the tip of the head and tail, the so-called pole cells, have been strongly implicated in head and tail fate specification and likely initiate the expression gradients of the tail and head signals within the body musculature (Blassberg et al., 2013; Chen et al., 2013; Oderberg et al., 2017; Reuter et al., 2015; Vogg et al., 2014). Furthermore, the recent discovery that specific layers of the body wall musculature express axis-specific signals is intriguing in light of the stereotypic arrangement of the fibres along the A-P and M-L axes (Scimone et al., 2017). Although the actual tissue distribution of the muscle-expressed BMP and Wnt ligands has not yet been determined, their dramatic influence on planarian anatomy and cell fate choices strongly suggests that at least some of them can permeate the neoblast-containing mesenchyme (Witchley et al., 2013). Hence, a conceptual framework is beginning to emerge whereby the expression patterns of patterning signals in the body wall musculature translate into location-specific signalling environments in the mesenchyme that ultimately mediate location-specific fate choices of neoblast progeny (Reddien, 2018; reviewed by Rink, 2018).

The mechanisms and principles by which patterning signals influence neoblast fate choices are currently an important focus in the field. In the case of BMP4-mediated D-V patterning, the presumptive BMP signalling gradient has been shown to cause D-V gene

Development (2019) 146, dev167684. doi:10.1242/dev.167684

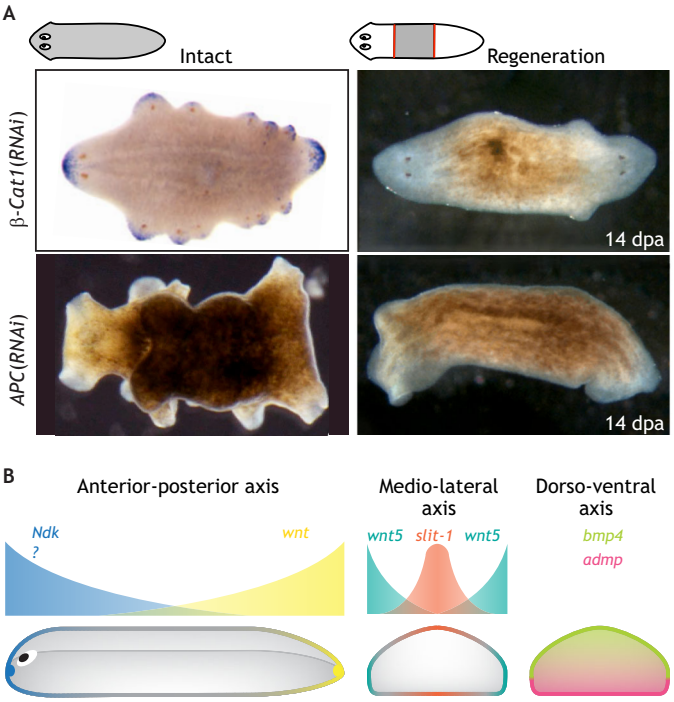

Fig. 5. Planarian patterning systems. (A) Morphological consequences of experimentally perturbing Wnt signalling in intact worms (left) or in regenerating trunk pieces (right). Inhibition of canonical Wnt signalling via *Smed-β-catenin-1(RNAi)* forces head formation, triggering either tail-to-head conversion and the appearance of multiple ectopic heads (marked by *Smed-sfrp-1* via *in situ* hybridization) in intact animals, or double-headed formation in regenerating trunk pieces. Activation of Wnt signalling by *Smed-APC(RNAi)* forces tail formation, causing either loss of all anterior structures and global posteriorization in intact animals, or double-tail formation during trunk piece regeneration. (B) Schematic depicting currently known primary patterning signals and their deployment along the indicated cardinal body axes. Note that the depicted signalling gradients are largely hypothetical extrapolations from the expression patterns of the respective genes, as only the canonical Wnt signalling gradient has been experimentally demonstrated so far (see text for details).

