

Planarian stem cells: a simple paradigm for regeneration

A. Aziz Aboobaker

Evolutionary Developmental Biology Group, Centre for Genetics and Genomics, University of Nottingham NG7 2UH, UK

Planarians are capable of profound regenerative feats dependent upon a population of self-renewing adult stem cells called neoblasts. The key features of neoblasts are their capacity for indefinite self-renewal, their totipotency and the ability of their progeny to interpret differentiation and polarity signals and correctly replace lost structures after tissue damage. Regeneration in planarians offers a paradigm for understanding the molecular and cellular control of the repair and regeneration of animal tissues, and could provide valuable insights for the safe use of stem cells to repair damaged, diseased and ageing human tissues with little or no regenerative capacities. Here, I review recent progress in understanding neoblasts in regeneration and the growing potential this research has to be broadly informative for human biology.

What can we learn from a regenerative life history? The tale of a stem cell

Currently, the molecular mechanisms behind regenerative phenomena remain obscure, largely because highly regenerative organisms do not have the short lifecycles and high fecundity suited to classical genetics; therefore, the amount of genetic, and subsequent molecular, research on these species has been relatively small.

Regenerative phenomena in various organisms are now proving to be molecularly tractable [1,2]. Among the bilaterians, planarians probably represent the most prodigious regenerators, able to replace all body tissues and cell types facilitated by a population of adult somatic stem cells [3–6] called neoblasts. These neoblasts comprise 20% or more of the cells in an adult worm. They are distributed throughout the mesenchyme, are absent from the pharynx and regions in front of the photoreceptors [3–7] and are defined as the only proliferative cells in the body. Neoblasts give rise to progeny that replace older differentiated cells (Figure 1a) and allow planarians to grow and degrow in response to changing nutrient conditions, while maintaining constant tissue and cell proportions [4,8,9].

In response to wounding, neoblasts proliferate in a characteristic spatial and temporal manner. First comes a broad response to wounding and then a more spatially localized distribution of proliferation to replace missing tissues [10]. The progeny of neoblasts are postmitotic and accumulate across the site of wounding to form a regenerative blastema where they differentiate and integrate, replacing distal missing tissues while existing tissue remo-

dels (Figure 1b). Damaged and aged cells within each tissue are constantly replaced by differentiating progeny of the neoblast pool, with wholesale regeneration from small tissue fragments the extreme example. Significantly, asexual planarians rely on this regenerative ability as a means of reproduction because they reproduce by transverse fission along the anteroposterior (A/P) axis [4].

The homeostatic maintenance of planarian tissues and regeneration after injury requires the acute control of proliferation, cell death and autophagic processes [11–13]. Early data from the planarian model suggest that the pathways controlling proliferation, growth and degrowth are likely to be conserved, and planarians represent an excellent model system for studying the basic aspects of these pathways, many of which are directly involved in cancer biology, in a regenerative and highly homeostatic context. Neoblasts have also been suggested as a potential model system for understanding the proliferative control of stem cells, which is relevant to human cancers [14–16]. For example, the study of planarian orthologs of the human tumor suppressor PTEN shows that RNAi-mediated knockdown leads to neoblast hyperproliferation and abnormal outgrowths through the overactivation of the TOR signaling pathway [14]. Studying this and other conserved disease-relevant genetic pathways in planarians will provide novel regulatory insights with direct relevance to our own tissue homeostasis control.

Planarians have also been suggested as a possible model system for understanding ageing. In mammals, dividing cells eventually undergo replicative senescence, a contribution to organismal ageing [17]. This occurs because the telomeres at chromosome ends undergo progressive shortening after each round of DNA replication until reaching a critical length that induces senescence. Failures in the induction of senescence in human malignancies allow transformed cells to keep dividing [18]. Neoblasts must have overcome the end replication problem indefinitely to facilitate their immortality. Whether this is through canonical telomeres and telomerase activity or whether it is a property of all neoblasts or confined to a selection of truly immortal populations remains unknown.

