Advanced Review

The history and enduring contributions of planarians to the study of animal regeneration

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Having an almost unlimited capacity to regenerate tissues lost to age and injury, planarians have long fascinated naturalists. In the Western hemisphere alone, their documented history spans more than 200 years. Planarians were described in the early 19th century as being 'immortal under the edge of the knife', and initial investigation of these remarkable animals was significantly influenced by studies of regeneration in other organisms and from the flourishing field of experimental embryology in the late 19th and early 20th centuries. This review strives to place the study of planarian regeneration into a broader historical context by focusing on the significance and evolution of knowledge in this field. It also synthesizes our current molecular understanding of the mechanisms of planarian regeneration uncovered since this animal's relatively recent entrance into the molecular-genetic age. © 2012 Wiley Periodicals, Inc.

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PLANARIANS AND THEIR HISTORICAL CONTEXT

The study of regeneration has a rich, intertwined history with experimental embryology. In the 17th century, naturalists contemplated two ancient paradigms for thinking about embryology: preformationism versus epigenesis. Preformationism contended that animals were already formed in miniature at the time of conception and simply expanded in size over the course of development. In contrast, epigenesis stated that animals were built piece by piece during development, guided by some intrinsic information housed in the undifferentiated embryonic cells. The rediscovery of animal regeneration at the end of the 1600s cast serious doubt upon the validity of preformationism. As naturalists began gathering more insights into both regeneration and embryonic development, epigenesis eventually took center stage as one of the most important principles of biology.[1]

The earliest known description of animal regeneration came from Aristotle around 350 BCE. Among other things, he described that the tails of lizards regenerate.[2] In 1686, Thévenot, Perrault, and Duverney revived this finding.[3] This rediscovery of regeneration created a wave of excitement, and 18th century naturalists began experimenting on any animals they could find to determine if this was a common phenomenon across the tree of life. de Réaumur showed that arthropods could lose appendages such as limbs and subsequently regenerate them.[4] Trembley systematically demonstrated that hydra, a member of the cnidarian phylum, regenerates after transection.[5] Bonnet proved that annelid worms regenerate,[6] and Spallanzani described the regenerative abilities in a variety of invertebrates such as snails and vertebrates like salamanders and frog tadpoles.[7]

The birth of the study of planarians is most frequently associated with Pallas, who encountered them while exploring the Ural mountains in the late 18th century. There, he observed that these animals regenerate missing body parts after fissioning.[8] However, other early reports of planarians exist. Trembley described feeding pieces of planarians to hydra in

Additional Supporting Information may be found in the online version of this article.

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his monograph in 1774.[5] Müller described a number of planarian species in 1773, but erroneously grouped them with the trematode genus *Fasciola*.[9] Woodcut prints of land planarians can be found in Japanese encyclopedias dating as far back as the 17th century[10,11] (Figure 1), while written descriptions of the animals long precede that.[12,13] In fact, the oldest known reference to planarians comes from the Chinese text *Yu-Yang Tsa-Tsu* written around 860 AD by T'uan. He describes the animal 'T'u-K'u' (likely the land planarian *Bipalium*), and hints at its regenerative abilities by saying that it can 'easily separate into several pieces' when touched.[12–14]

Over the course of the 19th century, more than a dozen different European[15–25] and American[26–33] biologists—including Darwin himself—continued to study these animals and demonstrated that the robustness of regeneration was common across planarian species. Indeed, in Dalyell's eloquent words, planarians appear to be 'almost immortal under the edge of the knife,' making them tantalizing animals for study.[16]

Work continued on planarians for the rest of the 20th century, as it did in other animal models of regeneration.[34] However, in all fields, progress toward a mechanistic understanding of regeneration was hampered by an incomplete understanding of basic cell biology and genetics, in addition to a lack of tools for experimentation. Only recently have significant advances in molecular biology, genetics, and sequencing technologies reignited interest in planarians and other regenerative organisms. Today we are poised to tease apart the molecular mechanisms of regeneration, and unlock the mysteries of this biological phenomenon that have fascinated so many for so long.

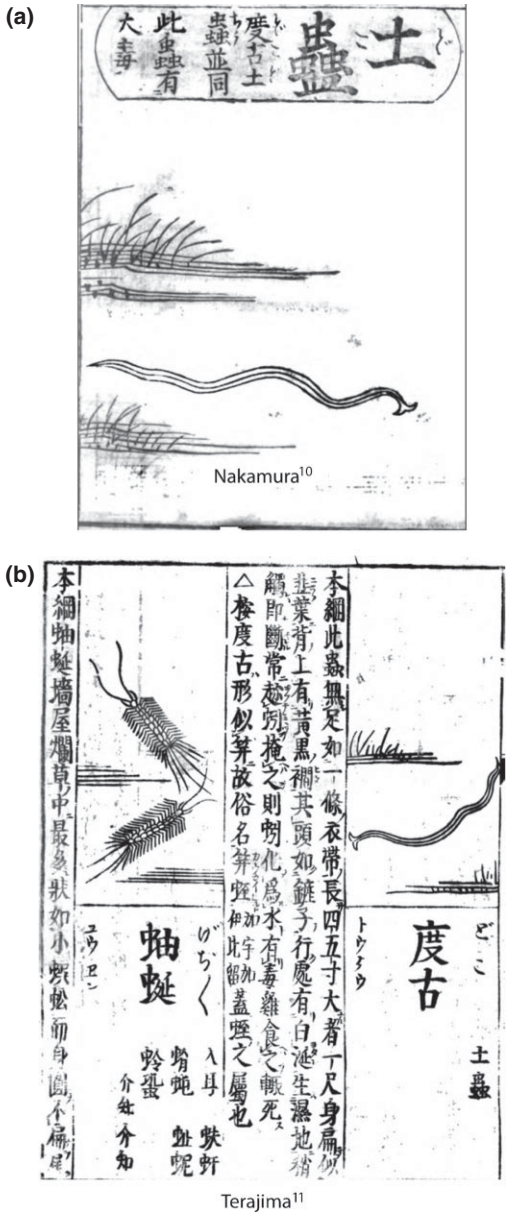

FIGURE 1 | Ancient Japanese texts describe planarians and their ability to fission. (a) Wood-print reprinted from the Japanese encyclopedia *Kinmô-Zui* by Nakamura, 1666 (Reprinted from Ref 10). Image depicts a striped land planarian (likely *Bipalium*). The caption indicates that 'it is very poisonous and similar to another soil insect (nematode) previously described'. (Translation assistance provided by Dr. Tamaki Suganuma, Nobuo Ueda, and Shigeki Watanabe.) (b) Wood-print reprinted from the illustrated Japanese encyclopedia *Wakan Sansai-Zue* by Terajima, 1713 (Reprinted from Ref 11). Image depicts a striped land planarian (likely *Bipalium*) in the right column. The vertical text is translated to read:

*'''Doko'' or ''Toku''. The animal has the shape of a Japanese belt in general appearance and is without legs. It measures up to 12 to 15 cm in length; a large specimen attains about 30 cm. The body is flattish in shape as a leaf of leek. There are yellow and black folds on the dorsal surface. The animal has a head shaped like a Japanese forceps. . .**If the animal is touched, fission may occur**. . .'.*[13]

WHY STUDY REGENERATION IN PLANARIANS?

Planarians Are Masters of Regeneration

Today's popular model organisms were selected for a simple reason: they are biological exaggerations. Morgan selected the fruit fly and Sydney Brenner, the nematode worm because their exaggerated reproductive biology made them ideal for performing forward genetic screens. Likewise, planarians are ideal for regeneration studies because they undergo amazing feats of restorative and physiological regeneration.[35]

Planarians undergo restorative regeneration in response to almost any type of injury. An upregulation of cell proliferation forms a mass of unpigmented

newly differentiating cells, called a blastema. From this blastema emerges many of the tissues lost to injury, producing a fully restored worm in as little as 1–2 weeks[28,31] (Figure 3(a) and (b)). This restorative response involves rebuilding anatomy *de novo*—a process Morgan called 'epimorphosis'. It also involves remodeling the pre-existing tissues and integrating them with the newly made anatomy so that the animal regains its proper proportions and restores function to its organs. Morgan termed this type of remodeling 'morphallaxis'[35] (Figure 3(c)).

