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The known, unknown, and unknown unknowns of cell-cell communication in planarian regeneration

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ABSTRACT

Planarians represent the most primitive bilateral triploblastic animals. Most planarian species exhibit mechanisms for whole-body regeneration, exemplified by the regeneration of their cephalic ganglion after complete excision. Given their robust whole-body regeneration capacity, planarians have been model organisms in regenerative research for more than 240 years. Advancements in research tools and techniques have progressively elucidated the mechanisms underlying planarian regeneration. Accurate cell-cell communication is recognized as a fundamental requirement for regeneration. In recent decades, mechanisms associated with such communication have been revealed at the cellular level. Notably, stem cells (neoblasts) have been identified as the source of all new cells during planarian homeostasis and regeneration. The interplay between neoblasts and somatic cells affects the identities and proportions of various tissues during homeostasis and regeneration. Here, this review outlines key discoveries regarding communication between stem cell compartments and other cell types in planarians, as well as the impact of communication on planarian regeneration. Additionally, this review discusses the challenges and potential directions of future planarian research, emphasizing the sustained impact of this field on our understanding of animal regeneration.

Keywords: Planarians; Stem cells; Regeneration; Cell communication

INTRODUCTION

Organisms exhibit the remarkable capacity to regenerate and replace lost tissue, although this ability varies distinctly across species (Bely & Nyberg, 2010; Goldman & Poss, 2020). Given the multifaceted biological dimensions of regeneration, it is crucial to define the explicit processes involved. Several models have been pivotal in regeneration research, including fish and axolotls, which can regenerate organs and structures, and hydras and planarians, which can regenerate their entire bodies (Darnet et al., 2019; Reddien, 2022; Vogg et al., 2019). Certain mammalian models, including mouse with regenerative digit tips and deer with regenerative antlers, have also been employed in such studies (Qin et al., 2023; Takeo et al., 2013). Although the momentum of regeneration research waned during the 20th century, recent advances in genetics and molecular biology have reignited interest in the field. Planarians provide an excellent opportunity to address emerging questions related to whole-body regeneration. These animals are noted for their remarkable abilities in wound healing, body patterning, tissue remodeling, and adult stem cell maintenance, with the additional advantages of short regeneration time and easy laboratory upkeep (Newmark & Sánchez Alvarado, 2022). This paper provides a comprehensive review of planarian regeneration, as well as a framework for understanding communication between stem cell compartments and other cell types in planarian regeneration.

HISTORICAL PERSPECTIVE OF CELLULAR BASIS OF PLANARIAN REGENERATION

Planarians, classified within the class Turbellaria and phylum Platyhelminthes, are simple free-living organisms distinguished by bilateral symmetry, three germ layers, and distinct organs composed of multiple cell types (Newmark & Sánchez Alvarado, 2002). Their remarkable regenerative ability was first documented more than 240 years ago (Newmark & Sánchez Alvarado, 2002; Randolph, 1897). While initial research faced considerable methodological and experimental constraints, the robust regenerative ability of planarians prompted scientific curiosity and the development

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of different hypotheses and concepts. Planarian studies can be categorized into two defining eras: experimental zoology and molecular genetic analysis, as shown in Figure 1.

Planarian regeneration studies commenced with descriptions by Randolph (1892), introducing the idea of neoblasts based on traditional tissue staining of the annelid worm *Lumbriculus* during regeneration. Between 1898 and 1905, Thomas Hunt Morgan used the term morphogenesis to describe planarian regeneration and aimed to validate "morphallaxis" in these organisms — a process involving the remodeling of old or existing tissues without cellular proliferation (Agata et al., 2007). These foundational studies influenced subsequent research, emphasizing the importance of polarity as a field of study, and paving the way for understanding the roles of other tissues in planarian regeneration (Morgan, 1901). In contrast, Charles M. Child, who dedicated significant time to regeneration research, approached planarian regeneration from a metabolic gradient perspective and pioneered a quantitative method to assess head regeneration capacity along the body axis. Many recent studies have referenced these hypotheses, especially regarding polarization, as discussed below (Child, 1913; Morgan, 1905). Such studies have provided foundational insights into planarian regeneration, including the origin of cellular regeneration material, polarity, gradient metabolism, and components of blastema organization.

Anterior wound sites in planarians have been extensively examined, revealing a sequence of events, including muscle contractions, morphological changes in the epidermis that smooth the injury site, followed by mitotic neoblast progression, and finally, ganglia regeneration. However, the specific signals that regulate cellular fate determination for blastema formation are unknown (Baguñà et al., 1994; Chandebois, 1979; Hori, 1991; Nentwig, 1978; Turner, 1935). The role of neoblasts as a potential differentiated cell source has been a topic of contention for nearly a century. In the early 1900s, Bardeen & Baetjer (1904) noted the pronounced effects of X-rays on planarian regeneration, with Dubois & Wolff (1947) later using X-ray exposure to demonstrate the totipotency of neoblasts. Building upon these studies, Baguñà et al. (1989a) treated planarians with a combination of neoblast transplantation and X-ray exposure, demonstrating that blastemal formation was primarily attributable to neoblasts, thereby challenging the prevailing "dedifferentiation theory".

In addition to the recognized role of neoblasts in regeneration, diverse outcomes have been observed in experiments involving transplantations and amputations in various planes. For example, previous research showed that after removing middle segments and transplanting the foreparts to tail segments, an unpigmented, intercalated body part formed between the transplanted segments within 44 days, underscoring the reliance of regeneration on existing tissues (Brøndsted, 1955). As such, many investigators have explored how original tissues initiate blastema formation, with a particular focus on activating and inhibitory signals. While irradiation studies established that depletion of neoblasts impeded regeneration, the involvement of the nervous system in this context remained contested. Specifically, Child and Watanabe posited that the nervous system negatively affected