The planarian model also has the potential to offer an integrated understanding of how diseased, damaged and ageing tissues can be regenerated. For planarian regeneration, neoblasts must: i) have an indefinite capacity for self-renewal and homeostasis of the neoblast population; ii) maintain pluripotency and the production of progeny that can differentiate into all other cells and tissues; and iii) correctly interpret polarity signals and positional cues to

Corresponding author: Aboobaker, A.A. (aziz.aboobaker@nottingham.ac.uk).

ensure the functional integration of new tissues with old. These are the same mechanisms that must be manipulated for successful stem cell-based regenerative therapies. Induced pluripotency has great potential to provide a source of stem cells for regenerative medicine [19], but using these cells safely to repair or replace tissues will require understanding how signals in existing tissue affect stem cell differentiation and how they can be manipulated to facilitate functional integration. The planarian system – where regeneration is *de rigueur* – provides an ideal opportunity to study these signals.

In this review, I will describe recent progress in characterizing molecular pathways in neoblasts using functional genomic approaches and the development of molecular markers. I will then discuss advances in our understanding of how these molecules contribute to neoblast maintenance, differentiation, axial polarity and positional information and the integrative control of regenerative events in planarians.

A growing catalog of neoblast-associated genes: telling mother and daughter apart

An important starting point for investigating neoblast biology is the identification of actively expressed genes. These serve as candidate genes for regulating important aspects of neoblast biology and as markers of neoblasts and their undifferentiated progeny. One simple but important tool in planarian biology has been the use of gamma irradiation to remove proliferative neoblasts. Microarray-based expression analyses in two different species comparing normal and irradiated worms produced a catalog of genes potentially involved in neoblast biology [20,21], limited however by the relatively small numbers of planarian genes represented on the arrays. A more recent study using next-generation sequencing in the *Schmidtea mediterranea* transcriptome identified 2 300 transcripts (out of 25 000) that were significantly downregulated by irradiation [22]. Not surprisingly, the set of genes defined by these studies is enriched for those regulating the cell cycle (neoblasts are the only proliferative cells), but also for genes known to regulate developmental mechanisms in other animals. These might be candidate genes for maintaining pluripotency and directing neoblast differentiation. However, without markers that differentiate between neoblasts and daughter cells it is difficult to specify roles for these genes in neoblast maintenance and self-renewal versus differentiation.

An elegant study using the microarray expression analyses of an irradiation time course, and the subsequent double-labeling of cells with BrdU [23] and potential markers of neoblast progeny, identified the first markers that label neoblasts and neoblast daughter cells independently [21]. Combining this approach with the large set of genes identified by the comparative RNAseq of normal and irradiated animals should allow the identification of more neoblast and neoblast progeny markers [21,22]. Using known markers in RNAi knockdown studies will allow genes of interest to be more clearly assigned to roles in neoblast maintenance and/or the control of neoblast differentiation (Figure 1c). Two studies investigating the function of the planarian homologs of the tumor suppressor p53

and the chromatin remodeling component CHD4 have already shown the utility of neoblast progeny markers [24,25]. The RNAi of these conserved genes showed that they were required for neoblasts to facilitate regeneration and tissue homeostasis. In addition, looking at the dynamics of proliferating neoblasts and neoblast daughter cells separately showed that fewer early neoblast progeny are produced followed by a later depletion of neoblasts. Thus, these genes could be assigned temporally specific roles in the correct production of neoblast progeny (early differentiation) with a later effect on neoblast maintenance.