In addition to restorative regeneration, planarians display physiological regeneration, repairing anatomy as it naturally ages. In the absence of an injury, these animals constantly undergo impressive levels of cell proliferation to replace old tissues. Like many cnidarians, annelids, echinoderms, and ascidians, planarians can maintain physiological regeneration for decades without losing the ability to regenerate or developing cancer.[36,37] This not only makes planarians useful for asking questions about regeneration. It also makes these long-lived animals tantalizing subjects for aging research, which has so far included studies of the mechanisms of planarian telomere maintenance[38] and the function of genes that affect longevity in other organisms.[39–41]

Planarians Are Sufficiently Complex in Anatomy and Behavior

Planarians are protostomes and members of the Lophotrochozoan clade. They are triploblastic and thus have tissues derived from all three germ layers (ecto-, meso-, and endoderm). While they have simpler body plans than vertebrate model systems, they have long been recognized to have discrete organ systems and behaviors amenable for regeneration studies (Figure 2(a)).

Planarians eat (Supporting Information Video S1) and defecate (Supporting Information Video S2) through a muscular feeding tube, or pharynx, which connects to the gut[43–46] (Figure 2(b) and (c)). A blind gut with one anterior branch and two posterior branches occupies much of the body cavity[47–49] (Figure 2(c)). The animals possess an organized nervous system composed of two anterior cephalic ganglia and two parallel nerve cords that run ventrally along the length of the body (Figure 2(c)). A pair of dorsal photoreceptors is connected to the nervous system by axons that make up the optic chiasm[50,51] (Figure 2(d)). They possess motile cilia on their ventral epithelium that enables them to glide across surfaces[52–56] (Figure 2(e)). Their body plan is peppered with protonephridia, organs that facilitate osmoregulation and may ultimately prove to be homologous rather than analogous to the vertebrate kidney[57–59] (Figure 2(f)). While much work has focused on regeneration in asexual planarians, sexual strains also exist as cross-fertilizing hermaphrodites, regenerating ovaries and testes after amputation or starvation.[60–64] Planarian tissues also have intricate domains of molecularly discrete cell populations, yielding a plethora of markers to assess wound responses and general organization of the body plan[65] (Figure 2(g)). Finally, these animals display complex behaviors including negative phototaxis, fissioning in response to stimuli like changes in population density, and even cannibalism[16–18,66–69] (Supporting Information Video S3). Thus, planarian anatomy and behavior provide a sufficiently complex palette for studying regeneration.

An Expanding Molecular Toolkit

Planarian studies prior to the end of the 20th century were plagued by a lack of cellular resolution. Investigators relied principally upon basic histology, electron microscopy, and the visualization of gross anatomy under a transmitted light microscope to assess the regenerative response. They had little means of distinguishing cellular identity or tracing the lineage and movement of cells over time. They also had few ways to perturb the animals, and frequently resorted to treating planarians with pharmacological agents or toxins which had unknown mechanistic effects. Ultimately, the lack of experimental tools hampered the understanding of planarian biology.

Within the last two decades, our ability to visualize planarian tissues has improved drastically. Unlike Morgan and his inability to visualize the cellular and molecular events underpinning regeneration (Figure 3(a) and (b)), we can now assess each step of the regenerative response with extensive panels of markers. We can detect changes in gene expression and protein function, yielding a much sharper picture of the unfolding morphological and cellular dynamics of regeneration.[70–72] For instance, it is possible to visualize the regeneration of endodermally and ectodermally derived organ systems like the gut and brain, respectively (Figure 3(c)). Cellular activities that are not necessarily associated with organogenesis can also be assessed, such as the reestablishment of anterior domain identities after amputation (Figure 3(d)).

In addition, the genome of the species *Schmidtea mediterranea* has been sequenced,[74] to which EST, transcriptome, proteome, and small RNA datasets can be mapped.[75–87] Genome microarrays have been generated to identify genes important for

