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Emerging patterns in planarian regeneration

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Summary

In the last decade, the planarian has become an increasingly tractable invertebrate model for the investigation of regeneration and stem cell biology. Application of a variety of techniques and development of genomic reagents in this system have enabled exploration of the molecular mechanisms by which pluripotent somatic stem cells called neoblasts replenish, repair, and regenerate planarian tissues and organs. Recent investigations have implicated evolutionarily conserved signaling pathways in the re-establishment of anterior-posterior, dorsal-ventral, and medial-lateral polarity after injury. These studies have significantly advanced our understanding of early events during planarian regeneration, and have raised new questions about the mechanisms of stem cell-based tissue repair and renewal.

Introduction

Freshwater planarians are non-parasitic flatworms (phylum Platyhelminthes). They are able to repair and replace tissue that is lost or damaged after injury, as part of normal cellular turnover, or during growth and degrowth in response to changing nutritional availability [1–4]. These impressive restorative abilities are conferred, in part, by a population of pluripotent somatic stem cells, called neoblasts, that are distributed throughout the mesenchyme of the planarian body (Fig. 1A,B), and that differentiate into tissues and organs lost to injury. The molecular mechanisms underlying planarian regeneration and neoblast dynamics have until recently been poorly understood. However, the application of molecular and functional genomics technologies has proceeded rapidly in planarians (Box 1), allowing increasingly systematic analyses of a growing array of developmental [1,4–10] and physiological processes [11,12], as well as other problems [13,14].

In order for injured planarians to regenerate in response to almost any amputation [3], robust mechanisms must exist to re-establish anterior-posterior (A–P), dorsal-ventral (D–V), and medial-lateral (M-L) axes after wounding. In the last two years, a number of investigations have revealed roles for evolutionarily conserved signaling pathways in these critical early events. Here, we survey these studies and discuss some of the unresolved questions that have emerged.

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Recent technical developments have made planarians experimentally tractable

Planarians possess a rich history as an experimental subject, with rigorous studies dating back to the late 1800s [1–3,7]. Starting in the 1990s, the application of molecular cloning, immunohistochemistry, and other modern techniques initiated the re-examination of long-standing questions derived from nearly a century of surgical, physiological, histological, and ultrastructural investigations [1,10,15,16].

Experimental tractability of planarians has vastly increased in the past ten years due to a number of achievements. The selection of a European species, *Schmidtea mediterranea* [17], as a model planarian was made because of its small genome, diploid complement of chromosomes (four), and extant asexual and sexual strains [1]. Transcriptome characterization has been fruitful in *S. mediterranea* as well as the Japanese species *Dugesia japonica*: ~78,000 *S. mediterranea* expressed sequence tags (ESTs) and ~7500 *D. japonica* ESTs have been deposited in NCBI, the products of both published and unpublished efforts [18–20]. *S. mediterranea* is also the first planarian species to have its genome sequenced (Washington University Genome Sequencing Center, St. Louis, MO); annotated genomic, transcriptomic, and phenotypic data are available in a publicly accessible database [21,22]. Furthermore, gene expression can be assayed *in situ* [18,20, 23,24], neoblasts can be labeled, isolated, and analyzed using a variety of methods [25–29], and assessment of gene function by RNA interference can be accomplished by injecting, soaking, or feeding [30–33]. These developments represent a powerful toolkit that has enabled the identification and functional analysis of genes that regulate axial patterning and neoblast dynamics during regeneration.

Planarians maintain and regenerate anterior-posterior polarity

As the only mitotically active somatic cells, neoblasts proliferate at sites of injury to generate the blastema, a mass of cells that differentiates into the tissues and organs lost by amputation [1]. In order for these newly generated cells to be patterned correctly, mechanisms must exist to reset polar axes after wounding; for example, if a tail is amputated, the positional identity of “posterior-most” must be re-established in order for a new tail to regenerate properly. This fact was appreciated by T. H. Morgan, whose hypotheses regarding morphogenetic gradients and polarity were formulated in part to explain the appearance of two-headed planarian regenerates: extremely thin transverse fragments would sometimes re-grow heads at both cephalic and caudal amputation sites [1,3,34–37] (Fig. 2A).