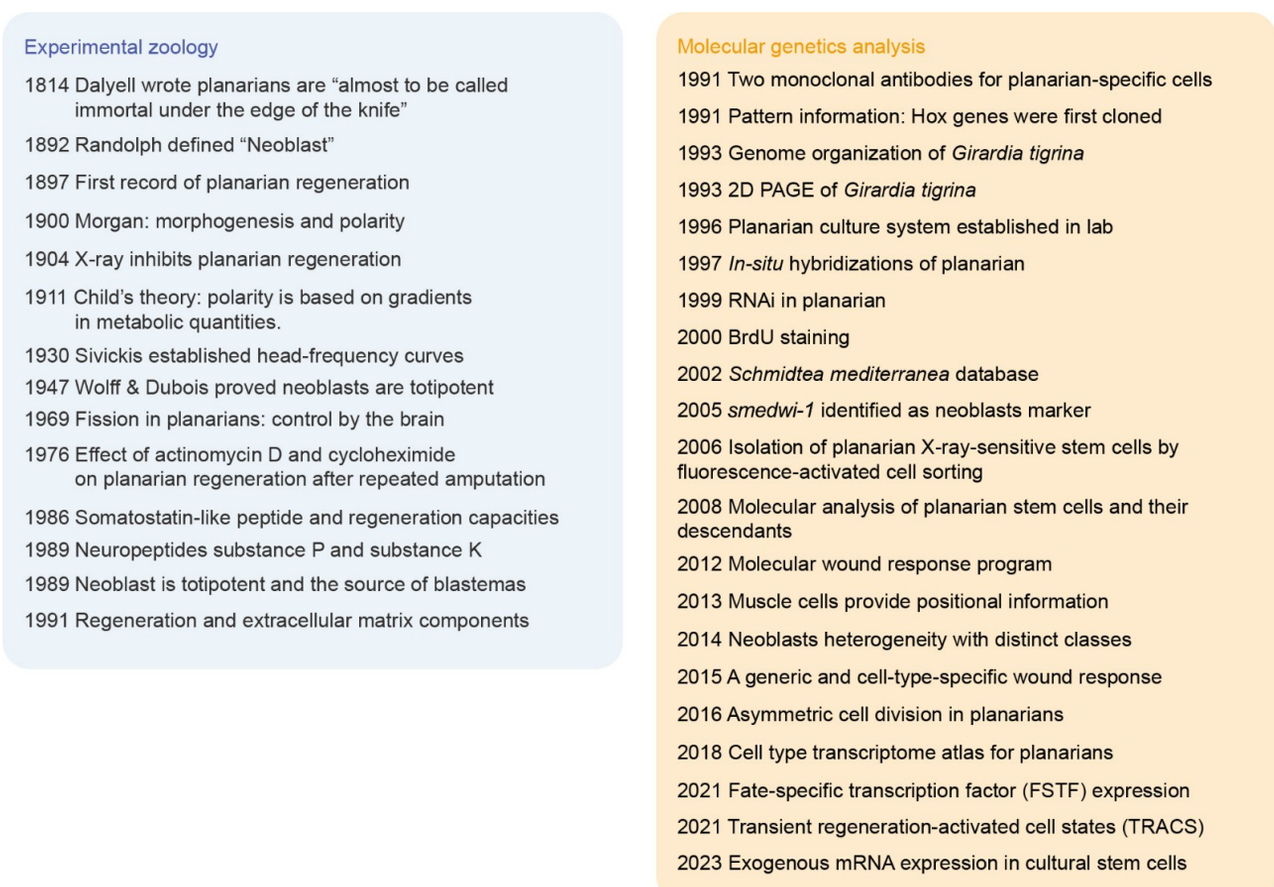

Figure 1 Key milestones in the history of planarian regeneration studies

head regeneration, whereas other research findings contradicted this assertion (Bardeen, 1902; Child & Watanabe, 1935; Morgan, 1905). The specific factors causing inhibition and their potential effects on neoblasts remained unidentified. The seminal work of Child laid the foundation for the study of planarian physiology in the context of regeneration (Child & Watanabe, 1935). Later research validated the metabolic processes necessary for blastema formation, revealing that externally introduced ribonucleic acids, amino acids, and even pH levels could influence the rate and extent of regeneration. Furthermore, specific chemical agents were identified as disruptors of DNA, RNA, and protein synthesis in planarians, subsequently affecting their regenerative capabilities (Baguñà et al., 1994).

While researchers have long recognized the complex interplay between neoblasts and other factors in planarian regeneration, it was not until the 1990s that cellular and molecular studies on planarians began to flourish, facilitated by the establishment of culturing methods for *Dugesia japonica* and *Schmidtea mediterranea*. Notably, the field saw rapid advancement with the introduction of various molecular tools, including RNA interference (RNAi), bromodeoxyuridine (BrdU) cell labeling at the S phase of the cell cycle for lineage tracing, fluorescence *in situ* hybridization for gene expression analysis, fluorescence-activated cell sorting (FACS), and modern genomic technologies (Newmark & Sánchez Alvarado, 2000; Pearson et al., 2009; Sánchez Alvarado & Newmark, 1999; Sánchez Alvarado et al., 2002; Solana et al., 2012). Due to their impressive regenerative abilities and tissue maintenance through stem cell-mediated self-renewal, planarians have emerged in recent years as an excellent model for studying regeneration, stem cell biology, and tissue homeostasis. The utilization of molecular tools has provided researchers with robust methodologies to investigate the underlying mechanisms, thus driving our understanding of planarian biology.

HETEROGENEITY AND INTRINSIC REGULATION OF PLANARIAN ADULT STEM CELLS: NEOBLASTS

Planarians contain multiple complex organ systems, including the neuronal, intestinal, and epidermal systems, which are differentiated from neoblasts during homeostasis and regeneration. Initial studies into the cellular nature of planarians predominantly relied on histological and morphological analyses. The concept of a neoblast was first defined by Randolph (1892) based on studies of the annelid worm *Lumbriculus*. Characterized by large oval nuclei, neoblasts are regarded as specialized embryonic cells responsible for the formation of new mesoderm after worm fission (Randolph, 1897).

Phylogenetic analysis of planarian species based on the 18S rRNA gene has underscored the significance of planarian or free-living flatworms in metazoan regeneration studies (Sánchez Alvarado, 2006). However, not all planarian species exhibit uniform regeneration ability. For example, species such as *Procotyla fluviatilis* and *Dendrocoelum lacteum* demonstrate limited regeneration ability in their posterior regions (Liu et al., 2013; Sikes & Newmark, 2013), suggesting the requirement of a molecularly tractable organism for planarian regeneration research.

Both *S. mediterranea* and *D. japonica* have become central to planarian regeneration research due to their stable diploid genomes and amenability to laboratory cultivation. Sánchez Alvarado et al. (2002) identified and investigated ~ 3000 genes that displayed differential expression during early regeneration. Inspired by the seminal work of Fire and Carthew, in which double-stranded (ds) RNA treatment was found to inhibit gene function in *C. elegans* and *Drosophila* (Fire et al., 1998; Kennerdell & Carthew, 1998), Sánchez Alvarado & Newmark (1999) tried to obtain loss-of-function planarians via dsRNA injection and soaking. Together with successful whole-mount *in situ* hybridization experiments with antibodies, they showed that dsRNA delivery decreased transcript levels and, subsequently, protein levels corresponding to target genes (Cebrià et al., 1997; Sánchez Alvarado & Newmark, 1999). Sánchez Alvarado et al. (2002) then sequenced the *S. mediterranea* genome, opening the field of planarian regeneration research to the molecular era. Neoblast-specific labeling was also shortly achieved based on the expression of *piwi* genes (Reddien et al., 2005; Rossi et al., 2006).