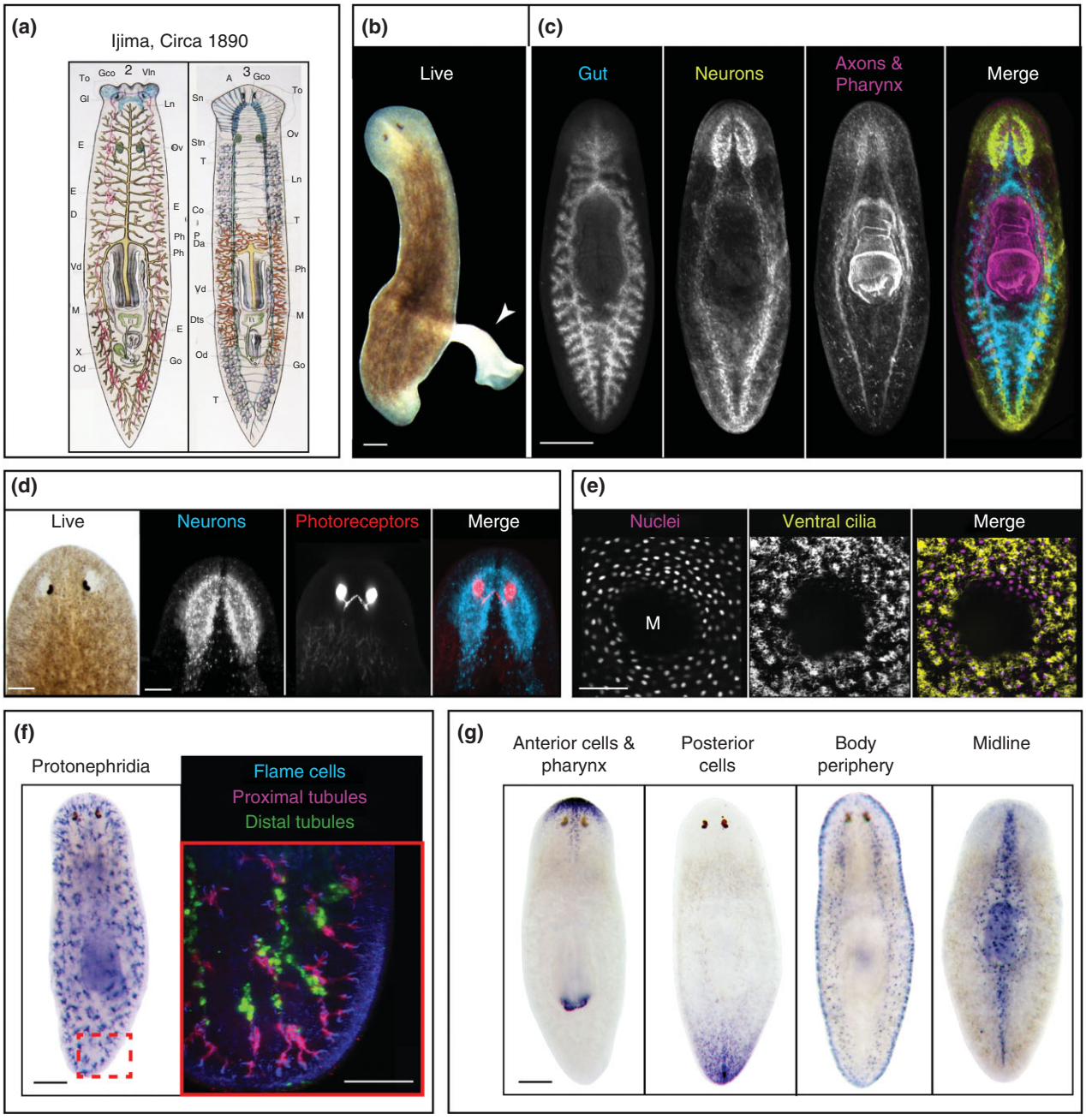

FIGURE 2 | Planarian anatomy is sufficiently complex for regeneration studies. (a) Two depictions of planarian anatomy adapted from Leuckart's zoological wall chart series entitled 'Vermes,' circa 1890.[42] (image obtained from MBLWHOI Library, Rare Books Archive). (b) A live planarian extruding its pharynx (*arrowhead*). Scale bar 200 μm. (All animals depicted in Figures 2–7 are the asexual strain of *Schmidtea mediterranea* unless otherwise noted.) (c) Overlay of gut (blue, *Smed-porcn-1*), neurons (yellow, *Smed-PC-2*), axons, and pharynx (magenta, anti-α-tubulin antibody). Scale bar 200 μm. (d) *Left panel*: Head of a live planarian. Photoreceptors are darkly pigmented. *Right panels*: A different specimen showing neurons of the cephalic ganglia (blue, *Smed-PC-2*), photoreceptors, and commissural visual axons (red, anti-arrestin antibody; a kind gift of Dr Kiyokazu Agata). Scale bars 200 μm. (E) Tufts of ventral cilia (yellow, anti-acetylated-tubulin antibody) projecting from epithelial cells (nuclei: magenta, TOPRO-3) facilitate swimming. Image focused around opening to the pharynx cavity (M, mouth). Scale bar 50 μm. (F) *Left panel*: Protonephridia, which compose the excretory system (*Smed-innexin-10*). Scale bar 200 μm. *Right panel*: Close up of tail tip of a different specimen. Confocal maximum projection of protonephridial system, including flame cells (blue, anti-α-tubulin antibody), proximal tubules (magenta, *Smed-innexin-10*), and distal tubules (green, *Smed-CAVII-1*). Scale bar 50 μm. (Images provided by Hanh Thi-Kim Vu.) (g) Markers labeling distinct body regions. Left to right: anterior cells and distal tip of pharynx (*Smed-sfrp-1*), posterior cells (*Smed-wnt11-2*), body periphery (*Smed-wnt5*), and midline (*Smed-slit-1*).

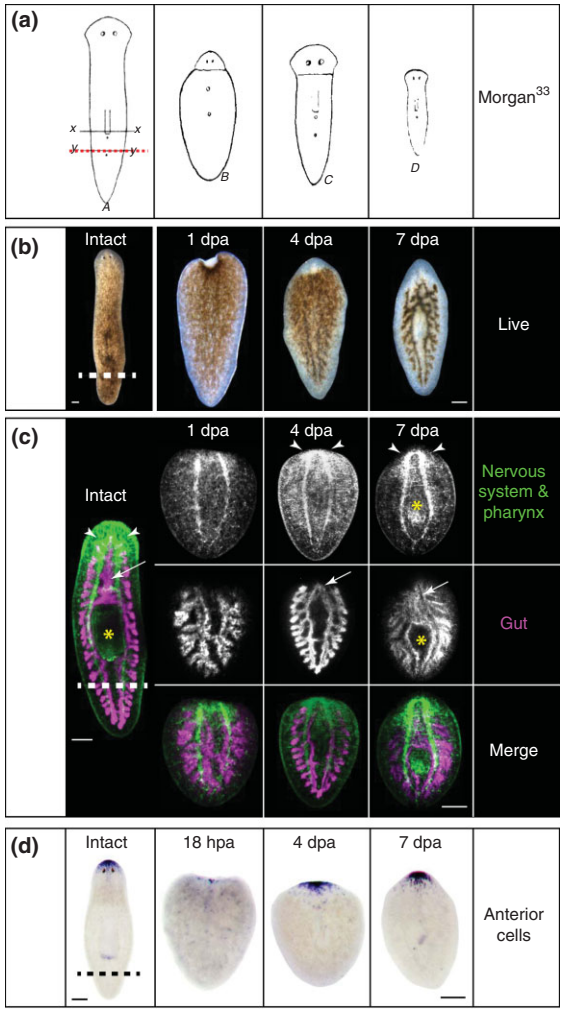

FIGURE 3 | Upon injury, planarians regenerate lost tissues, re-establish scale and proportion, and maintain axial polarity. (a) Morgan amputated an adult planarian (red dashed line) and observed it regenerate missing anatomy ('epimorphosis') and re-establish proper body proportions ('morphallaxis'). (Modified from the original as first published in Ref 33). (b) A live intact planarian was amputated (white dashed line), and the regenerating tail fragment is shown at 1, 4, and 7 dpa. Scale bar 200 μm. dpa: days post amputation. (c) The cephalic ganglia (arrowheads), pharynx (yellow asterisk), and anterior gut branch (arrow) regenerate by 7 dpa. An intact planarian (left) was amputated (white dashed line) and regenerating tail fragments were stained at timepoints indicated (right) for nervous system, pharynx (green, anti-α-tubulin antibody) and gut (*Smed-porcn-1*). Scale bars 200 μm. (Reprinted with permission from Ref 73). (d) The A/P decision is made by 1 dpa, preceding tissue regeneration and anatomical remodeling. Regenerating tail fragments stained at timepoints indicated for marker of anterior cell identity (*Smed-sfrp-1*). Scale bars 200 μm. hpa: hours post amputation. (Reprinted with permission from Ref 73. Copyright 2010 Elsevier)

regeneration.[59,88–91] High-throughput RNAi screens can be performed to characterize gene function.[92–94] Fluorescence activated cell sorting (FACS) is used to identify and isolate discrete cell populations, which subsequently can be used for such purposes as single-cell gene profiling or functional transplantation studies.[95–101] All of these tools, coupled with the ability to perform lineage tracing using BrdU, have opened the door for rigorous study of the molecular mechanisms underlying planarian regeneration.[102]

PATTERNING THE PLANARIAN BODY AXIS

Polarity Is Maintained during Regeneration

One of the earliest uses of the term 'polarity' in reference to body plan regeneration can be found in the work of Allman to describe the tubularian's propensity to regrow a head from anterior-facing wounds and not posterior ones.[103] The mechanisms that establish and maintain polarity are fundamental questions shared by the fields of regeneration and embryology. How do cells know they are different from other cells? How are these differences translated into proper specification of the body axes and subsequent organogenesis? How is polarity re-established in the face of unexpected perturbations—whether that perturbation is a blastomere ablation in an embryo or an appendage amputation in an adult?

During regeneration, adult planarians maintain the polarity of their body axes. A small piece of tissue removed from the flank of the animal conserves the original orientation of the anterior–posterior (A/P), dorsal–ventral (D/V), and medial–lateral (M/L) axes[28] (Figure 4(a)). Additional experiments from the earlier part of the 20th century demonstrated that the juxtaposition of tissues from different regions of the animal—such as the transplantation of anterior tissue to posterior regions—triggers abnormal regeneration, including the formation of an ectopic body axis[104,105] (Figure 4(b)). These results suggest that planarian tissues possess some type of intrinsic positional and polarity information. During regeneration, this starting information must be read and interpreted correctly such that the proper structures are made in the right location. Determining how polarity is re-specified and maintained is critical for understanding the mechanisms of animal regeneration, and we are now discovering that some of the genetic toolkit used to establish polarity in an embryo may also play similar roles in maintaining polarity during regeneration.