In metazoans, Wnt proteins signal through their receptors via β-catenin-dependent (“canonical”) and -independent (“non-canonical”) pathways to regulate embryonic A–P axis formation as well as a variety of other processes [38–41]. Recently, three groups reported that in planarians, RNAi-mediated knockdown of *Smed-β-catenin-1* causes the regeneration of a “posterior head” after tail amputation, replicating the two-headed phenotype that Morgan observed [34,35,42] (Fig. 2A). These animals regenerate posterior cephalic ganglia (the brain) and eyes [34,35,42], and even intestinal branches regenerate to form a single primary branch, characteristic of anterior, not posterior, patterning [34,42] (Fig. 2B). Ectopic anterior structures also appear in the uninjured regions of regenerating tail pieces [34] (Fig. 2C), in response to small lateral incisions [35] (Fig. 2D), or even in the lateral flanks of uninjured animals [34, 35,42], indicating a role for β-catenin not only during regeneration, but also during normal homeostatic tissue maintenance. Furthermore, Gurley et al. [34] indirectly demonstrated that upregulation of β-*catenin* may be sufficient to promote posterior identity, since knockdown of *Smed-APC-1* (Adenomatous Polyposis Coli, a β-catenin inhibitor) results in a reciprocal phenotype: the regeneration of posterior tissues after head amputation (an “anterior tail”).

Candidate upstream regulators of β-catenin were identified by Petersen and Reddien [35], who reported that the expression of several *Wnt* genes is re-established in small clusters of posterior cells within 4–5 days after amputation. In a subsequent study, Adell and colleagues reported that *Smed-wntP-1*, *Smed-wnt11-2*, and *Smed-evi/wntless* (necessary for Wnt secretion) are required to varying degrees for proper specification of posterior fate [43]. Additionally, in both *D. japonica* and *S. mediterranea*, knockdown of *wntA* results in posterior expansion of the brain [43,44].

Wnt signaling pathways have been implicated during early stages of limb and tail regeneration in zebrafish, *Xenopus*, and axolotl [45–48], during which their disruption inhibits the initiation of regeneration, blastema growth, and proliferation of progenitor cells. By contrast, in planarians blastema formation and growth appear to be normal in *wnt* and β-*catenin* knockdown animals, suggesting that the primary role of these genes during planarian regeneration is to regulate repatterning [34,35,42,43,49]. Taken together, the evidence suggests that Wnt signaling (through β-catenin-dependent mechanisms) promotes posterior specification and/or suppresses anterior identity in both intact and injured planarians. Since Wnt/β-catenin-mediated regulation of A–P axis formation has been studied primarily in the context of embryogenesis, observations in planarians (and other regenerating invertebrates such as *Hydra*) support suggestions that such a role is ancient and broadly conserved [34,35,42,43, 49–53].

Questions pertaining to the role of Wnt/β-catenin signaling in planarians remain. For example, both β-*catenin* and *APC* are transcribed rather ubiquitously [34,35,42] and are upregulated in both anterior and posterior blastemas within 24 hours after amputation [34]. Since activation of these genes might occur as a nonspecific response to injury, assessment of the spatial and temporal regulation of APC, Wnt, and β-catenin proteins (for example, in which cells β-catenin translocates to the nucleus) ultimately will be needed to understand the mechanistic details of A–P axis re-establishment [50]. Additionally, other factors may regulate A–P polarity. For example, treatment with a pharmacological inhibitor of gap junctions can, like β-*catenin* knockdown, cause regeneration of posterior heads [54]. Likewise, a number of planarian Hox genes are expressed differentially along the A–P axis, although functional roles for these genes have not yet been reported [55–57]. Thus, although β-*catenin* is required to confer posterior identity, multiple pathways may interact to regulate A–P axis formation and patterning [34].