For a long period, neoblasts were viewed as a homogenous population. Through the application of single-cell transplantations, Wagner et al. (2011) determined that clonogenic neoblasts (cNeoblasts) can differentiate into all cell types. However, the percentage of successfully rescued donor worms was relatively low, suggesting that neoblast heterogeneity or donor worm niche should be considered.

To elucidate neoblast lineage development, Eisenhoffer et al. (2008) combined X-ray irradiation assays and genome-wide microarrays to identify genes expressed in neoblasts and their descendants and further categorized cells into three groups based on *in-situ* hybridization and BrdU-labeled cell tracing experiments. Van Wolfswinkel et al. (2014) explored neoblast heterogeneity using single-cell RNA-sequencing (scRNA-seq) and identified multiple subclasses of neoblasts with distinct lineages, yielding novel insights into the neoblast specialization model, which underlies the dynamic regulation of neoblast populations throughout the cell cycle. In more recent years, studies indicated that specialized neoblasts uniquely express cell-fate-specific transcription factors (FSTFs) in the S/G2/M phases and maintain their pluripotency through asymmetric division (Raz et al., 2021). These findings challenged what had been understood about the properties of previously categorized lineage-specific neoblasts and directed future studies into the cues that alter the rate of cell fate switches (Molinaro & Pearson, 2016; Pearson, 2022; Tanaka & Reddien, 2011; Zeng et al., 2018). Histone modification, a key mechanism for epigenetic gene expression regulation, impacts chromatin accessibility. Research into these modifications has facilitated the identification of possible enhancer-like elements in patterning positions (Neiro et al., 2022; Pascual-Carreras et al., 2023). These studies have and will continue to provide novel insights into how adult pluripotent stem cell populations maintain their potency in response to tissue regeneration demands.

Beyond gene regulation, post-transcriptional regulatory mechanisms play vital roles in many aspects of neoblast regulation. These mechanisms include alternative splicing, alternative polyadenylation, translational control, mRNA modification, and binding with micro (mi)-RNAs and PIWI-interacting (pi)-RNAs (Sasidharan et al., 2013). Alternative splicing of *bruli* in neoblasts enhances the inclusion of neoblast-specific exons rather than differentiated cell exons (Guo et al., 2006). In contrast, *mbnl* regulates alternative splicing in differentiated cells (Solana et al., 2016). Alternative

polyadenylation in the 3' untranslated region (UTR) of mRNA can determine cell fate, with highly proliferative cells containing shorter 3' UTRs and differentiated cells tending to contain longer 3' UTRs (Lakshmanan et al., 2016). In a planarian transgenesis experiment, the addition of a UTR from a particularly abundant transcript can promote the translation of exogenously delivered mRNAs (Hall et al., 2022).

N6-methyladenosine (m6A), one of the most abundant mRNA modifications, has also been the subject of recent investigations in planarians. Cui et al. (2023) demonstrated that depletion of the m6A methyltransferase regulatory subunit *wtap* causes regeneration defects by up-regulating the cell-cell communication ligand *grn* and cell cycle-related genes *cdk7* and *cdk9*. Upon disruption of the m6A pathway via methyltransferase complex gene knockdown, Dagan et al. (2022) revealed that m6A is essential for regulating planarian neoblast differentiation through a process that mediates nucleosome remodeling and deacetylase (NuRD) activity. Despite the expression of nearly all m6A-related genes in neurons and certain unidentified cell types, the exact mechanism through which m6A modification governs cell cycling via the NuRD complex within neoblasts remains elusive. Such modulation may differ from the regulatory mechanisms of adjacent cells. In addition to the protein encoded by *piwi-1*, the planarian neoblast markers PIWI-2 and PIWI-3 also regulate mRNA stability through the piRNA pathway (Kim et al., 2020). This pathway has been investigated in the germline and stem cells of other animal species, potentially highlighting conserved stem cell regulatory mechanisms.

Neoblast heterogeneity has been identified through single-cell RNA sequencing (scRNA-seq) and lineage prediction experiments. Subsequent studies have explored the associations between DNA content and other characteristics, such as RNA content and mitochondrial status, further clarifying this heterogeneity. For example, Molinaro et al. (2021) characterized a population of planarian RNA^{low} neoblasts with low transcriptional activity and slow cycling during homeostasis but that respond to injuries and enter the cell cycle via the mTOR signaling pathway. Using mitochondrial staining, Mohamed Haroon et al. (2021) reported that neoblasts with low mitochondrial mass and activity correspond to pluripotent stem cells, whereas those with high mitochondrial mass correspond to differentiated cells. Adult stem cells of planarians demonstrate a remarkable ability to differentiate into all cell types during homeostasis and regeneration, confirming them as pluripotent stem cells that enable the organism to achieve whole-body regeneration (Raz et al., 2021; Wagner et al., 2011). In contrast, adult stem cells in other species typically exhibit a more limited regenerative capacity, restricted to specific tissues, with their population and regenerative potential declining with age. The regulatory mechanisms that allow planarians, but not organisms such as humans, to maintain their regenerative capacity have remained a long-standing puzzle. Research insights will help elucidate how planarians accomplish whole-body regeneration, potentially informing strategies to promote tissue regeneration and healthy aging in humans.