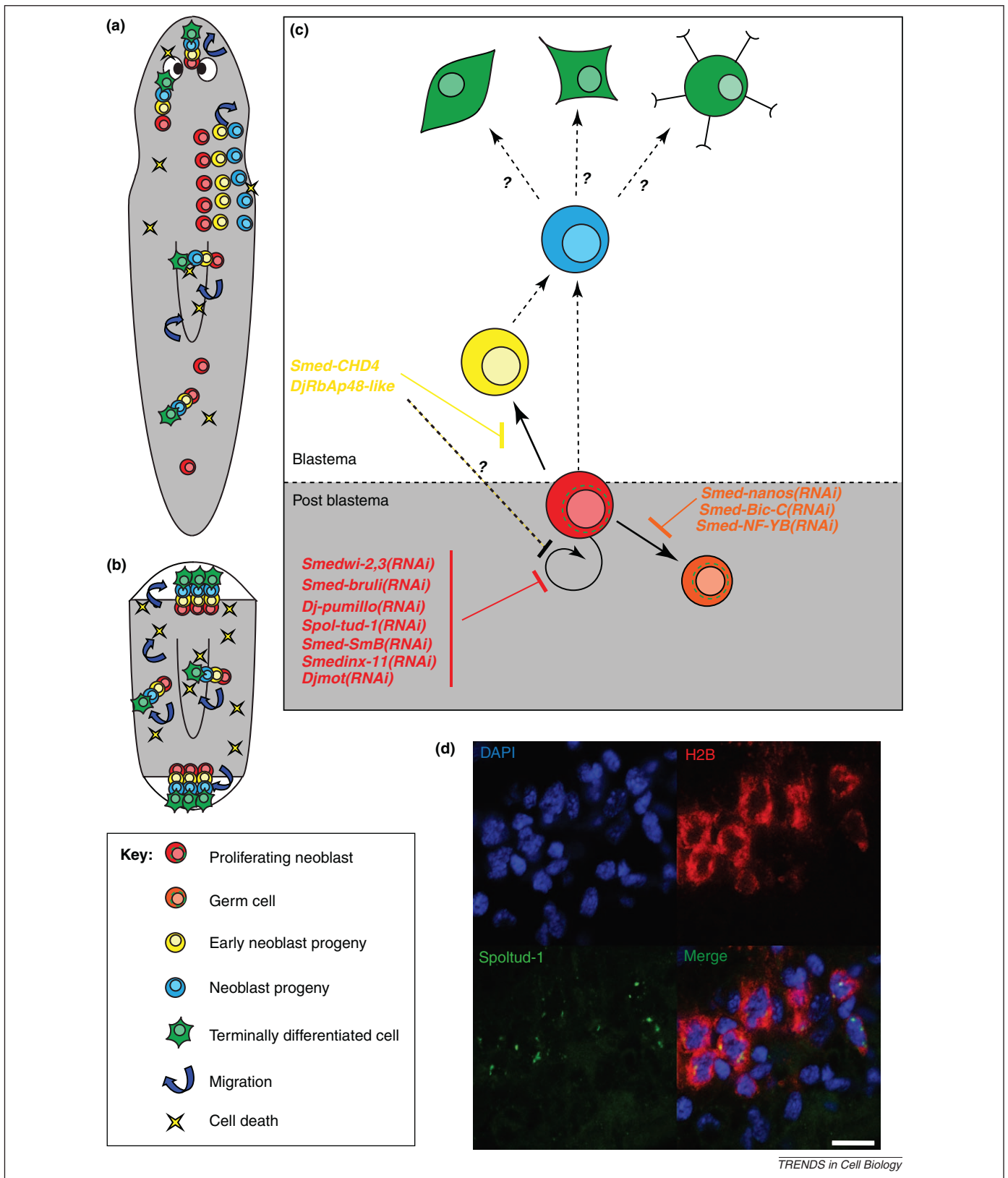
Understanding how the interplay and balance between stem cell maintenance and differentiation is controlled and how it can be manipulated is a key area of research for regenerative biology, and planarians provide an ideal system in which to study this. Markers that label the precursors of specific differentiated lineages are needed to understand how neoblasts replace the different specialized cell types present in planarians. For instance, *Smed-nanos* labels germline stem cells in planaria and is required for the formation of the germline [26]. Making definitive links between neoblast progeny and specific differentiated lineages will require live labeling techniques and will probably depend on the development of robust transgenic technologies. Even so, it is encouraging that what we already know about the molecular requirements for neoblast maintenance, pluripotency and differentiation depicts a remarkable molecular conservation with components required for these processes in mammals and other animals.