Bone Morphogenetic Protein regulates dorsal-ventral patterning

Dorsal-ventral (D–V) patterning is also important for the organization of the planarian body plan. Cephalic ganglia, nerve cords, the majority of ciliated epithelial cells, and the mouth opening are ventral, whereas eyes, testes, as well as specific populations of pigment and secretory cells are located in stereotypical positions along the D–V axis.

Bone morphogenetic protein (BMP) signaling regulates D–V polarity in metazoans, promoting ventralization in vertebrates, and dorsal identity in invertebrates [58]. In planarians, the first *bmp* homolog was isolated from *D. japonica*, where its expression in a dorsal stripe of midline cells suggested a potential role in D–V or midline patterning [59]. Three recent studies conducted in *D. japonica* [60] and *S. mediterranea* [61,62] assessed the roles of several BMP pathway genes. These included a *bmp2/4/decapentaplegic* homolog [60–62]; *Smed-smad1 and Smed-smad4-1*, members of a family of transcription factors that transduce BMP and Transforming Growth Factor β (TGF-β) receptor signaling [61,62]; and *smedolloid-1*, the *S. mediterranea* homolog of *tolloid*, an extracellular metalloprotease that potentiates BMP signaling by inactivating Chordin/Short Gastrulation [61]. These groups reported ventralization phenotypes such as the disappearance of dorsal markers accompanied by the ectopic dorsal expression of ventral markers [61,62], dorsal duplication of cephalic ganglia

and ventral nerve cords [60–62], as well as the duplication of lateral body margins (the "D–V boundary") [60,62] (Fig. 3A). Perhaps most dramatic was the propensity of animals to swim on their (formerly) dorsal surfaces, accompanied by a nearly complete conversion of the dorsal epithelium to a ventral-like, ciliated epidermis [61]. As with disruption of A–P patterning (above), many phenotypes were observed in both regenerating and uninjured animals, revealing a role for this signaling pathway in the maintenance of D–V patterning during normal tissue homeostasis.

Other phenotypes suggest that BMP signaling may play roles beyond the regulation of D–V patterning. A role in medial-lateral (M-L) patterning is supported by the fact that normally, *bmp* expression shifts toward a lateral plane of injury, suggesting an early role in resetting the position of the midline [59,61]. Consistent with this idea, *bmp*, *smad*, and *smedolloid* knockdown animals fail to re-express lateral body margin markers after longitudinal amputations [61], and lateral blastemas in these animals are severely reduced or absent [61, 62]. Furthermore, in animals that are cut transversely (i.e. in which heads or tails are amputated), disruption of BMP signaling causes the production of indented anterior and posterior blastemas [61,62]; in these misshapen blastemas, visual axon projections across the midline are often absent or otherwise aberrant [61]. Even more dramatically, in long-term *bmp* and *smad* RNAi experiments in uninjured animals, photoreceptors and their projections to the brain are often inappropriately duplicated on either side of the midline [60–62]. Consistent with these phenotypes, BMP signaling has been shown to regulate midline patterning during development in other organisms, for example in the mouse telencephalon [63], in the zebra fish heart and viscera [64], and during axon guidance [65].

Although indented anterior/posterior blastemas and reduced/absent lateral blastemas may be the output of disrupted early midline repatterning [61,62], these phenotypes also suggest that BMP signaling might regulate neoblast proliferation. *Smed-smad4-1* knockdown does not affect neoblast proliferation 24 hours after transverse amputation [32]; the effects of *Smed-bmp4-1* knockdown on early proliferation have not been reported [32,61]. However, knockdown of either of two planarian *msx* (muscle segment homeobox) transcription factors, *Djmsh1 or Djmsh2*, delays the formation of the head blastema and interferes with the normal dynamics of neoblast proliferation after injury [66]. Vertebrate MSX proteins are often coexpressed with BMPs during development and regeneration, mediating some of their activities [67–70]. Furthermore, recent evidence suggests that BMPs may function mitogenically during vertebrate limb, tail, fin, and retina regeneration [69,71,72]. Thus, the possibility that BMP signaling regulates proliferation in planarians warrants further investigation.