DYNAMICS AND DIVERSITY OF CELL-CELL COMMUNICATION IN PLANARIAN REGENERATION

Precise observations of dynamic regulation among different cell types and tissues, such as eyes, epidermis, muscles, intestines, and neurons, have highlighted key signaling pathways, including the *Hox*, fibroblast growth factor receptor (FGFR), and Wnt signaling pathways, as regulators of anterior and posterior axes in planarians (Cebrià et al., 2002; Petersen & Reddien, 2008, 2009). Wenemoser et al. (2012; 2010) identified two mitotic neoblast peaks after wounding, pioneering the exploration of transcriptional responses to regeneration initiation in different cell types, thus providing spatial context to wound-response genes. Comprehensive single-cell transcriptional profiling has facilitated systematic categorization of planarian cell types and has enabled classification of previously discovered genes based on cell-type-specific expression (Cheng et al., 2018; Fincher et al., 2018; Forsthoefel et al., 2020; Van Wolfswinkel et al., 2014; Witchley et al., 2013; Wurtzel et al., 2015; Wurtzel et al., 2017). Witchley et al. (2013) demonstrated that genes associated with position control are localized in the sub-epidermis and expressed by muscle cells. Specific genes, such as *wnt1* and *notum*, have been identified as wound-response genes, present in diverse cell populations in addition to the epidermal cells and neoblasts (Wurtzel et al., 2015). Recent studies have indicated that injury-induced post-mitotic cells from the muscle, epidermis, and intestine regulate neoblast populations and functions (Benham-Pyle et al., 2021). However, the mechanisms by which these cells perceive injury signals and recruit neoblasts to the appropriate position for blastemal formation are largely unknown. Collectively, these results emphasize the importance of cell-cell communication in influencing regeneration patterns and neoblast proliferation and differentiation.

Over the past two decades, both the advancement of novel techniques and established foundational knowledge have elucidated many of the cellular and molecular mechanisms underpinning planarian regeneration. Previous reviews have comprehensively discussed the core principles of regeneration processes, including wound response, positional information, and polarity control, as summarized in Figure 2 (Molina & Cebrià, 2021; Reddien, 2018, 2022). Extensive communication occurs among diverse somatic tissues, which is discussed below in relation to neoblasts. Cellular interactions among different cell types are summarized in Figure 3. However, many fundamental questions remain unaddressed; for example, which signaling pathways direct the initial specialization of neoblasts (i.e., switch cell fates)? Which cell types provide the permissive niches that regulate the planarian stem cell population? Understanding the mechanisms guiding planarian neoblast specification in response to external cues may provide insights into broader stem cell biology.

Communication between neoblasts and somatic cells

The niche, or surrounding environment, determines stem cell fate. Although scRNA-seq has provided insights into gene function and signaling pathways in planarians, our comprehension of stem cell regulation by other cell types remains incomplete. Low engraftment efficiency in cell transplant experiments has suggested the existence of a niche environment for transplanted neoblasts (Raz et al., 2021; Wagner et al., 2011). While signals that activate stem cell proliferation and differentiation throughout planarian regeneration have been investigated, the mechanism by which these signals operate and the specific cell types responsible for signal transduction are yet to be determined. Here, we

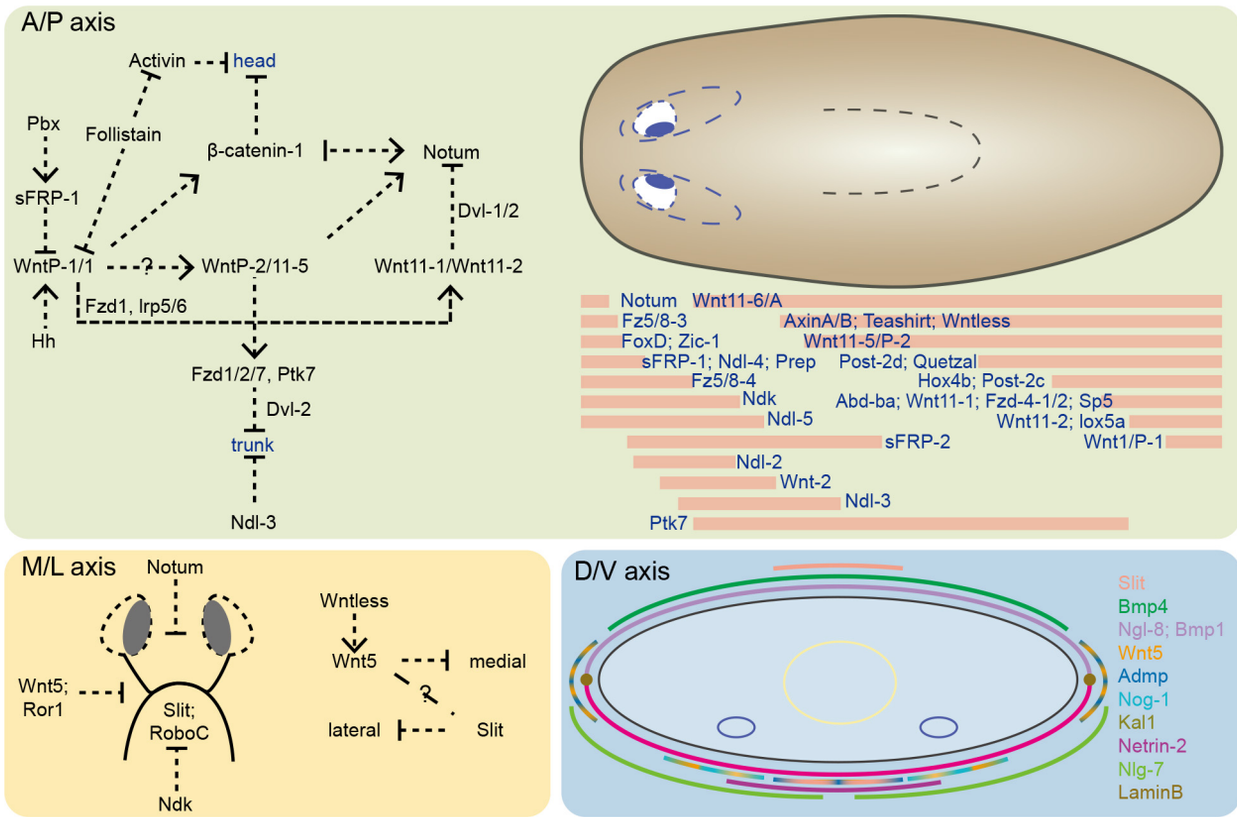

Figure 2 Diagrams showing planarian positional information at different levels

Upper panel, signal transduction and regional expression patterns along anterior-posterior axis. Lower left, biochemical regulation of eye regeneration and pattern control of medium-lateral axis. Lower right, expression pattern of positional control genes along dorsal-ventral axis. Reproduced with modifications from Scimone et al. (2016), Reddien (2018), and Scimone et al. (2020).

discuss the interactions between neoblasts and several somatic cell types during planarian regeneration.