expression zonation within neoblasts (Wurtzel et al., 2017). Together with the observation of *bmp4(RNAi)*-induced ventralization of newly differentiating neoblast progeny, rather than 'reprogramming' of terminally differentiated cells (Wurtzel et al., 2017), this suggests that the position of a neoblast and the consequent combination of patterning signals that it is exposed to restricts the lineage choices that its progeny can make. In the case of A-P-patterning, Wnt signals induce the expression of Wnt target genes in neoblasts, including planarian Hox homologues and other transcription factors (Reuter et al., 2015; Stückemann et al., 2017). In conjunction with the tail-to-head Wnt signalling gradient, a neoblast situated in the high Wnt environment of the tail consequently expresses a different complement of transcription factors than do neoblasts located in the low Wnt environment of the head (Reuter et al., 2015). Although the underlying gene regulatory circuits and networks remain to be elucidated, the conversion of a Wnt signalling gradient into transcription factor expression gradients in stem cells provides a working hypothesis that could help explain a key aspect of positional information in planarians and, more generally, why neoblasts in the tail do not make eye progenitors.

Regeneration of the planarian body plan

Given the importance of signalling gradients for the maintenance of planarian steady-state anatomy, restoration of the signal patterns in regenerating tissue pieces becomes a key prerequisite for successful regeneration. An interesting observation in this respect is that trunk fragments, or any other type of tissue fragments, always regenerate the head and tail along the orientation of the original body axes (e.g. Fig. 1A). This demonstrates that planarian tissues are intrinsically polarized and that tissue polarity in turn instructs the direction of regeneration. Although the mechanistic basis of planarian tissue polarity remains unclear, the Wnt inhibitor *notum* has been identified as a key polarity effector (Petersen and Reddien, 2011). *notum* is selectively expressed at anterior-facing wounds within ∼3 h of amputation, and *notum(RNAi)* animals regenerate a tail instead of a head, consistent with the necessity and sufficiency of Wnt inhibition for head formation (as discussed above). The anterior-specific expression of *notum* and many other aspects of the early regeneration response do not require neoblasts (Gurley et al., 2010; Wenemoser et al., 2012). However, neoblasts are instrumental in pattern regeneration as they are necessary for pole regeneration. Pole cells become specified in the wound vicinity and congregate at the tip of the blastema by day 3 after amputation (Gurley et al., 2010; Hayashi et al., 2011; Oderberg et al., 2017; Scimone et al., 2014; Vásquez-Doorman and Petersen, 2014; Vogg et al., 2014). In the absence of pole formation, e.g. in *pbx(RNAi)* animals, small blastemas form, but they fail to specify head or tail identity and also do not acquire a midline (Blassberg et al., 2013; Chen et al., 2013; Scimone et al., 2014). Thus, pole cell regeneration is likely an essential aspect of pattern regeneration. The fact that the poles mark the origin of the head and tail gradients, the distal-to-proximal regeneration of the Wnt gradient out of the blastema (Stückemann et al., 2017), and the ability of transplanted head tip cells to initiate head outgrowth (Oderberg et al., 2017) are all consistent with a role for pole cells as pattern initiators.

Overall, planarian regeneration can thus be envisaged as being guided by similar conceptual principles as those that govern steady-state turn-over (Reddien, 2018; reviewed by Rink, 2018). Polarity cues acting as an additional aspect of tissue-resident positional information generate a unique signalling environment at the wound site that encodes wound type and orientation. The signalling environment, in turn, gates critical lineage choices of arriving neoblast progeny, including the tightly gated access to the pole cell lineage and the likely canonical Wnt signalling-dependent choice between head and tail pole formation. Interaction of pole cells with the general body wall musculature then re-initiates gradient formation and thus restores the 'regeneration' of positional information in the tissue piece.

Conceptual problems that can be addressed by studying planarian regeneration

Given that *Hydra*, salamanders, zebrafish and a range of other emerging models all vie for the 'master of regeneration' title (Galliot, 2012; Gemberling et al., 2013; Holstein et al., 2003; Tanaka and Reddien, 2011), what is it that planarians can bring to the table? First, the complete regeneration of a triploblastic body plan from arbitrary tissue pieces within 2 weeks, and the availability of a broad range of molecular tools, simply provide convenient experimental access to the many remaining fundamental challenges of regeneration – 'fundamental' because they pertain to a general understanding of the phenomenon of regeneration, and 'challenges' because we are so far lacking a mechanistic understanding in any model system. Second, the many quirks of planarian physiology offer unique perspectives on a broad range of important problems in the current biomedical research landscape. Below, we provide a subjective selection of some such fundamental challenges.