An alternative explanation for some phenotypes is that they occur secondarily to D–V axis disruption [61,62]. For example, Molina et al. [62] have proposed that the inappropriate differentiation of cells with ventral identity in dorsal regions may lead to ectopic D–V interactions, causing some phenotypes such as dorsal outgrowths and abnormal blastema formation. This explanation is consistent with studies in which experimental juxtaposition of dorsal and ventral tissues induces proliferation, blastema formation, and secondary axes [3, 73–75]. Further complicating interpretation, variability among *bmp*, *smad*, and *smedolloid* phenotypes was reported. This may reflect the overlapping and non-overlapping roles of BMP pathway components [60–62], some of which (for example, members of the *noggin* family of BMP pathway inhibitors) have been identified only recently [76]. Given that cross-talk between signaling systems regulating A–P, D–V, and M-L polarity is likely [48,50,58,77,78], further analysis will be required to understand the multiple roles BMP signaling may play during regeneration.

On an interesting side note, Reddien and colleagues also reported that in thin lateral regenerates, *bmp*-expressing cells appear even in irradiated animals [61]. Stem cell-independent induction of gene expression has also been reported for a *noggin-like* gene in *D. japonica* [79]. Additionally, irradiation of either host or donor tissue does not abrogate the production of ectopic outgrowths from D–V reversed transplants [75]. These results all suggest that differentiated cells are capable of initiating or altering the expression of positional cues without input from the neoblasts, a capability that may allow rapid repatterning in response to injury [61].

Medial-lateral patterning and the central nervous system

Some planarian organs, most notably the central nervous system (CNS) and the gonads, are duplicated laterally on either side of the midline. However, the mechanisms by which bilateral symmetry is maintained and regenerated are poorly understood. In the cephalic ganglia of *D. japonica*, homologs of *orthodenticle* and *orthopedia* homeobox-containing genes are expressed in non-overlapping medial-lateral (M-L) domains, although the functional significance of these expression domains is unknown [23,80].

Cebrià and colleagues recently identified two planarian genes whose function is critical for proper patterning and regeneration at the midline [81,82]. Slit is a secreted extracellular ligand for the Roundabout (Robo) family of receptors, functioning in vertebrates and invertebrates as a midline repulsive cue for axons and dendrites [83]. Knockdown of *Smed-slit* causes regeneration of cephalic ganglia and photoreceptors that are collapsed at the midline, as well as inappropriate fusion of posterior intestinal branches (Fig. 3B) [81]. Ectopic neural tissue also appears over time at the midline in uninjured *Smed-slit(RNAi)* animals, suggesting a role for Slit in the normal homeostatic maintenance of CNS organization [81]. *Smed-slit* is expressed in both dorsal and ventral midline cells, whereas *roboA* is expressed in the brain, ventral cords, and early in the brain "primordia," cells within the blastema thought to be among the first neurons to differentiate after amputation [5,6,81,82]. Thus, the regeneration of collapsed neural tissue (and the appearance of ectopic midline neuronal tissue in intact animals) may reflect the inappropriate migration or differentiation of neurons that normally respond to the midline as a repulsive boundary. Intriguingly, however, *Smed-roboA* knockdown results in the growth of supernumerary pharynges with reversed A–P orientation, dorsal cephalic outgrowths, and a reduced (or absent) anterior commissure connecting the two cephalic ganglia (Fig. 3C) [82]. This production of ectopic tissue (pharynges and cephalic outgrowths) in *roboA (RNAi)* animals correlates with the failure of the regenerated cephalic ganglia to re-establish proper connectivity with the pre-existing ventral cords [82]. In other regenerating organisms, deviation of nervous tissue can cause the growth of ectopic structures, and in urodele amphibians, regeneration is dependent on the nervous system [5,82,84]. *Smed-roboA(RNAi)* phenotypes support the possibility of a similar role for the planarian CNS in the general coordination of patterning [5,82]. The identification of additional ligands and/or receptors will be required in order to understand why *slit* and *roboA* phenotypes are not identical, and why *roboA* knockdown causes reduction of the anterior commissure.

How are neoblasts instructed by patterning cues?