Epidermal cells

The planarian epidermis was initially delineated into three subdivisions, i.e., ciliated epidermis, non-ciliated epidermis, and dorsal-ventral boundary epidermis. Subsequent scRNA-seq analysis of planarian neoblast heterogeneity revealed a specific cluster of neoblasts indicative of epidermal progenitors, designated as the ζ class (Van Wolfswinkel et al., 2014; Wurtzel et al., 2015). Lineage development of these cells has been extensively studied through spatial and temporal analysis, resulting in the identification of key cell-stage-specific markers. The ζ class marker, *zfp-1*, indicates epidermal progenitor cells before they progress to *prog-1*+ early progenitor cells or *AGAT*+ late progenitor cells. Subsequent mature cells can be identified by the expression of *PRSS12*, *laminB*, and *rootletin* (Wurtzel et al., 2017). Interestingly, *AGAT-1*+ cells synthesize creatine, which is subsequently taken up by muscle cells and neurons, and express many genes involved in metabolic processes (Tu et al., 2015). Recent studies have indicated that *AGAT-1*+ cells play roles in the wound response and regulation of stem cell proliferation (Benham-Pyle et al., 2021). Moreover, inhibition of epidermal gene expression in *egr5*-, *AGAT-1*-, and *p53*-RNAi worms increases neoblast proliferation, suggesting a feedback mechanism between the epidermis and neoblasts (Benham-Pyle et al., 2021; Pearson & Sánchez Alvarado, 2010; Tu et al., 2015). In response to positional cues, the epidermis envelops wound sites and specifically expresses wound-induced genes upon injury. Scimone et al. (2022) analyzed wound epithelialization and found that the *equinox* gene is expressed in planarian epidermal wounds shortly after injury. They posited that the *equinox*-encoded protein product is secreted to mediate cell-cell communication between the muscle and epidermis and is critical for initiating blastema formation. Thus, these results broaden our understanding of stem cell regulation in epidermal injury.

Muscle cells

Based on positional features, planarian muscles can be classified into two major types, i.e., enteric muscles surrounding the gastrovascular cavity, and circular, diagonal, and longitudinal body wall muscles providing structural support for locomotion. Witchley et al. (2013) identified positional control genes (PCGs) and found that the *bmp* (involved in dorsal-ventral axis maintenance), *notum*, and *wnt1* (*wntP-1*) (involved in anterior and posterior polarity modulation, respectively) genes are expressed in muscle cells. *Bmp4* inhibition leads to progressive ventralization in planarians and abolishes epidermal *kal1*+ and *equinox*+ cells, suggesting that muscle-derived *bmp4* can modulate neoblast specialization during epidermal development (Scimone et al., 2022; Wurtzel et al., 2017). *Wnt1* is a wound-induced gene regulated by *follistatin* and *notum* signals (Petersen & Reddien, 2009). After inhibition of *wnt1*, worms display an anterior pattern at the posterior-injured face, with abnormal expression of ectopic eye-progenitor *ovo*+ cells (Petersen & Reddien, 2011). Recent studies of two *wnt11* genes (*wnt11-1* and *wnt11-2*) found that signaling through *dvl* suppresses *notum* expression in posterior-facing wounds, thus revealing the diverse roles of *wnt* signals in planarians (Gittin &

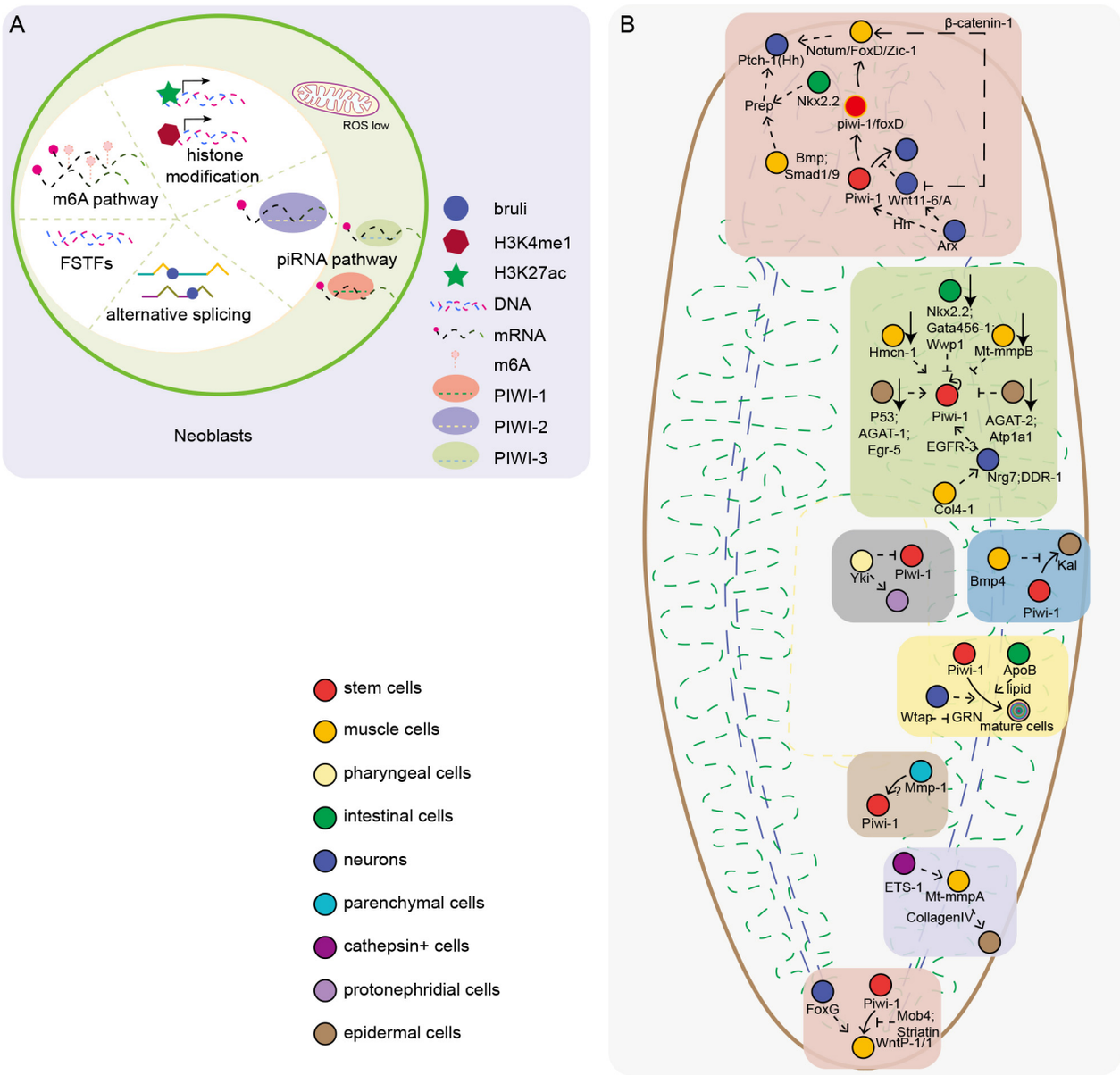

Figure 3 Regulation of neoblast specification

A: Gene expression in neoblasts regulates cell fate determination. B: Communication mechanisms between somatic cells and neoblasts.