Historical Views of Regeneration Polarity

Over the centuries, investigators have proposed diverse mechanisms to explain the phenomenon of polarity. Some of these models had distinctly

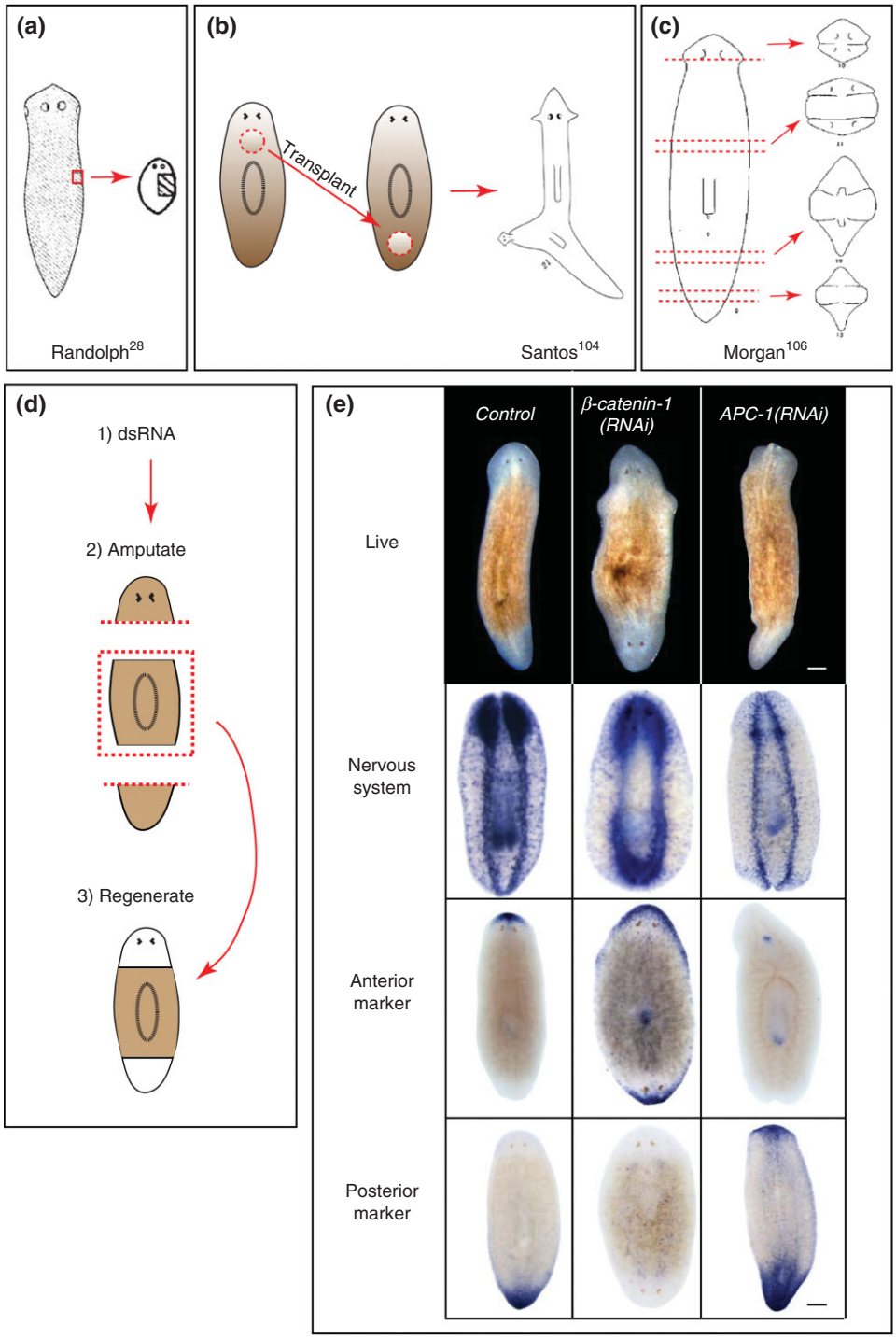

FIGURE 4 | Anterior–posterior polarity. (a) Randolph showed that a small piece of tissue amputated from the flank of the body (left, red box) maintains axial polarity during regeneration (right). (Reprinted with permission from Ref 28). (b) Transplanting tissue from the anterior region of one planarian to a posterior region of another (left) results in outgrowth of a new body axis (right). (Reprinted with permission from Ref 104).(c) Thin transverse amputations (left, red dashed lines) cause heteromorphic regeneration, resulting in double-headed (top) or double-tailed (bottom) regenerates. (Modified from the original as first published in Ref 106). (d) RNAi strategy employed for Figures 4–6. Animals were (1) fed dsRNA to knockdown a gene of interest, (2) amputated, and (3) allowed to regenerate. (e) Wnt/β-catenin signaling controls A/P polarity. Live images and fixed animals stained for the nervous system (*Smed-PC-2*), anterior cell identity (*Smed-sfrp-1*), and posterior cell identities (*Smed-fz-4*). Controls regenerate normally. *Smed-β-catenin-1*(*RNAi*) causes a head to regenerate from posterior blastemas. *Smed-APC-1*(*RNAi*) causes a tail to regenerate from anterior blastemas. Scale bars 200 μm. (Live images provided by Dr Kyle A. Gurley and Dr Jochen C. Rink.)

preformationist undertones. Bonnet hypothesized that 'germs' exist in the earthworm that contain a fully formed miniature head or tail. Upon amputation, fluid flow transports 'head germs' anteriorly and 'tail germs' posteriorly so that a head and tail sprout at the proper locations.[6,107] Weismann, a declared preformationist, extended his theory of the germ–plasm to explain regeneration. He proposed that preformed cells containing an 'idioplasm' facilitate the reconstitution of the limbs of salamanders and newts. 'Idioplasm' is organic material that predetermines the reconstitution of the limb, regardless of cues from the environment or the regenerating appendage. As cells divide, portions of the nuclear 'idioplasm' are lost, and the division progeny are left with only enough 'ids' to produce the next most distal cells in the limb. Thus, this predetermined regeneration program ensures that distal structures are never regenerated before more proximal ones.[108]

Other hypotheses regarding regeneration polarity were more grounded in the ideals of epigenesis, proposing that polarity came not from preformed germs or 'ids,' but instead developed progressively out of some instructive cues intrinsic to the cells and tissues. Bardeen argued that the pre-existing anatomy of a planarian exerts mechanical forces that constrain the location in which new anatomy can physically fit. He also argued that the nervous system was key in dictating polarity.[66,109] Pflüger proposed that the chemical composition of the pre-existing tissue's cut surface establishes polarity. Each tissue laid down during regeneration provides a chemical signal that instructs the fate of the next layer laid down on top of it, and regeneration thus proceeds in a proximal to distal direction.[110] Child thought that gradients of metabolic activity guide regeneration. He believed that anterior tissues have higher metabolic rates and, thus, display 'physiological dominance' over more posterior tissues, establishing A/P polarity early on in regeneration.[111] Brøndsted, heavily influenced by Child, proposed that unknown effectors establish A/P polarity through a time-graded regeneration field. This field exposes 'high points' in a planarian blastema where regeneration of the head occurs faster and more vigorously than in other regions, subsequently releasing factors that inhibit head formation in more posterior areas.[112,113] (For additional examples of regeneration polarity theories, see Refs 4,114–118.)

While most of these hypotheses have been proven insufficient to fully explain regeneration polarity or the defects resulting from experimental manipulation, Morgan's theory has best withstood the test of time. Based upon meticulously documented regeneration experiments performed in a wide variety of animals, Morgan observed that 'something in the piece itself determines that a head shall develop at the anterior cut surface and a tail at the posterior cut surface. This "something" is what we call polarity.'[119] He hypothesized that polarity results from some type of physical and/or chemical gradient along the body axes.[120–122]

A/P Polarity in Planarians

Early attempts to better understand axial polarity in planarians centered around perturbing regeneration through surgical means. The abnormal, surgically produced regenerates were referred to as heteromorphoses. Before the use of chemicals, irradiation, electric fields, or RNAi, heteromorphoses provided key insights from which hypotheses could be made about the mechanisms underpinning regeneration. Heteromorphoses of the A/P axis were described early on in studies of planarian regeneration, since the head was an easily recognized structure. Most notably, Morgan observed that transverse amputations producing short cross-pieces frequently regenerated bipolar heads or tails[31,33,106] (Figure 4(c)). Coupled with similar regeneration defects from experiments on earthworms and tubularians, Morgan suggested that a regenerate might interpret a gradient of chemical or physical information along the body axis to maintain proper axial polarity. Very thin slices of tissue could have too shallow of a gradient to be deciphered, causing the production of a head from posterior wounds by default.