Roles for Wnt/β-catenin, BMP, and Slit-Robo signaling in A–P, D–V, and M-L patterning, respectively, have been established by the studies outlined above. When axial patterning or repatterning is disrupted, the most common result is production of ectopic differentiated tissues -- anteriorized central nervous and digestive systems (*wnt*/β-*catenin* knockdown), dorsal neurons and ectopic photoreceptors (BMP pathway disruption), and supernumerary pharynges and dorsal outgrowths (*roboA(RNAi)*). These phenotypes imply that neoblasts are instructed to differentiate inappropriately, raising a number of questions. How do neoblasts integrate

positional information? Do they respond directly to positional cues, for example to a gradient of secreted Wnt and/or BMP proteins? Or do the neoblasts respond indirectly to the axial identity of differentiated tissues, for example to signals secreted by severed nerve cords? In an uninjured animal, is neoblast fate restricted regionally? That is, do neoblasts themselves have positional identity, or do extracellular cues act only during or after lineage restriction? Many of the genes involved in re-establishment of polarity in planarians (β-*catenin*, *APC*, *bmp2/4*, *slit*, *roboA*) are upregulated in blastemas [34,35,42,43,62,81,82], in which early markers of neoblast differentiation are also expressed [85]. At least one of the 10 planarian Wnt receptor genes (*Smed-frizzled-4*) is expressed in the blastema [34]; the identity and expression pattern (s) of BMP/TGFβ receptors has not been reported. Identification of cells that co-express these genes may provide clues to the genetic programs that incorporate positional information with the control of neoblast dynamics.

Upregulation of patterning genes in the blastema also suggests that upstream components of these signaling pathways, that is, those that link wound healing with blastema growth and patterning, remain to be identified [3]. In planarians, knockdown of *Djmsh1* or *Djmsh2* (mentioned above) causes an overall reduction in the level of *Djbmp* mRNA [66], although a specific role in the blastema has not been demonstrated. Similarly, in fetal mouse digits, both *Msx1* and *Msx2* homeobox transcription factors are required for *Bmp4* expression [70]. Also, transgenic expression of *noggin* in the regenerating *Xenopus* tadpole tail prevents upregulation of *wnt3a* and *wnt5a*, suggesting the possibility that BMP signaling may regulate Wnt expression [48]. In planarians, the drastically different phenotypes in *wnt(RNAi)* and *bmp (RNAi)* regenerates would seem to argue against such a hierarchy, but the regulatory relationships between these pathways have not yet been investigated.

Conclusions

The potential to inform our understanding of both stem cell and cancer biology has led to renewed interest in the molecular basis of regeneration in a variety of organisms [1,3,4,13, 84,86–90]. Recent studies have revealed that planarians maintain and regenerate polar axes by employing evolutionarily conserved patterning mechanisms. Deeper understanding of the utilization of positional information in regenerating organisms may provide additional insights into the functions of secreted factors in adult stem cell niches [91,92], and may inform efforts to direct the differentiation of embryonic or induced pluripotent stem cells *in vitro*. In addition to determining the extent to which genetic programs of regeneration recapitulate those of metazoan embryogenesis [45,67,93], a second major goal is the identification of novel regulators [87]. Recently, larger scale approaches have identified hundreds of planarian genes that are expressed in dividing and differentiating neoblasts [85,94], and that are required for blastema growth, proliferation, and patterning during planarian regeneration [32]. Although many questions remain, future investigations of this intriguing invertebrate will continue to provide new perspectives on the initiation and maintenance of polarity, and the coordination of somatic stem cells by positional cues in both regenerating and fully developed tissues.

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in situ hybridization; not all genes are coexpressed, indicating heterogeneity of the stem cell population.