Petersen, 2022). The Wnt signaling pathway often coordinates with other pathways, such as the transforming growth factor beta (TGFβ) and FGFR signaling pathways (Arnold et al., 2019; Scimone et al., 2016); however, whether these interactions regulate stem cell behavior requires further investigation.

In addition to positional control gene expression, Cote et al. (2019) studied the components and expression levels of the planarian matrisome and proposed that muscle cells also serve as a source of secreted extracellular matrix (ECM), such as collagens and core glycoproteins. All 21 planarian genes predicted to encode collagens are expressed in muscle cells, including 11 fibrillar collagens (colfs), seven predicted type IV collagens (COLIVs/col4s), and three multiplexin collagen family members. Following sublethal radiation, RNAi of *colf-2/7/8* or *col4-1/2/3/4* increases neoblast density, supporting the hypothesis that ECM is released by muscle cells and can modulate the neoblast environment. Chan et al. (2021) proposed that *col4-1* inhibits *nrg-7* in neuronal cells by interacting with the *DDR-1* receptor. In addition, nrg-7 is a ligand of EGFR-3 in neoblasts, regulating asymmetric cell division and self-renewal (Lei et al., 2016). These results suggest a relationship among planarian muscle cells, neurons, and neoblasts. In addition to ECM cellular expression analysis, Cote et al. (2019) also determined that *plc*, *hmcn-1*, the extracellular collagen chaperone *SPARC*, and *P4H4* (an enzyme that stabilizes collagen structure) are expressed in muscle cells. *Hemicentin-1* (*hmcn-1*) encodes a highly conserved ECM glycoprotein and is expressed explicitly in body wall muscle cells. Furthermore, *hmcn-1* inhibition results in ectopic neoblasts and differentiated cells outside the muscle layer (Cote et al., 2019). Matrix metalloproteinases (MMPs) are a large family of regulatory enzymes that function in ECM degradation and facilitate diverse cellular processes. Dingwall & King (2016) explored the roles of MMPs in planarian stem cell biology and found that *mmpB* is expressed in dorsal-ventral muscle fiber. Notably, *mmpB* inhibition reduces body size and the proliferative cell population. Thus, these

discoveries highlight the critical roles of muscle cells in regulating neoblasts.

Neurons

The planarian nervous system comprises the cephalic ganglia and two longitudinal ventral nerve cords. Several neuronal cell types produce neurotransmitters. Wyss et al. (2022) used scRNA-seq analysis to distinguish cell types in the planarian nervous system, and identified eight types of enriched neurons, i.e., peptidergic, cholinergic, glutamatergic, GABAergic, dopaminergic, glycinergic, serotonergic, and octopaminergic neurons, based on neurotransmitter marker gene expression. However, almost all single neurons can express more than one neurotransmitter, suggesting that neurotransmitter networks require individual neuron flexibility for proper neurogenesis in planarians (Wyss et al., 2022). *Nkx2.1* and *arx* are expressed in cholinergic, GABAergic, and octopaminergic neurons; these neuronal types are implicated in the release of the hedgehog (*Hh)* ligand, regulation of neoblasts, maintenance of normal proliferation levels, and promotion of homeostatic neurogenesis (Currie et al., 2016b). In addition, *arx*+ cells also reduce neoblast specialization via the Wnt signaling pathway, increasing neuron production. *Arx*+ cells can transduce *wnt11-6* signals to the surrounding stem cells via feedback machinery dependent on *notum* inhibition (Hill & Petersen, 2015). Several studies have indicated that neurons regulate polarity along the ventral nerve cords. Gene regulatory networks (GRNs) built by modeling transcription factor interactions with enhancers suggest that *ptch-1*+ neurons transduce signals to maintain polarity along the axis (Neiro et al., 2022). Additionally, the *FoxG* and the G-protein subunits *Gαq1* and *Gβ1-4a* are predicted to be upstream regulators of *wnt1*, required for planarian posterior identity specification and anterior re-establishment, respectively (Jenkins & Roberts-Galbraith, 2023; Koinuma et al., 2003; Pascual-Carreras et al., 2020, 2023).

In 1989, Baguñà et al. (1989b) reported that neuropeptide substances P and K can promote neoblast proliferation via tachykinin receptors. Peptidomics and functional genomics have characterized neuropeptides in both asexual and sexual planarians. The identities of P and K homologs in planarians and specific interactions between neurons and neoblasts remain unclear, although it has been suggested that treatment with antagonists of substance P decreases neoblast proliferation and migration near neurons (Baguñà et al., 1989b; Bautz & Schilt, 1986; Rossi et al., 2012). Interestingly, epidermal growth factor (EGF) signals are reported to activate cell proliferation. Lei et al. (2016) showed that signals released from neurons can mediate asymmetric cell division via the *egfr-3* receptor. These discoveries provide sufficient evidence to support a critical model of nerve factors in communicating with stem cells.

Intestinal cells

The planarian intestine comprises one anterior and two posterior gut branches, which elongate into secondary, tertiary, and quaternary branches. The complex morphology of the intestine surrounding planarian neoblasts is considered a niche that modulates neoblast behavior. Knockdown of several intestine-enriched transcription factors, such as *nkx2.2* (expressed in phagocytes) or *gata4/5/6-1*, causes defects in intestinal integrity and regeneration due to reduced blastema formation and decreased neoblast proliferation (Flores et al., 2016; Forsthoefel et al., 2012). The HECTE3 ubiquitin ligase family gene *wwp1* is highly expressed in the intestines and plays roles in both intestinal integrity and neoblast maintenance (Henderson et al., 2015). Barberán et al. (2016) proposed that loss of *egfr-1* induces hyperproliferation and expansion of neoblast progenitors, suggesting a role of the intestine in modulating the niche environment. Forsthoefel et al. (2020) employed laser capture microdissection to analyze intestinal cells, discerning the roles of phagocytes, goblet cells, and basal cells within intestinal branches. They ascertained that goblet cells are potentially linked to lipid metabolism, protein processing, ECM organization, and innate immunity, while phagocytes may be pivotal for nutrient uptake and storage. Recently, the same group connected the functions of intestinal cells to the regulation of neoblasts, with *apolipoprotein b* orthologs enriched in intestinal cells to regulate stem cell progeny differentiation and regeneration in planarians via lipid metabolism (Wong et al., 2022). These findings provide evidence of a metabolic switch during regeneration and demonstrate the essential role of lipid regulators in supporting communication with stem cells.