Neoblast maintenance and RNA metabolism: what happens in the chromatoid body (CB)?

Functional studies have revealed that many genes with roles in stem cells and germ cells in other animals have functions in neoblasts and early neoblast progeny [27–38]. Many of these genes are associated with RNA binding and metabolism and are localized in neoblast CBs or the CBs or germ plasm of other animals.

CBs are characteristic electron-dense perinuclear structures that resemble the germ granules associated with the germline lineages of animal embryos, with which they share conserved molecular constituents [37,39–41]. These structures are rich in conserved RNA–RNA-binding protein complexes and are major sites of cytoplasmic RNA metabolism [37,39]. Planarian CBs largely disappear during neoblast differentiation, except for in germline lineages where they seem to be maintained and in neural lineages where they presumably reappear after differentiation (see Figure 1d for planarian CB labeling in neoblasts) [32]. A growing body of evidence in planarians suggests CBs have a central role in regulating neoblast biology.

Members of the piwi/Argonaute gene family are expressed in mammalian germline CBs [42,43] and are required for the production of piRNAs. Three planarian orthologs, called *smedwi-1*, *-2* and *-3*, are all expressed in neoblasts [28,29]. The subcellular localization of *smedwi-2* transcripts shows perinuclear localization reminiscent of CBs [44]. Both *smedwi-2* and *-3* are required for regeneration and homeostasis [28,29], with RNAi-mediated knockdown causing a decrease in the production of piRNAs [28,45]



and a loss of neoblasts [28,29]. In addition, *smedwi-2(RNAi)* might result in the production of neoblast progeny that cannot differentiate properly [29]. piRNAs regulate transposon silencing and are involved in both post-transcriptional and epigenetic gene regulation; defects in either of these might explain the loss of neoblasts observed in *smedwi-2* and *-3(RNAi)* animals [46,47]. In this scenario, neoblast maintenance and differentiation could fail because of the mutagenic effect of activated transposons.

Although it is not directly established that planarian piwi/Argonaute homologs are present in planarian CBs, other proteins required for neoblast maintenance are present, including Smed-SmB, a planarian member of the LSm superfamily of RNA-binding proteins [48]. *Smed-SmB(RNAi)* leads to a drop in the number of CBs prior to the loss of neoblast proliferation and the loss of neoblast marker expression [36]. This suggests that components in the CB act to maintain neoblast maintenance and self-renewal. In support of this, Spoltud-1, an RNA-binding protein and tudor homolog from the species *Schmidtea polychroa*, is also expressed in CBs and is required for neoblast maintenance [32]. Significantly, *Spoltud-1(RNAi)* has a relatively long latency in its phenotype; impaired regeneration is caused by a loss of neoblasts at 7 weeks post-RNAi treatment and after three full rounds of regeneration. Recent data from both mice and flies have shown that tudor family proteins bind piwi family proteins at symmetrical dimethyl arginines and are required for normal levels of piRNAs and piwi-mediated transposon silencing [49]. One possible reason for the latency of the *Spoltud-1* phenotype is that it reflects a gradual decrease in the amounts of piRNAs [50]. This interaction is highly conserved across animals, and understanding its significance and the role of piRNAs in neoblast biology will be an important future direction for understanding germline and stem cell maintenance [47,49,50].

Two other RNA-binding proteins – DjPum, a pumilio homolog in planarian *Dugesia japonica* and Smed-bruno-like (bruli), a bruno/arrest homolog from *S. mediterranea* – are expressed in neoblasts and are required for neoblast maintenance [27,31]. The RNAi knockdown of these genes leads to a loss of neoblasts, their homologs are expressed within the germ plasm of other animals and in *Drosophila melanogaster* their homologs are essential for different stages of germline development [37,39]. This suggests that DjPum and bruli are likely to be planarian CB components.