RNAi screens have uncovered phenotypes recapitulating Morgan's double head and double tail defects, lending support to his gradient hypothesis (Figure 4(d)). The Wnt/β-catenin pathway, which is involved in many developmental processes across metazoa including establishing polarity along the primary axis,[123,124] is required for A/P polarity in planarians.[125,126] Knockdown of the pathway's core transcription factor *Smed-β-catenin-1* results in anteriorization of the body axis, causing a head to regenerate from a posterior wound instead of a tail (Figure 4(e)). This anteriorized phenotype is also produced by knockdown of upstream ligands *Dj/Smed-wnt1* and *Smed-wnt11-5*, receptor-associated agonists *Smed-dvl-1* and *Smed-dvl-2*, and the transmembrane protein required for secretion of WNTs *Smed-evi/wntless*. In contrast, upregulation of β-CATENIN-1 activity by knockdown of such inhibitors as *Smed-APC-1*, *Smed-notum*, *Smed-axinA*, and *Smed-axinB* elicits the opposite phenotype in which tails regenerate from anterior wounds[125–132] (Figure 4(e)). These results suggest that during regeneration, graded levels of β-CATENIN activity along

the body plan regulate the anterior-versus-posterior fate choice. β-CATENIN activity must be sufficiently high in posterior blastemas to facilitate tail regeneration and sufficiently low in anterior blastemas to produce a head. Likewise, this signaling system must be acting during physiological regeneration, as knockdown of *Smed-β-catenin-1* anteriorizes uninjured animals too.[125–127] Whether there is a posterior-to-anterior gradient of β-CATENIN nuclear localization is unknown. However, numerous posteriorly expressed Wnt ligands and anteriorly expressed Wnt inhibitors suggest that there may indeed be such an activity gradient.[73,125,126,128,129,131]

The Hedgehog pathway, well characterized during the development of many animals,[133] is also important for A/P polarity in planarians. RNAi of pathway activators *Dj/Smed-hh*, *Dj/Smed-gli-1*, and *Smed-smo* decreases Hh signaling and results in loss of posterior regeneration. In contrast to this 'tailless' phenotype, increased Hh signaling through RNAi of pathway inhibitors *Dj/Smed-ptc* and *Dj/Smed-sufu* causes defects in anterior regeneration. In these animals, a tail regenerates instead of a head at anterior wounds. Thus, high levels of Hh signaling are required to properly specify posterior tissues, while lower levels are required for specifying anterior tissues. Furthermore, the Hh pathway may act upstream of the Wnt/β-catenin pathway by modulating the expression of *Dj/Smed-wnt1*, which likely signals through β-CATENIN to specify posterior fates.[52,128,130,134,135]

Additional parallel or convergent pathways are known to participate in the establishment and maintenance of A/P polarity. Simultaneous knockdown of putative gap junctions *Smed-innexin-5*, *-12*, and *-13* produces double heads.[136] RNAi of the LIM homeobox transcription factor *Djislet* causes a tailless phenotype.[131] Knockdown of the TALE class homeobox transcription factor *Smed-prep* causes cyclopic and headless phenotypes.[137] Graded membrane voltage, based at least in part on high intracellular calcium levels in anterior wounds, also plays a role in establishing A/P polarity in planarians.[138–142] At this point, however, it is unclear how all these collective signals are integrated to properly reestablish the A/P axis.

D/V Polarity in Planarians

As in other regenerative animals, an amputation in a planarian brings dorsal and ventral tissues into close contact at the wound site.[143] Grafting experiments in animals including newts,[144,145] arthropods,[146] and annelids[147] have suggested that signaling between tissues from different regions of the dorsoventral (D/V) axis—such as the interaction induced by wound closure—might be an early trigger for regeneration. Classical planarian experiments have also supported this idea. In particular, Santos grafted plugs of planarian tissue into a host in either normal D/V orientation or inverted orientation (Figure 5(a)). In the former case, the tissue healed and the animal appeared normal. In the latter case, a blastema formed at the interface between the graft and host tissues and large cup-shaped protrusions emerged at the graft site. In at least one case, Santos even observed an ectopic planarian developing from the graft with inverted D/V orientation to the host's body axis (Figure 5(a)). This suggested that the graft not only retained its original D/V polarity after transplantation, but the juxtaposition of dorsal and ventral tissues somehow triggered the formation of a new body axis.[104,105]

Seventy years after Santos' initial observations, investigators are revisiting his experiments with modern tools. Histological analyses and expression studies show that Santos' inverted transplants indeed maintain their original D/V polarity after grafting. In addition, the boundary between the host and graft tissues of inverted transplants ectopically expresses a body edge marker, while noninverted control transplants do not.[148] While future studies are needed to determine whether an ectopic body axis is truly forming from these protrusions and how this is accomplished, these results do suggest that the closer positioning of dorsal and ventral tissues after an injury and wound healing might be an important aspect of the regenerative response of planarians, helping promote blastema formation and specification of a new body edge.[149]

Recent RNAi screens have uncovered numerous genes important in establishing and maintaining D/V polarity in planarians. So far, all genes identified in these screens are components of the BMP pathway, a branch of the TGF-β signal transduction cascade, which has a conserved role in organizing the D/V axis in diverse metazoans.[124] Reduction of BMP signaling by RNAi of *DjBMP/Smed-bmp4*, *Smed-smad1*, *Smed-smad4*, *Smed-admp*, and *Smed-noggin-like-8* ventralizes animals during both restorative and physiological regeneration (Figure 5(b)). Collectively, these ventralized defects include a dorsal duplication of the brain and ventral nerve cords, ectopic dorsal expression of ventral markers, and growth of dorsal cilia that enable the animals to swim in an inverted fashion on their dorsal side.[94,150–154] Likewise, increasing BMP signaling through knockdown of putative inhibitors *Smed-noggin-1* and *Smed-noggin-2* causes the opposite dorsalized phenotype in which