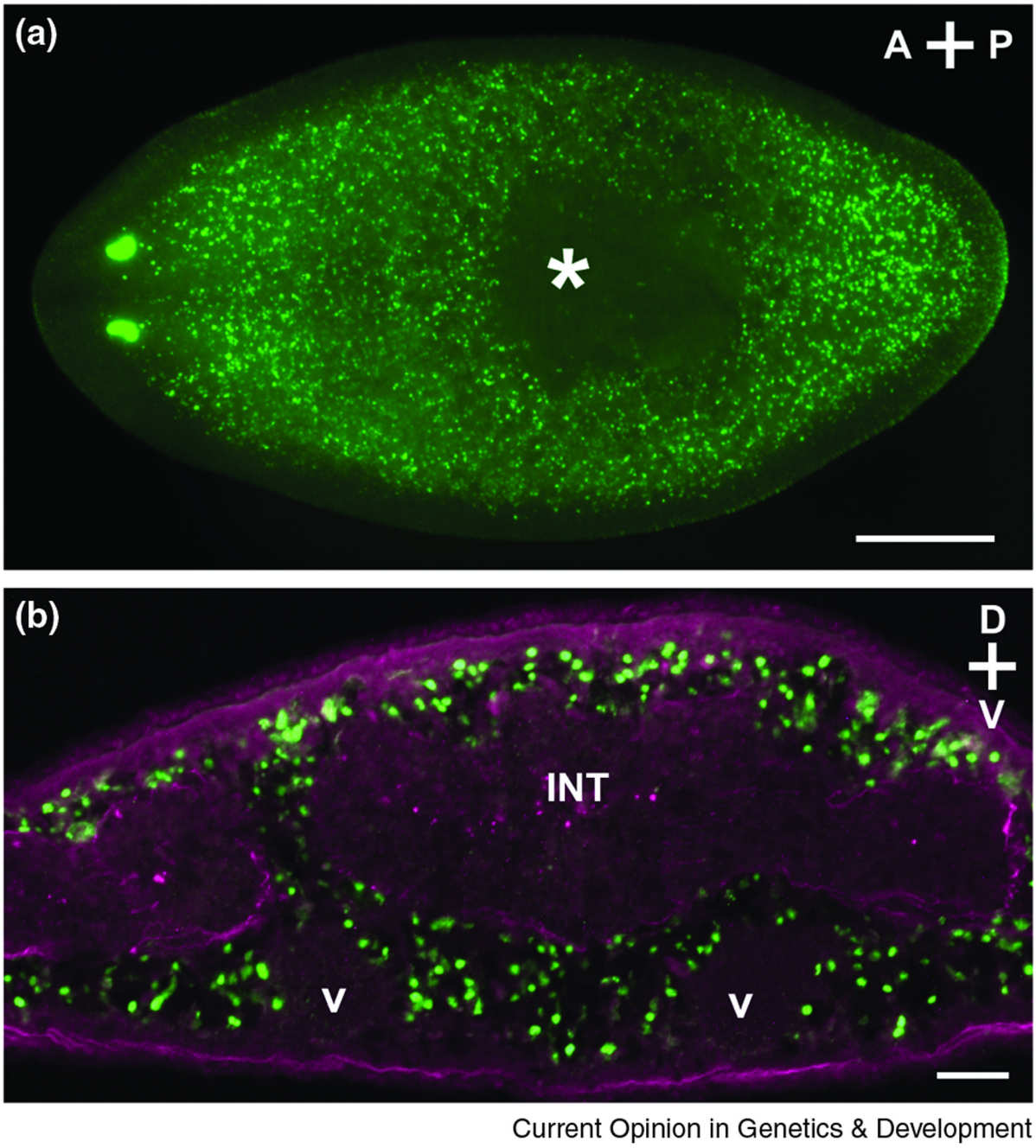

Figure 1. Planarian neoblasts

(A) Distribution of neoblasts in an intact animal 24h after BrdU incorporation (green). S-phase cells do not reside anterior to the photoreceptors or within the pharynx (asterisk), and fragments amputated from these regions do not regenerate [1,25]. Dorsal view; anterior is to the left. Scale bar, 0.5 mm. (B) In cross section (anterior to the pharynx), neoblasts (green) are distributed mesenchymally around differentiated tissues such as the intestine (INT) and ventral nerve cords (V). Enteric and outer body wall muscles are labeled in magenta. Scale bar, 0.05 mm.