Other tissues

In addition to more widely studied tissues, phagocytic activity has been discovered in *cathepsin*+ pigment, glia, and dendritic cells (Scimone et al., 2018). Recent studies have indicated that *ETS-1*, expressed in *cathepsin*+ cells, regulates the ECM regulator *mmpA*, balancing the degradation and synthesis of muscle-secreted collagen IV and modulating the development of epidermal progenitors (Dubey et al., 2022). Together, *cathepsin*+ cells play an essential role in regulating neoblast specialization, consistent with their phagocytic characteristics and ECM regulation. However, a systematic understanding of this cell type remains to be achieved.

Stem cells are distributed throughout the planarian body, except the anterior and pharynx, making the interface between stem cell compartments and parenchymal cells essential for homeostasis and tissue remodeling. Hori (1991) used electron transmission microscopy to examine planarian tissues and observed that flexible parenchymal cells are closely associated with regenerating cells during regeneration, suggesting signal transduction via gap junctions or ECM. Recent spatial transcriptomic analysis of planarian whole-body regeneration revealed a strong link between *MMP-1*+ secretory cells and stem cells, highlighting diverse interactions between stem cells and their microenvironment (Benham-Pyle et al., 2023).

Communication among somatic cells

Somatic cell signal transduction regulates their growth, but elements of the cell-cell communication cascade also determine neoblast cell fate, especially for muscle cells (as mentioned above). *Wnt11-2*+ muscle cells receive signals from *wnt1*+ cells to guide tail regeneration (Pascual-Carreras et al., 2021). There also exists a robust connection between neurons and muscle cells in planarians, although the associated molecular mechanisms are unclear. In the context of eye regeneration, *notum*+/*frizzled 5/8-4*+ muscle cells can collectively define the precise positioning of trajectories between the eyes, synchronously regulated by the medium-lateral axis and *notum*+ neuron cells (Hill & Petersen, 2015, 2018; Scimone et al., 2020). However, certain questions remain to be addressed, such as whether communication is specified in certain regions and whether these regulations are conserved in other animals.

CELL-CELL COMMUNICATION FOR ASEXUAL REPRODUCTION AND EMBRYOGENESIS IN *S. mediterranea*

Triclad flatworms can reproduce sexually or asexually. Sexual reproduction via fertilization is common among multicellular animals. However, asexual reproduction mediated by somatic multi- or pluripotent stem cells can also occur in many invertebrate species. Planarians are remarkable regarding their regenerative abilities, but different planarian species exhibit varying degrees of regenerative capacity, with even closely related species or members of the same species from other locations demonstrating different capabilities (Krichinskaya, 1986; Liu et al., 2013; Sikes & Newmark, 2013). Moreover, the mode of reproduction in planarians is dynamic. While certain species, such as *S. mediterranea*, have evolved into stable biotypes with asexual or sexual reproduction, others can switch between both. At present, the relationship between sexual and asexual reproduction and regeneration is yet to be resolved.

In planarians, spontaneous fission occurs in the post-pharyngeal area (Hori, 1991; Hori & Kishida, 1998). After fission, the two fragments can independently regenerate into an intact worm. However, the regeneration processes that occur after fission are different from those that occur after surgical manipulation. In 1959, Pedersen stated that neoblasts accumulate in the posterior part of the body prior to division, which causes rapid regeneration (Pedersen, 1959). In contrast, Kenk reported that the fission fragment accomplishes head formation through morphallaxis as an internal remodeling process (Kenk, 1937; Best et al., 1969). To further explore fission behaviors, Child studied the fission phenomenon from a physiological perspective, leading to prominent research related to size-dependent fission (Child, 1903; Brøndsted, 1955). Notably, planarian size was found to be responsive to nutrient uptake relative to the activity of the insulin signaling pathway and *sirtuin-1* (Malinowski et al., 2017; Miller & Newmark, 2012; Ziman et al., 2020). Child also noted that the presence of the head inhibits fission, further confirmed by Baguñà's experiments (Baguñà et al., 1989b; Child, 1932). However, neoblast proliferation also contributes to fission. Sakurai et al. (2012) discovered that a homolog of the *D. japonica* membrane protein P2X modulates the neoblast proliferation response to nutrient uptake, with knockdown of the gene encoding this protein found to increase fission. Therefore, the hypothesis raised by Child that there is a metabolic gradient along the axis warrants further investigation (Child, 1911).

Recent studies have revealed that the *Hox*, Wnt, and TGFβ signaling pathways coordinate to regulate size-dependent behaviors (Arnold et al., 2019; Arnold et al., 2021). By examining the functions and expression patterns of genes involved in the Wnt and TGFβ signaling pathways, Arnold et al. (2019) showed that *pkd1L-2*+/*gabrg3L-2*+ mechanosensory neurons display a decreasing angle relative to planarian width with increasing body size, thus inhibiting fission behaviors. In addition, *post2b* induces gland cells associated with parenchymal and epidermal cells to secrete a compound that anchors the posterior end of a worm to a substrate to initiate fission (Arnold et al., 2021). These findings indicate that the Wnt and TGFβ signaling pathways interact with the central nervous system to modulate fission frequency. Concurrently, the *Hox* gene regulates the fission plate and associated behaviors via a secretion pathway, as illustrated in Figure 4.

Planarian embryogenesis proceeds via anarchic cleavage development, encompassing eight distinct stages, with multiple fertilized zygotes and yolk cells accumulating within a capsule after mating. Embryogenesis was extensively studied in the early 19th century, with a comprehensive review provided by Martín-Durán et al. (2012). In adult planarians, neoblasts serve as adult stem cells, responsible for orchestrating cell differentiation, including germ cells. Such findings raised questions regarding whether neoblasts are the same as blastomeres and, if not, when and how are pluripotent neoblasts generated during embryogenesis. Through transplantation and *in-situ* hybridization, Davies et al. (2017) showed that neoblasts developed from embryonic stem cells around stage 5. Stem cells isolated from this stage can