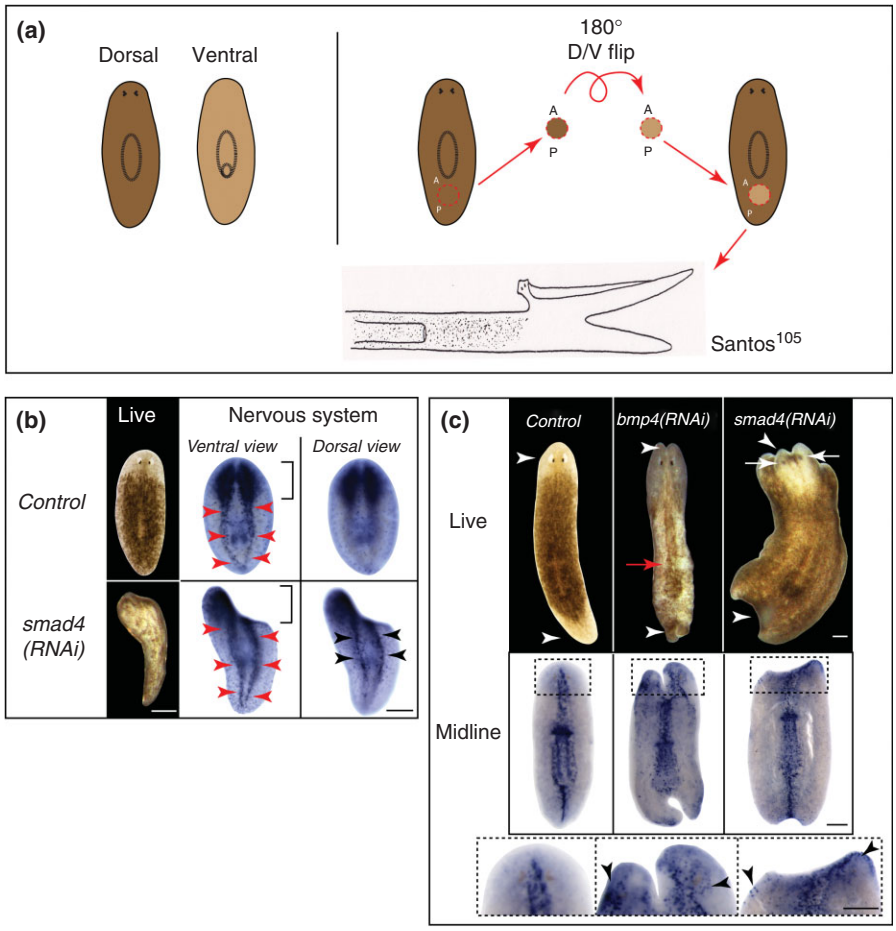

FIGURE 5 | Dorsal–ventral polarity. (a) Santos showed that flipping the D/V orientation of a tissue plug without altering the A/P orientation results in the outgrowth of an ectopic body axis. This ectopic growth has inverted D/V polarity compared to the main body's D/V axis. Only the tail region is pictured in Santos' sketch; the main body's head is to the left, out of view. (Modified from the original as first published in Ref 105). (b) BMP signaling controls D/V polarity. Control tail fragments form a blastema (bracket) and regenerate nerve cords localized ventrally only (*Smed-PC-2*, red arrowheads; compare ventral vs. dorsal views). *Smed-smad4*(*RNAi*) causes a loss of blastema formation, regeneration of the cephalic ganglia (compare brackets), and growth of ectopic dorsal nerve cords (black arrowheads; compare ventral vs. dorsal views). Scale bars 200 μm. (c) BMP signaling is required for blastema formation and organization of the midline. Control animals form anterior and posterior blastemas (white arrowheads) and regenerate a midline (*Smed-slit-1*). *Smed-bmp4*(*RNAi*) causes midline indentations in the blastemas, dorsal ruffling (red arrow), and ectopic expression of a midline marker (black arrowheads). *Smed-smad4*(*RNAi*) causes a loss of blastema formation (white arrowheads), photoreceptor regeneration in old tissue (white arrows), and ectopic expression of a midline marker (black arrowheads, compare insets). Scale bars 200 μm.

animals ectopically express dorsal markers on their ventral side.[153,154]

In addition to identifying a molecular foothold for studying the regulation of the D/V axis during planarian regeneration, these defects hint that an interaction between dorsal and ventral tissues juxtaposed during wound closure may indeed be important for regeneration, as Santos proposed from his grafting experiments[104,105] (Figure 5(a)). All ventralized RNAi phenotypes examined thus far display reduced or absent blastemas (Figure 5(b) and (c)) and a loss of expression of body edge markers at the wound site. Perhaps critical signaling events between properly specified dorsal and ventral tissues organize or permit downstream events in regeneration. While such an interaction could explain the BMP pathway blastema phenotype and the formation of a second body axis observed by Santos, these spatially introduced signaling events have yet to be confirmed.

M/L Polarity in Planarians

Randolph and Morgan both described a variety of perturbations in M/L regeneration.[28,31,33,60] One of the most striking experiments involved a simple midline incision in either the anterior or posterior

region of the animal. This incision did not fully cut the animals in half, and the wounds were allowed to heal back together. While some animals simply healed, this midline incision triggered a duplication of the M/L axis in others. These animals became wider and sprouted ectopic pharynges and photoreceptors lateral to the pre-existing ones (Figure 6(a) and (b)). While this experiment has yet to be revisited with molecular markers, their results suggest that the M/L axis is tightly regulated during regeneration. Simple wounding may cause the animal to reassess the integrity of the M/L axis, and trigger cells to take on different positional identities as if the animal had been cut through and through.

RNAi screens have identified numerous genes important for regulating anatomical patterning with respect to the M/L axis in planarians. This includes the Slit/Netrin repulsion–attraction signaling system. Among other developmental processes, SLIT and NETRIN ligands cooperatively regulate the migration of axonal projections across the midline, with SLIT repulsing axons as NETRIN attracts them.[155,156] Similarly, *Dj/Smed-slit-1* is required to maintain the planarian midline during restorative and physiological regeneration. RNAi knockdown causes a collapse of lateral tissues toward the midline, including the cephalic ganglia, nerve cords, photoreceptors, optic chiasm, and posterior gut branches[157,158] (Figure 6(c)). While it is curious that knockdown of the SLIT receptor, *Dj/Smed-roboA*, does not fully recapitulate these midline defects, it does cause aberrant crossing and fasciculation of the axons of the optic chiasm, in addition to a lateral displacement of the ganglia and reduction of the anterior commissure. This suggests a defect in M/L patterning.[158,159] In contrast to the *slit-1*(*RNAi*) phenotype, the most penetrant defects associated with knockdown of Netrin signaling via *Smed-netR*(*RNAi*) and *Smed-netrin2*(*RNAi*) are a lateral expansion of the cephalic ganglia, reduction in anterior commissure, and disorganization of the axons of the ventral nerve cords.[160] These contrasting defects in M/L patterning, coupled with simultaneous double RNAi for *Dugesia japonica* Slit-Netrin signaling components,[158] suggest a Slit-Netrin signaling synergy may help direct various events in M/L patterning.

The Wnt pathway is also involved in midline patterning in planarians. *Smed-wnt11-2*(*RNAi*) causes a failed extension of *Smed-slit-1* cells at the tip of the regenerated tail, resulting in abnormal looping of the ventral nerve cords and inappropriate midline crossing of the posterior gut branches.[73,128] In contrast, knockdown of *Smed-wnt5* yields a phenotype very similar to the defects observed by Randolph and

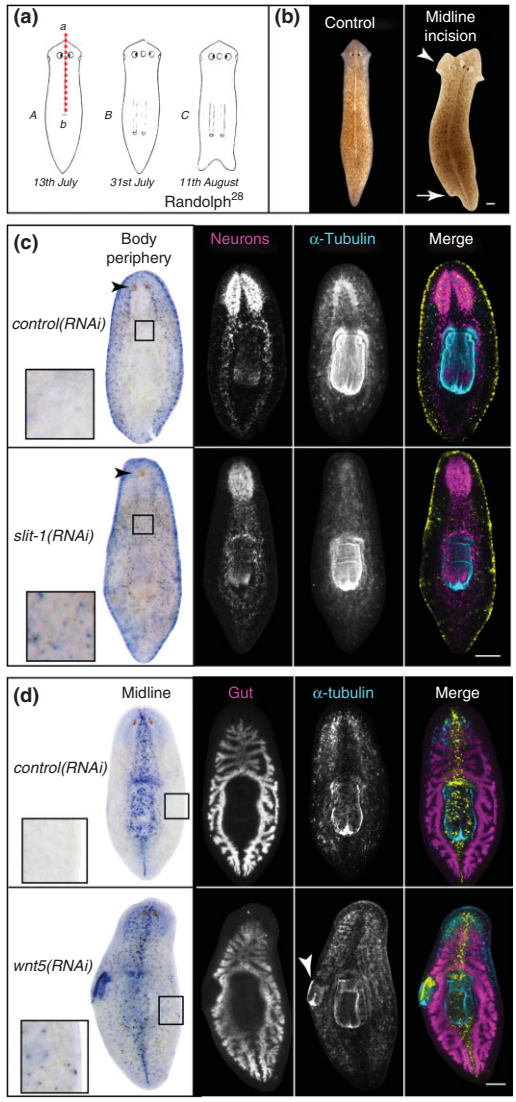

FIGURE 6 | Medial–lateral polarity. (a) Randolph showed that a midline incision (red dashed line) that is allowed to heal together causes, with some frequency, a duplication of midline structures. (Modified from the original as first published in Ref 28). (b) Live images 14 days after the midline incision depicted in 6A. Although the tissue was allowed to heal back together, the head is duplicated at the site of the incision (white arrowhead). The tail is forked (white arrow), even though the posterior was never injured. Species *Dugesia sanchezi*. Scale bar 200 μm. (c) *Smed-slit-1* maintains the M/L axis. Compared to *control*(*RNAi*), *Smed-slit-1*(*RNAi*) causes the cephalic ganglia, nerve cords (magenta, *Smed-PC-2;* blue, anti-α-tubulin antibody), photoreceptors (black arrowhead), and markers for the body periphery (*Smed-wnt5;* compare insets) to collapse toward the midline. Scale bar 200 μm. (Reprinted with permission from Ref 73). (d) *Smed-wnt5* maintains the M/L axis. Compared to *control*(*RNAi*), *Smed-wnt5*(*RNAi*) causes the lateral expansion of the axon tracts, and the formation of an ectopic lateral pharynx (blue, anti-α-tubulin antibody; white arrowhead) flanked by gut branches (magenta, *Smed-porcn-1*). Expression of a midline marker expands laterally (*Smed-slit-1;* compare insets). Scale bar 200 μm. (Reprinted with permission from Ref 73. Copyright 2010 Elsevier)

Morgan after midline incisions: a lateral expansion of medial structures toward the body periphery, in addition to the growth of lateral, ectopic pharynges[56,73,128] (Figure 6(d)). In light of these phenotypes, the M/L axis must be as tightly regulated during planarian regeneration and homeostasis as the A/P and D/V axes are.