In summary, the CBs of neoblasts contain many RNA-binding proteins associated with the formation of the germline in other animals. Together these data suggest that, although neoblasts are adult somatic stem cells, they might have similarities with germline stem cells. Work is now needed to look deeper at planarian CBs with respect to RNA metabolism and how different RNA-binding proteins interact to ensure the long-term stability of the neoblast cell population. Of particular importance will be relating CB RNA metabolism to the control of gene expression involved in stem cell maintenance, pluripotency and differentiation.

Data from planarians have been central to the suggestion that *vasa*, *piwi*, *tudor*, *pumilio*, *nanos* and *bruno* homologs are part of a germline multipotency program (GMP) conserved across animals [51]. This GMP is also active in some multipotent somatic cells, such as neoblasts and multipotent somatic cells in other animals. One exciting possibility is that the GMP mediates its effects through the control of genome-wide epigenetic signatures [51]. These, in turn, would regulate chromatin structure and gene expression to ensure stem cell and germline maintenance and pluripotency. The manipulation of genes comprising the GMP might provide an alternative to current methods for inducing pluripotency in mammalian cells.

Future work in planarian CBs will provide insight into how stem cell maintenance and pluripotency might be controlled in the context of regenerative therapies. However, maintaining the capacity for the self-renewal and pluripotency of stem cells is only half the story of planarian regeneration. Neoblasts must also produce progeny able to differentiate into the correct cell type at the correct position along the body axis.

Chromatin organizers are implicated in regulating early stages of neoblast differentiation

Perhaps surprisingly, little progress has been made in identifying the genes required for neoblasts to differentiate down particular lineages. As mentioned above, the regeneration and differentiation of germline stem cells from neoblasts requires *Smed-nanos* [26], and a microarray expression-based functional genomic screen utilizing the *Smed-nanos(RNAi)* phenotype has identified the genes required for the differentiation of germline stem cells [52]. But little is known about the cell autonomous processes required to initiate neoblast differentiation to form and maintain the somatic tissues of planarians.

Two recent studies have demonstrated that conserved epigenetic regulatory proteins – Smed-CHD4, the *S. mediterranea* Mi2/CHD4 ortholog, and DjRbAp48-like, a *D. japonica* RbAp48 ortholog [24,53] – have roles in mediating the early stages of neoblast differentiation. Both of these genes are components of the widely conserved nucleosome remodeling and histone deacetylase (NuRD) complex, which is required for the epigenetic control of cell fates in multiple tissues of many organisms [54]. In mammals, this complex is formed by at least seven polypeptides: HDAC1 and -2, RbAp46 and -48, MTA1/2, Mi-2 and MBD3. When the NuRD complex is recruited to target genes, the N terminal tails of histones H3 and H4 become deacetylated, leading to a more compact chromatin structure normally implicated in transcriptional repression. Through this impact on chromatin state, the NuRD complex contributes to the control of differentiation state in several organisms and developmental scenarios [55].

The planarian NuRD complex components seem to be required for proper regenerative blastema formation and for neoblasts to increase proliferation in response to wounding/amputation and feeding. In the case of *Smed-CHD4(RNAi)*, defects were observed in the production of

for neoblast maintenance are also associated with CBs. Cells are stained with *anti-Spoltud-1* antibody [32] labeling neoblasts in the planarian *S. polychroa* (image courtesy of Dr Jordi Solana). CBs are perinuclear structures (green) that are present in neoblasts cells (labeled in red by the neoblast marker *Histone-2B*).

early stem cell progeny from neoblasts [24,53], supporting a role in neoblast differentiation [21,24]. However, these animals also lost their neoblast population fairly rapidly; 20 days after RNAi treatment the animals had almost no proliferating cells or neoblast marker expression. These kinetics are similar to what is seen after the loss of genes required for neoblast maintenance [29,31], suggesting that although daughter progeny were not correctly produced by proliferating neoblasts, neoblast maintenance might also have been affected. The presence or loss of early and late neoblast progeny were not assessed in *DjRbAp48(RNAi)* animals, but the phenotypes of these animals are similar to *Smed-CHD4(RNAi)* [53].