Development (2019) 146, dev167684. doi:10.1242/dev.167684

Regeneration specificity: sensing what's missing

The essence of regeneration is precise reformation of a tissue or body part that has been damaged or lost. Irrespective of whether we are considering limb regeneration in axolotls or whole-body regeneration in planarians or *Hydra*, the restoration of 'whole' from random pieces necessitates a latent ability of tissues to sense what's missing, i.e. whether to initiate the regeneration of head, tail, or lateral tissues in planarians. Although elements of the mechanisms underlying regeneration specificity have now emerged in many model systems (Tanaka, 2016; Vogg et al., 2016; Wehner and Weidinger, 2015), it is probably fair to state that a mechanistic understanding of 'sensing what's missing' has not been achieved in any system. In this regard, planarians provide a particularly powerful experimental paradigm, owing to their rapid rate of regeneration and near-complete experimental freedom over the shape, size or anatomical origin of the regenerating tissue fragments examined. Particularly pertinent current questions regarding planarian regeneration specificity include the cellular and molecular basis of 'tissue polarity' in the head/tail decision (discussed above), or how the animals manage to restrict the general regeneration response in the case of limited damage to specific organs (e.g. the pharynx or the eyes; Adler et al., 2014; LoCascio et al., 2017). Beyond planarians, permutations of the regeneration specificity challenge include, for example, the injury site-dependent formation of various limb elements during amphibian limb regeneration or the position-dependent variation in regeneration rate during zebrafish fin regeneration (Kujawski et al., 2014; Tanaka, 2016). Thus, the general conceptual challenge is to understand how remnants of positional information can encode and restore the whole structure from arbitrary starting points.

Organization versus self-organization

In sharp contrast to embryogenesis, regeneration does not initiate from a precisely defined environment (e.g. the fertilized zygote), but instead is orchestrated from the entirely random remnants of injuries. *A priori*, this rules out pre-positioned fate determinants (e.g. *bicoid* or *nanos* in *Drosophila* zygotes) and, more generally, the 'traditional' textbook manifestation of the morphogen gradient concept with the source as a pre-specified cell fate as organizing principles. Self-organizing Turing or similar reaction-diffusion patterns are conceptually very attractive in this sense, as they can account both for spontaneous pattern emergence and pattern regeneration (Gierer and Meinhardt, 1972; Turing, 1952). The anatomical autonomy of the planarian tail gradient and Wnt-mediated Wnt expression as one of the core mechanisms of planarian regeneration (Stückemann et al., 2017) are indeed consistent with a Turing mechanism, yet the instructive role of intrinsic tissue polarity is suggestive of organized, rather than spontaneous, pattern emergence. However, it is known that pharmacologically induced ectopic heads permanently re-programme the polarity of adjacent tissues, as revealed by subsequent amputations after drug wash-out (Oviedo et al., 2010). Hence, even though tissue polarity can organize pattern regeneration, pattern regeneration can also, in turn, organize tissue polarity. At a systems level, regenerative patterning in planarians is therefore self-organizing. The interplay between tissue polarity and pattern establishment mirrors the concept of 'guided self-organization' in developmental systems and organoids, which involves channelling the inherently random nature of self-organized systems into predictable outcomes via the specification of boundary conditions (Lancaster et al., 2017; Turner et al., 2016; Werner et al., 2016). Understanding how self-organization can result in the highly specific and reproducible outcome of planarian regeneration, yet essentially random tissue architectures in the case of organoid differentiation (Lancaster and Knoblich, 2014), therefore poses an interesting challenge for the coming years.

Size and shape control

The fact that small or large planarian tissue pieces regenerate into small or large planarians, or the observation that the size of a regenerating newt new limb is matched to the size of the regenerating animal (Tanaka and Reddien, 2011), represent striking manifestations of a fundamental unresolved challenge: understanding the mechanisms by which biological systems specify, gauge and restore spatial dimensions. One component problem of this challenge is to understand how pattern length scales are matched to tissue size dimensions. Here, the restoration of planarian body plan proportions from arbitrary starting points provides a powerful experimental paradigm that can be used to, for example, probe the mechanistic basis of downscaling the tail Wnt gradient to the much shorter dimensions of the tail piece (Gurley et al., 2010; Stückemann et al., 2017). The evident scaling of planarian patterning gradients is intriguing because diffusion-based patterning concepts typically imply fixed-length scales that arise from physicochemical systems parameters (e.g. diffusion and degradation constants) (Werner et al., 2016). How scalable systems might achieve the necessary adjustment of reaction rates to system size remains an important problem not only in regeneration, but also in development (Aguilar-Hidalgo et al., 2018; Ben-Zvi et al., 2011; Werner et al., 2015). Here, the uncoupling of pattern scaling from tissue growth during the early stages of planarian regeneration promises a uniquely specific model system to elucidate the underlying mechanisms.