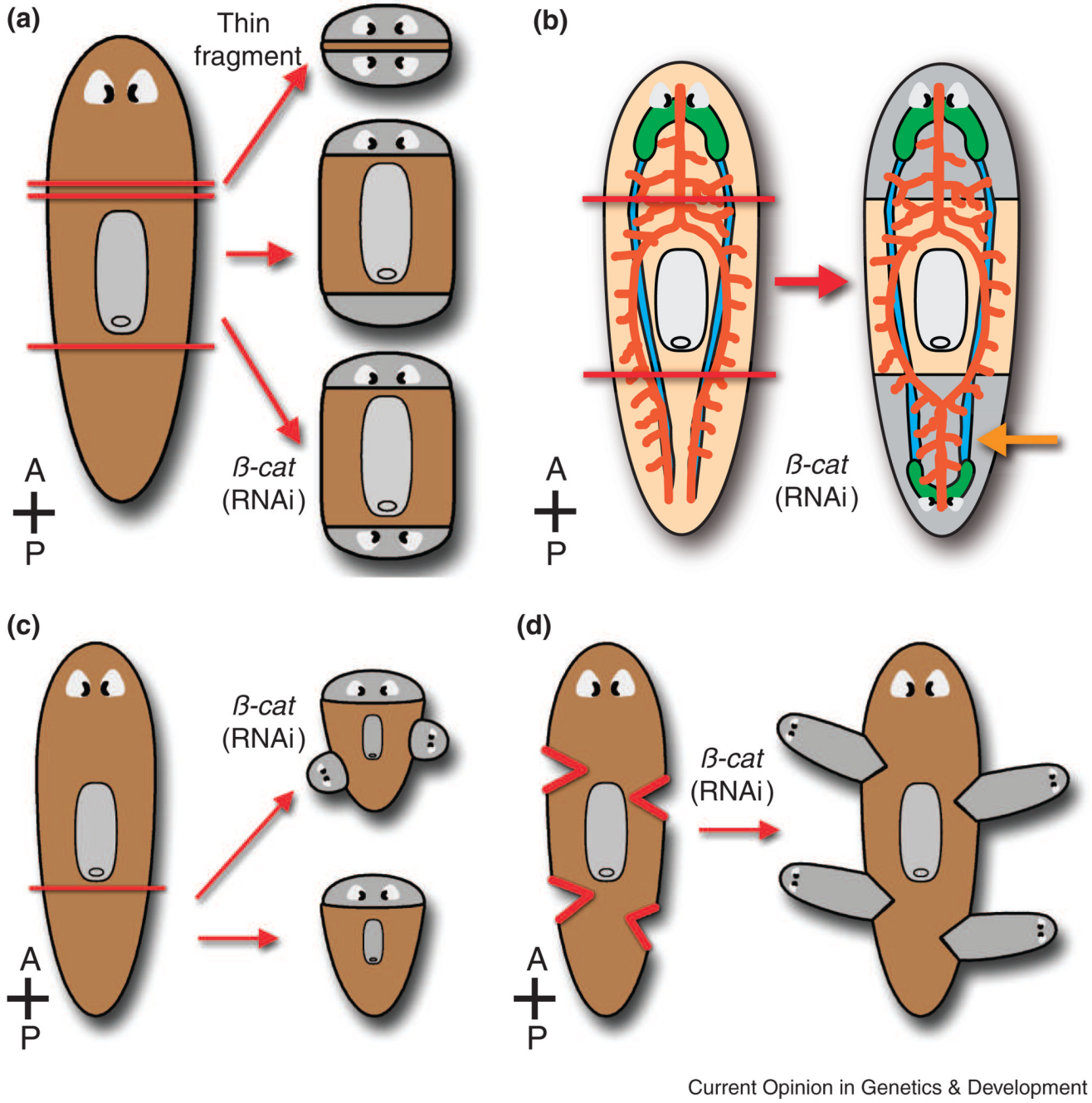

Figure 2. Patterning defects caused by β-*catenin* RNAi

(A) A transverse slice from a planarian normally regenerates an anterior head and a posterior tail (middle right). Morgan observed that a very thin transverse slice (top right) sometimes regenerates a head both anteriorly and posteriorly. Knockdown of β-*catenin* and other Wnt pathway genes also results in regeneration of a posterior head in trunk fragments (bottom right). (B) In β-*catenin(RNAi)* trunk regenerates, cephalic ganglia (green) and eyes are duplicated posteriorly, while intestinal morphology (orange) is anteriorized (orange arrow). (C) and (D) β-*catenin* knockdown also causes growth of ectopic heads along the flanks of regenerating tail pieces (C) or at lateral incisions (D). Gray, regenerated tissue (A–D). Red lines = amputation sites (A–D).

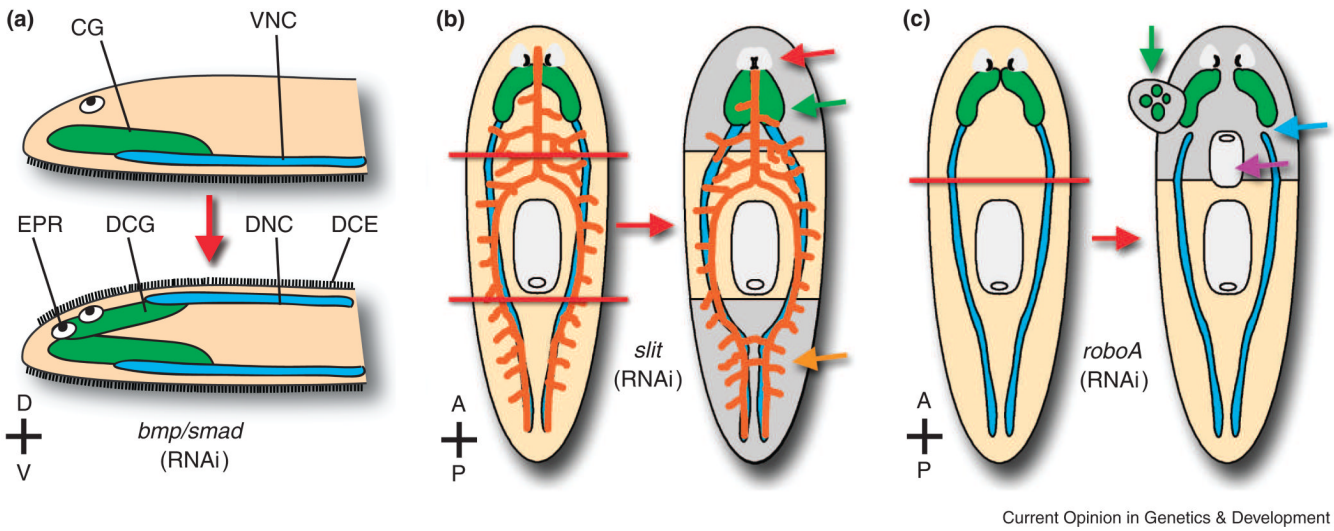

Figure 3. Patterning defects resulting from knockdown of BMP pathway, *slit*, and *robo* genes

(A) When BMP signaling is disrupted by RNAi, normally ventral tissues develop/regenerate dorsally. EPR, ectopic photoreceptor. CG, cephalic ganglia (green). DCG, dorsal cephalic ganglia. VNC, ventral nerve cord (blue). DNC, dorsal nerve cord. DCE, dorsal ciliated epithelial cells. (B) In trunk pieces (i.e. after amputation of both head and tail), *Smed-slit* knockdown results in the regeneration of cephalic neural tissue that is collapsed at the midline (green arrow), fused photoreceptors (red arrow), and posterior collapse of ventral cords (blue) and intestinal branches (orange and orange arrow). (C) After head amputation, *Smed-robo* knockdown results in regeneration of pharynges with reversed A–P polarity (purple arrow) and ectopic dorsal cephalic outgrowths (green arrow), correlated with lack of VNC/CG connectivity (blue arrow). Cephalic ganglia are also displaced laterally. Gray, regenerated tissue (B–C). Red lines = amputation sites (B–C).