The genome-wide epigenetic states of neoblasts are likely to affect maintenance, renewal and differentiation by impacting gene expression. Separating these roles will require identifying the components of epigenetic regulatory complexes that are specific to each process. For instance, RbAp48 and CHD4/Mi2 have broad molecular roles in many chromatin-modifying complexes, including Sin3, CAF-1 and dMec. All of these complexes affect cell proliferation and the maintenance of stem cell compartments [56,57]. It is possible that the effects of *Smed-CHD4* and *DjRbAp48* loss on proliferation, blastema formation and neoblast maintenance are the result of disrupting these alternate complexes, whereas their effects on differentiation are because of the loss of planarian NuRD complex activity. This distinction could be addressed by investigating a planarian homolog of the mammalian MBD3 protein, a unique component of the NuRD complex that has not been observed in other complexes.

The epigenetic states of neoblasts and neoblast progeny are likely to be highly dynamic and central to the differentiation process. Bringing to bear knowledge and tools for studying epigenetic modifications from other systems will be the first step in understanding this process in planarians. Early insights again highlight a promising level of molecular conservation between neoblast and mammalian stem cell differentiation. Ultimately, a detailed description of how the dynamics of epigenetic states change and control differentiation during planarian regeneration could aid in developing regenerative therapies. A key contribution from planarian research is likely to be in understanding how polarity signals in existing tissue might direct the epigenetic mechanisms controlling gene expression in neoblast progeny. It is these signals that coordinate the populations of neoblast progeny to regenerate functional missing tissues made up of several different differentiated cell types.

Directing stem cell differentiation in time and space to reestablish polarity, fate and function: old favorites in new packages

Perhaps the most rapid progress in the planarian model system has been made in understanding how the signals that pattern the positional identities of the planarian body plan are reestablished during regeneration. These signals direct neoblast progeny to replace distal missing tissues and remodel existing tissues to ensure the restoration and integration of all body systems. A detailed understanding of these processes provides a simple paradigm for how this

might be achieved in contexts where diseased, damaged or ageing tissues could potentially be repaired by stem cell-based therapies.

Conserved developmental pathways have been implicated in controlling regenerative polarity and the correct positional control of neoblast differentiation. For example, the BMP pathway is clearly implicated in correctly establishing the dorsoventral axis during regeneration, analogous to its role in the embryogenesis of other animals [58–60]. The patterning of the mediolateral axis and bilateral symmetry of the central nervous system also requires planarian homologs of molecules known to be involved in these processes during the embryonic development of other animals, such as *slit* [61], *robo* [62], *netrin* [63] and *Wnt-5* [64,65].

We know most about the formation of the A/P axis in planaria [66]. The Wnt signaling pathway, required across the metazoa for patterning the A/P axis during embryogenesis [67], plays a central role during both regeneration and homeostasis and is required for posterior regeneration and posterior fates [5,66,68]. Hedgehog (Hh) signaling is also required for the correct establishment of posterior identity by promoting the activity of Wnt signaling at posterior-facing blastemas [69,70]. In addition, experiments investigating the role of planarian gap junctions suggest that these structures might have a crucial and early role in proper A/P polarity at wound sites after amputation [71]. Although the gap junction component innexin-11 from *D. japonica* is required for stem cell maintenance [72], other innexins are required for A/P polarity [71]. Finally, a role for voltage-operated calcium influx in A/P polarity was uncovered in the human parasite *Schistosoma mansoni* [73]; however, little is known about how this relates to Wnt/Hh signaling or gap junction-mediated polarity.