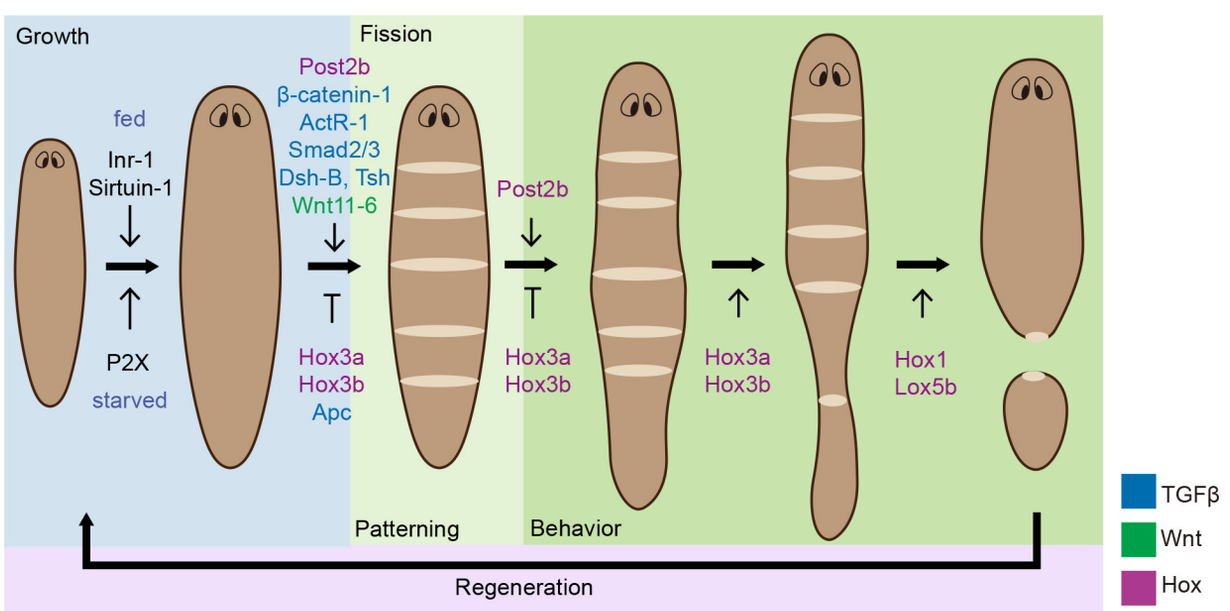

Figure 4 Molecular regulation of planarian asexual reproduction and growth

Reproduced with modifications from Arnold et al. (2021).

later rescue lethally irradiated worms. However, the ubiquitous expression of *piwi-1* and *h2b* during embryogenesis, functioning as stem cell markers, suggest that stem cell determination largely relies on the surrounding microenvironment. Recent discoveries of pluripotent stem cell origins in another regenerative worm, *Hofstenia miamia,* through lineage tracing may yield further insights into the formation of adult planarian stem cells (Kimura et al., 2022). Further studies should identify the compounds involved in signal transduction in blastomeres to modulate stem cell proliferation and differentiation and *ex vivo* self-organization.

LIMITATIONS AND PROSPECTS

Over the last two decades, studies on planarian regeneration have transformed from relatively simple histological observations to detailed analyses of molecular and cellular mechanisms. However, key questions remain to be resolved. For example, does a single type of pluripotent adult stem cell (cNeoblast) exist in planarians? What are the origins and identities of pluripotent adult stem cells? How do cells sense and transduce external stimuli? What signals regulate neoblast specialization, and how do they function? How do multiple cell types coordinate to remodel tissues? Why do planarians retain their regenerative capacity over individual lifespans and evolutionary time? What properties differentiate planarians from other species?

Although research has addressed neoblast heterogeneity and cell lineage development, systematic analysis of the regulation of neoblast specialization remains to be conducted (Barberán et al., 2016). Planarians possess an array of cell types and tissues, derivable from neoblasts during homeostasis and regeneration. Some of these cell types also serve as regulators of neoblast activity. Interactions among these diverse cell types, including neoblasts, have been explored in planarians based on conserved signaling pathways shared with mammals. These studies have offered insights into the factors contributing to the robust regenerative abilities of planarians, which are not mirrored in humans.

Analysis of the shared components of the *Hox*, Wnt, and TGF-β signaling pathways in planaria and other model organisms has also provided insights into the function of these genes beyond animal development. Evolutionarily conserved, these pathways collectively regulate body plan polarity and dictate cell fate. In planarians, 13 *Hox* genes are involved in anterior-posterior axis pattern during development (Arnold et al., 2021; Currie et al., 2016a). Various *wnt* genes in planarians also control global region patterning along the anterior-posterior axis, including head and tail determination (Arnold et al., 2019; Gittin & Petersen, 2022; Hill & Petersen, 2018; Pascual-Carreras et al., 2021; Petersen & Reddien, 2009; Reddien, 2022; Scimone et al., 2020). The TGF-β signaling pathway also regulates the dorsal-ventral axis and instructs epidermal cell functionality (Cloutier et al., 2021; Gaviño & Reddien, 2011; Gaviño et al., 2013; Reddien et al., 2007; Roberts-Galbraith & Newmark, 2013; Scimone et al., 2022). More importantly, research into planarian regeneration suggests the potential to adapt these signaling modalities to re-establish patterns within adult tissue contexts. Exploring how planarians re-establish patterning signals after tissue loss and assessing the additional functions of planarian proteins in diverse organisms may be a promising focus in the future (Pascual-Carreras et al., 2023).

Comparing regenerative mechanisms in planarians with those in other species, such as fin regeneration in fish (De Simone et al., 2021; Sun et al., 2022; Wang et al., 2020), tail regeneration in lizards (Alibardi, 2018), limb and brain regeneration in salamanders (Pan et al., 2023; Peng et al., 2021; Wei et al., 2022), and ear punch regeneration in mice (Tomasso et al., 2023), is also important for future research. Studying these diverse models will enable a deeper understanding of the common mechanisms and modules involved in the process of regeneration.

Transgenic gain-of-function planarian mutants have long been sought in research. Recent advancements have been made by two groups in methodological approaches for transgenic planarian studies, shedding light on gene editing and lineage tracing in these species (Hall et al., 2022; Lei et al., 2023). Anticipated developments in transgenesis and tissue culture systems will enable visualization of cell-cell interactions *in vivo* and *in vitro*, respectively (Lei et al., 2023). Moreover, emerging omics techniques will facilitate dynamic cell modulation, permitting analysis of planarians at the gene and protein to entire metabolic system level with two-dimensional and three-dimension resolution. Combining such tools in planarian will greatly enhance our understanding of stem cell biology and regeneration.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.J.C. and K.L. wrote the manuscript and constructed the figures. All authors read and approved the final version of the manuscript.

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