Finally, the TGF-β pathway is important not just for D/V axis organization, but also M/L organization. *Smed-bmp4* (which is expressed along the dorsal midline of the adult planarian) is upregulated at the wound edge during lateral regeneration. RNAi of some components of this pathway (*Smed-bmp4, Smedolloid-1,* and *Smed-smad4*) completely abolishes regeneration from amputations that bisect the animals down the midline.[152] RNAi of TGF-β pathway members that cause ventralization results in conspicuous midline indentations in anterior and posterior blastemas, suggesting that the midline does not regenerate properly[94,150–154] (Figure 5(c)). Furthermore, collapse of the nervous system and regeneration of supernumerary photoreceptors at the midline, as well as lateral ectopic pharynges are among other midline abnormalities reported thus far.[151] The perturbation of the M/L axis was molecularly verified in *Smed-admp*(*RNAi*) animals, which ectopically express the midline marker *Smed-slit-1* at the body periphery,[153] as well as in *Smed-smad4*(*RNAi*) regenerates (Figure 5(c), see Table 1 for summary of all polarity phenotypes described).

These results leave us with many questions. Does regeneration require establishment of one body axis before another can be specified? Or can an axis be specified independently of the other two, as in the case of zebrafish development?[161] Which cells provide polarity information and which cells interpret these cues? Are there organizing centers for body axis polarity analogous to those identified during embryogenesis? And how are all three axes integrated during regeneration? In order to understand how these animals regenerate in three dimensions, studies must focus on the timing of axis

TABLE 1 | Summary of Body Axis Patterning Phenotypes

		Anterior Regeneration Defects			Posterior Regeneration Defects			Dorsalization	Ventralization	Medial/Lateral Defects
Pathway	*RNAi* Knockdown	Two tails	Headless	Cyclopia	Reduced Tail	Tailless	Two heads			
Wnt	*Dj/Smed-ß-catenin-1*						X			
	Smed-dvl-1/2						X			X
	Smed-evi/wntless						X			X
	Dj/Smed-wnt1						X			
	Smed-wnt5									X
	Smed-wnt11-2				X					X
	Smed-wnt11-5						X			
	Smed-notum	X								
	Dj/Smed-APC-1	X								
	Smed-axinA	X								
	Smed-axinB	X								
Hh	*Dj/Smed-ptc*	X	X	X						X
	Smed-smo				X	X				
	Dj/Smed-Hh				X	X				
	Dj/Smed-sufu	X	X	X						X
	Dj/Smed-gli-1				X	X				
Tgf-β	*Smed-smad1*								X	X
	Smed-smad4								X	X
	Smed-bmp-1 (smedolloid-1)								?	X
	DjBMP/Smed-bmp4								X	X
	Smed-admp								X	X
	Smed-noggin-like-8								X	X
	Smed-noggin-1/2							X		
Slit/Netrin	*Dj/Smed-slit-1*			X						X
	Dj/Smed-roboA									X
	Smed-netrin-2									X
	Smed-netR									X
	Dj/Smed-roboA									X
	DjnetB									X (low penetrance)
	Djdcc									X (low penetrance)
	Djunc5A									X (low penetrance)
Miscellaneous	*Smed-innexin-5*						X			
	Smed-innexin-12						X			
	Smed-innexin-13						X			
	Smed-prep		X	X						X
	Djislet-1					X				

Data are summarized for A/P, D/V, and M/L patterning defects. Dorsalization and ventralization refers to ectopic expression of axis markers or regeneration of ectopic anatomy. Midline defects refer to any ectopic or missing anatomy at the midline. These include misguidance of the visual axons that cross the midline; expansion or collapse of midline structures like the brain, nerve cords, photoreceptors, or pharynx; loss of neural connectivity at the midline; ectopic expression of midline or body periphery markers; reduction in lateral regeneration, or midline indentations in the blastema. (See text for references and additional details for each phenotype.)

specification and the manner in which these axis decisions affect signaling cascades required for subsequent organogenesis.[49,58,59,64,162,163]

NEOBLASTS: CELLULAR AGENTS OF PLANARIAN REGENERATION

A Century-Long Debate Regarding the Cellular Agents of Regeneration

After the amazing regenerative abilities of planarians were discovered, the search for the cellular source of this phenomenon ensued. Surprisingly, many key insights predate the use of the molecular-genetic tools recently applied to study planarians.

The histological analysis of planarian tissues under the light microscope in the late 19th century allowed biologists to identify a subset of parenchymal cells that undergo cell division, as evidenced by the presence of mitotic figures. This proliferating population of 6–12 μm ovoid-shaped cells possesses large decondensed nuclei and scant, basophilic cytoplasm (Figure 7(a)). These cells were correctly identified as the main source of new tissues.[23–25,30] Over the years, many names were ascribed to these cells, including *verästelten bindegewebszellen* (branching connective tissue cells),[164] *bildungszellen* (forming cells),[23] *stammzellen* (stem cells),[24] *stoffträger* (support material),[165] *ersatzzellen* (replacement cells),[30] *cellules libres du parenchyme* (free cells of the parenchyma),[166] *regenerationszellen* (regeneration cells),[167] and *wanderzellen* (migratory cells).[168] Eventually, the term *neoblast* permanently designated these cells, a name applied by Randolph to describe the cells responsible for regeneration in the earthworm *Lumbriculus*.[169–171]

Biologists initially tried to integrate what they knew about embryogenesis with what they were learning about neoblast-based regeneration. At first, these cells were referred to as an 'embryonic stock,' likened to blastomeres that persisted into adulthood to replenish injured or aging tissues.[116,173] Keller even suggested that neoblasts comprise a previously unidentified fourth germ layer.[24] Soon, however, some biologists challenged the idea that neoblasts were a persistent undifferentiated pool of cells, and suggested instead that they were derived from differentiated tissues that had dedifferentiated or transdifferentiated—a phenomenon termed metaplasia.[174–177] Still others thought that planarian regeneration and tissue homeostasis might involve a combination of these two phenomena.[109,178–181]

For much of the 20th century, the source of a planarian's regenerative abilities created a heated debate, especially as new scientific tools facilitated more sophisticated analyses. Some groups tried to specifically label differentiated cells versus neoblasts to determine what tissues they contributed to. Attempts were also made to visualize regenerated tissues using improved histochemical techniques. These experiments led a few researchers to believe they had observed various cell types dedifferentiate into neoblasts.[182,183] Others took advantage of differences in ploidy between the somatic and germline tissues, and reported that the germline could dedifferentiate or transdifferentiate into tissues such as muscle.[184] Some even argued that since dedifferentiation was the mechanism of regeneration identified in most other animals studied thus far, planarian biology must work the same way.[185]

However, one tool proved key to properly addressing this question. In the first half of the 20th century, it was demonstrated that ionizing radiation primarily kills neoblasts, causing the animals to lose the ability to undergo physiological and restorative regeneration[186,187] (Figure 7(c)–(e)). This simple manipulation suggested that metaplasia might not play a major role in regeneration, as the animals died even though the differentiated tissues appeared to be intact. To further test this, Wolff and Dubois used a lead block to shield portions of planarians at different positions along the A/P axis, resulting in the destruction of all neoblasts not covered by the shield. Subsequently, they amputated the animals and demonstrated that the length of time required for blastema formation was proportional to the distance of the lead shield from the wound.[171,187] These results suggested that the surviving neoblasts migrated to the wound to facilitate regeneration, as opposed to the dedifferentiation of local tissues.