A working model of A/P polarity establishment during regeneration can be proposed (Figure 2, and models presented in [65,66]); however, little is known about downstream events within neoblasts and neoblast progeny in response to polarity signals. Neoblast progeny in regenerative blastemas can express components of the Wnt and Hh signaling pathways but how this leads to the formation of anterior and posterior structures is not known [5,66]. Recently, a putative Hox cofactor, the TALE class homeobox *Smed-PREP*, was described as acting downstream of A/P Wnt signaling [74], and this provides a mechanistic connection between stem cell progeny and polarity signals. *Smed-prep* is expressed anteriorly and around the pharynx in whole animals and in anterior and posterior blastemas in neoblast progeny during regeneration. *Smed-prep(RNAi)* results in a loss of anterior fates, but without the adoption of posterior fate. Combinatorial RNAi with *Smed-βcatenin-1* indicates that *Smed-prep* activity is normally post-transcriptionally suppressed in the posterior blastema by Wnt signaling. The combinatorial RNAi of *Smed-prep* with *nou-darake* results in the ectopic expansion of brain ganglia reminiscent of the knockdown *nou-darake* alone [75], suggesting that *Smed-prep* is not required for neoblast progeny to differentiate into brain-fated neurons. Instead, *Smed-prep* might alleviate the suppressive influence mediated by *nou-darake*, allowing neoblast progeny within its anterior domain of expression to assume brain fates.