Planarians also offer an additional experimental approach to the shape and size challenge because of their general lack of a fixed body size. Planarians grow when fed and literally shrink when starving due to dynamic and food supply-dependent adjustments of total organismal cell numbers (Baguñà et al., 1990). In the case of *S. mediterranea*, the momentary size of a single worm fluctuates between ~0.5 mm and 20 mm in body length or <10,000 to 8,000,000 cells (Thommen et al., 2019). Planarians further display tremendous inter-species variations in body size, as illustrated by the giant 'shoe-sole-sized' planarians of Lake Baikal (Sluys et al., 1998) or the meter-long land planarians of Japan (Kawakatsu et al., 1982). Such a broad spectrum of inter- and intra-specific body size variations provides a further powerful pattern-scaling paradigm, but in addition it raises many further questions. For example, what accounts for the recently demonstrated body size-dependent lipid storage in planarians and the resulting near-universal ¾-law scaling of metabolic rate with mass (Thommen et al., 2019)? How can planarians establish centimetre-length signalling gradients, given that free diffusion is an unlikely signal propagation mechanism at such length scales? Or, what encodes the maximal body size of a species in the genome? Overall, planarians thus clearly provide unique experimental opportunities for probing the mechanistic basis of size and shape.

The evolution of regeneration: why some animals can, but others can't

Finally, this leaves the big question of why *Hydra*, planarians, axolotl and zebrafish can all regenerate lost body parts, but, for example, humans cannot. In the face of 'survival of the fittest', regeneration as the apparent exception rather than the rule seems deeply counterintuitive. Various hidden 'costs' of regeneration have been proposed, such as increased cancer susceptibility,

compromised immune system function or the risk of developing malformations (Alibardi, 2018; Egger, 2008; Godwin and Rosenthal, 2014), but all evidence so far remains correlative. A second related challenge is to understand whether the widespread distribution of regeneration competence in certain phylogenies reflects ancestral 'core mechanisms' that were lost in various lineages or whether regeneration competence evolved *de novo* in multiple branches of animal phylogeny (Sánchez Alvarado, 2000; Slack, 2017). The answer to this question is deeply relevant with respect to regenerative medicine in humans, as the former possibility promises a potential 'unlocking' of latent regenerative abilities, whereas the latter one would necessitate the conceptually even more challenging *de novo* engineering of the trait.

The complete spectrum of regenerative abilities across the planarian taxa, the demonstrated ability to cultivate many of these species in the lab (Vila-Farré and Rink, 2018), and the existence of well-developed model species make planarians a uniquely powerful model system that can be used to probe the evolutionary dynamics of regeneration. As already demonstrated by the 'rescue' of head regeneration in regeneration-deficient planarian species (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013), comparative approaches can further provide opportunities to diagnose and understand the mechanistic basis of regeneration defects. Given the rapidly evolving toolkit in planarians, the identification of potential base pair changes in enhancer elements or coding sequences as proximate causes of regeneration defects is now becoming increasingly feasible. Such a truly mechanistic understanding of regeneration defects could lead to a better understanding of what it takes to regenerate, and thus, eventually, to a systems-level understanding of regeneration.

Conclusions

Planarians have developed into a powerful model system for studying the mechanistic basis of regeneration, and the comparative analysis of regeneration-deficient planarian species is beginning to provide access to the evolutionary dimension of the trait. In addition, the adult pluripotent stem cells of planarians and their uniquely dynamic tissue architecture expose multiple fascinating phenomena to experimental scrutiny; for example, the self-organized assembly of entire organs, the specification of size and shape, and the maintenance of a dynamic steady state per se. Overall, the seemingly quirky biology of planarians reminds us that the handful of more or less haphazardly chosen 'classical' model species cover but a fraction of the fascinating complexity and diversity of biological mechanisms and, consequently, that much remains to be discovered.

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Competing interests

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