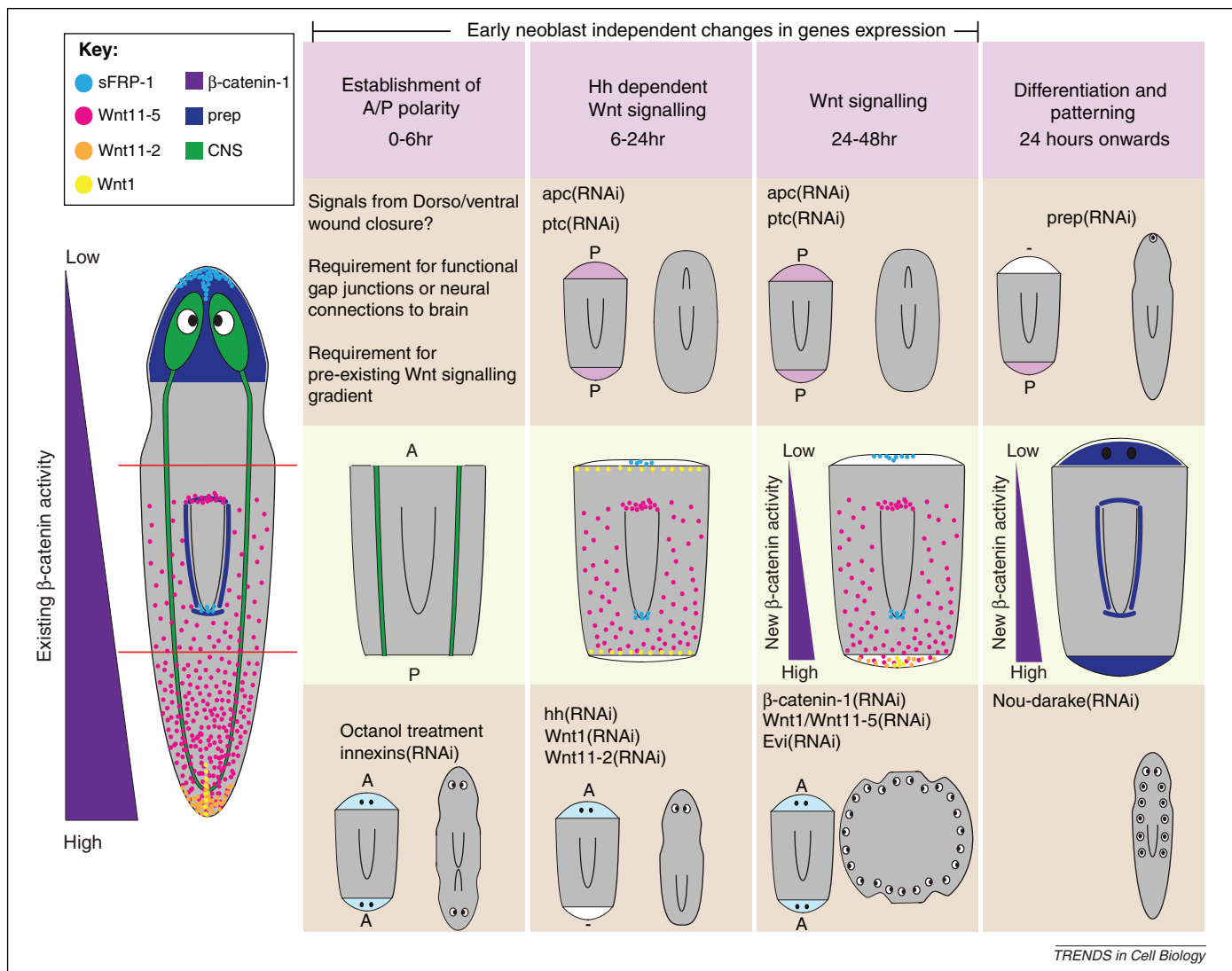


Figure 2. A summary of the events involved in early A/P polarity. The expression patterns of Wnt pathway genes suggest a gradient of Wnt signaling from the posterior to the anterior of intact animals, with some Wnts expressed in distinct posterior domains and sFRP molecules (Wnt antagonists) expressed in the anterior [65,70,78]. Early polarity after amputation is dependent on functioning gap junctions and/or the presence of brain tissue connected to the amputation site by intact ventral nerve cords [71]. In the absence of gap junction function, all cut sites regenerate brains (indicated by the presence of eyes). The RNAi phenotypes of Wnt and Hh pathway components indicate that Hh signaling acts to establish the expression of Wnt1 in the posterior after an early phase of wound-induced Wnt1 expression [69,70,79]. Later, Wnt11-5 expression is observed in the posterior, and is dependent on earlier Wnt1 expression [79]. The tailless phenotype observed in some RNAi phenotypes might be because of inefficient knockdown or might indicate differential roles in establishing polarity versus roles in elaborating posterior fate. Finally, we know that *Smed-prep* and *nou-darake* are both required for correct brain regeneration, with *Smed-prep(RNAi)* [74] leading to a loss of anterior structures and *nou-darake(RNAi)* [75] resulting in an expansion of the brain into more posterior regions of the body. Nomenclature for the Wnt pathway is that introduced by recent phylogenetic work [65].

These data provide the first clear connection between Wnt signals, putative fibroblast growth factor signals and the control of neoblast progeny differentiation to form functionally integrated organs. Future work will focus on mechanistic details surrounding neoblast differentiation and how neoblast progeny can interpret polarity and patterning cues to differentiate into the correct structures. Because early changes in expression patterns are also observed in irradiated animals, it seems that initial polarity and fate signals come from existing differentiated tissues and are neoblast-independent [65]. Whether these polarity cues are specific for tissues in animals with regenerative capacities is an important question. Do differentiated mammalian tissues contain polarity cues capable of correctly coordinating stem cell differentiation down alternate lineages? If not, can these signals be put in place? These questions require that we first have a detailed

knowledge of the possible cellular and molecular sources of polarity signals and how they coordinate stem cell differentiation in a regenerative scenario.

Concluding remarks

Considerable progress has been made in understanding the biology of the previously enigmatic neoblast stem cells, their progeny and the fundamentals of the regenerative process. We now have several clues about how early events in neoblast differentiation and maintenance are controlled (Figure 1) as well as a working model of how axial polarity and fate might be determined during regeneration (Figure 2). Nonetheless, detailed mechanistic descriptions are still missing. These will come to light as further transcriptomic and functional screens are performed [52,76] and combined with detailed biochemical analyses and the development of misexpression approaches.

One emerging theme from planarian research is that conserved genes and pathways direct neoblasts through the regenerative life history, suggesting that regeneration, even in naturally nonregenerative scenarios, will require the correct redeployment of well-known genetic networks. One caveat is that most research has focused on conserved genes and networks, and possible roles for novel flatworm genes have yet to be widely assessed. Hindsight suggests that these simple worms still offer a wealth of knowledge.

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