Fueled by improvements in cell labeling methods, cell culture, grafting techniques and microscopy, additional evidence mounted in support of neoblasts being collectively totipotent migratory stem cells.[188–194] Of note, Baguñà and colleagues took advantage of two different strains of *Schmidtea mediterranea* to identify the source of regenerated tissues. One of these strains is sexual while the other is asexual, and they can be distinguished at the cellular level by a distinct chromosomal translocation. Cell fractions enriched for either neoblasts or differentiated cells were isolated by serial filtration from sexual animals and injected into irradiated asexuals and vice versa. In both cases, only the neoblast-enriched fraction rescued irradiated animals. Furthermore, the host took on the sexuality and karyotype of the animal from which the cell fractions were isolated.[195]

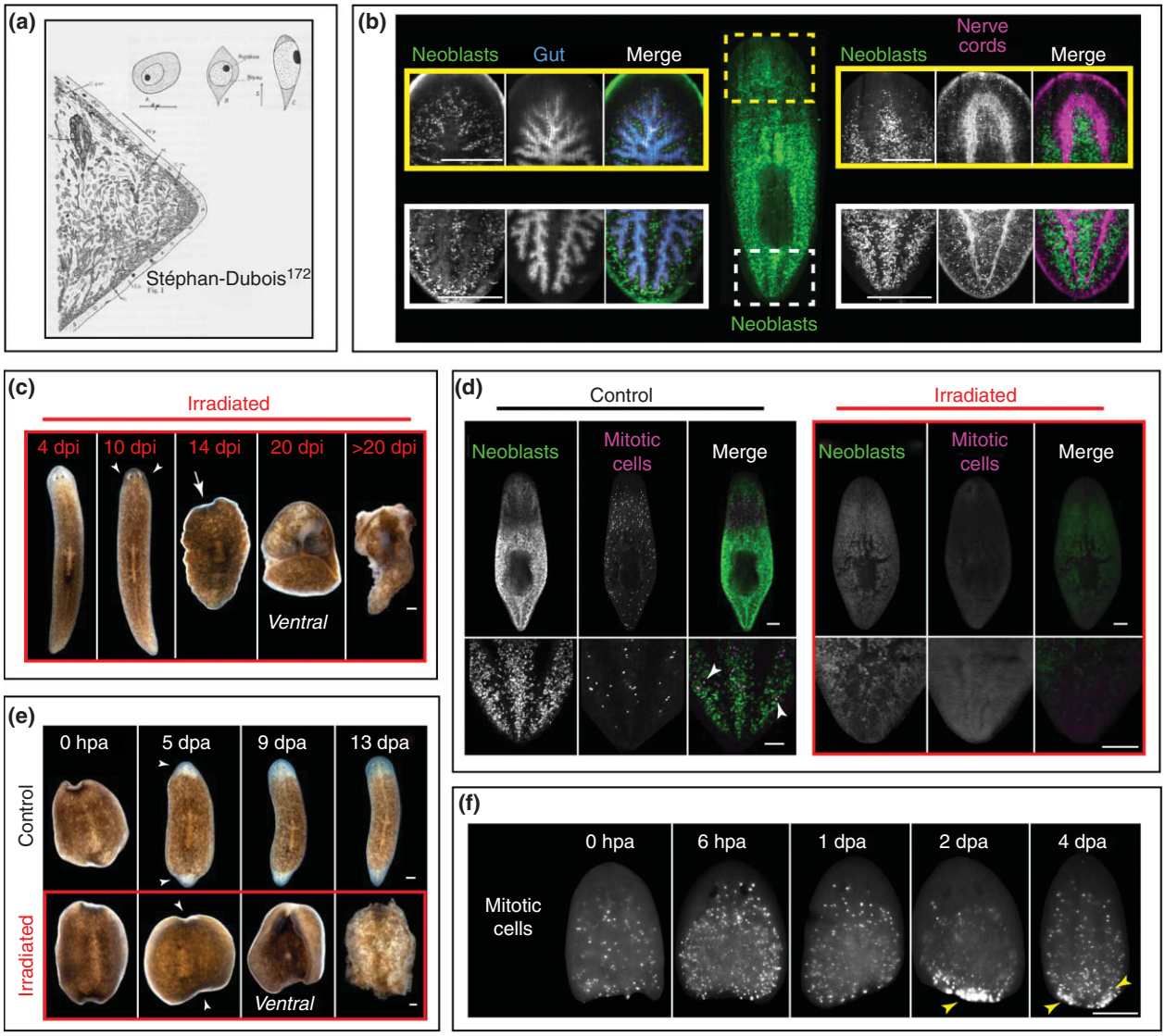

FIGURE 7 | Neoblasts as agents for regeneration. (a) Classical depiction of neoblasts by histology. (Modified from the original as first published in Ref 172). (b) Neoblasts (green, *Smed-piwi-1*) distributed throughout the parenchyma in between gut branches (blue, *Smed-porcn-1*) and in proximity to the nerve cords (magenta, anti-α-tubulin antibody). Images are confocal maximum projections. Scale bars 50 μm. (c) Irradiation disrupts physiological regeneration. Representative intact planarians at specified days post irradiation (dpi), exposed to 10,000 rads from a cesium source. Head regression is observed by 10 dpi, followed by ventral curling around 20 dpi. Lysis generally occurs after 20 dpi. Scale bar 200 μm. (d) Irradiation eliminates neoblasts and proliferation. *Left panels*: Neoblasts (green, *Smed-piwi-1*) are the only mitotic cells (magenta, anti-H3P antibody). White arrowheads indicate examples of colocalization in the tail of a different animal. *Right panels*: Neoblasts and mitotic cells are eliminated after irradiation by 3 dpi. Images are confocal maximum projections. Scale bars 200 μm. (Images provided by Dr Kyle A. Gurley). (e) Irradiation disrupts restorative regeneration. *Top panels*: Representative control trunk fragments displaying unpigmented regeneration blastemas by 5 dpa (white arrowheads). *Bottom panels*: Representative irradiated trunk fragments do not form blastemas (white arrowheads) or regenerate new tissues. Fragments curl ventrally and eventually lyse around 13 dpa. Scale bars 200 μm. (f) Amputation induces two waves of cell proliferation. Mitotic cells (white, anti-H3P antibody) are visualized in regenerating head fragments. A global burst in proliferation is observed within 6 hpa. By 2 dpa, a second proliferative burst occurs at the wound site (yellow arrowheads). Scale bar 200 μm.

Coupled with the observation that dividing cells migrate out of unirradiated tissue grafts into irradiated host tissues,[196–198] these results strongly suggested that neoblasts are a collectively totipotent, migratory stem cell population.

Modern Tools Demonstrate that Neoblasts Are Collectively Totipotent Stem Cells

In an effort to pinpoint the cellular source of regenerated tissues using modern molecular tools, early efforts focused on identifying genetic markers for neoblasts

(Figure 7(b)). These efforts have included cloning candidate stem cell and proliferation-dependent genes, generating EST libraries of regenerating animals, and testing antibodies against conserved proliferation-dependent histone modifications and cell cycle regulators.[45,71,96,199–205] Adaptation of FACS protocols enabled profiling and isolation of two side populations of cells mainly composed of neoblasts (termed 'X1s') and a mixture of neoblasts and their recent division progeny (termed 'X2s').[97] Also, after 35 years of attempts to incorporate modified thymidine analogs into proliferating neoblasts, BrdU was successfully optimized for use in planarians, facilitating the tracing of neoblasts and their division progeny.[102,206,207] Furthermore, the sequencing of the planarian genome[74] led to the development of microarrays to examine global gene expression changes after ablation of neoblasts by irradiation. These microarrays not only identified genes that define a molecular 'signature' for neoblasts.[89] They also revealed a number of categories of genes that disappear at different timepoints after irradiation and have distinct distributions of expression in the planarian body plan. By performing BrdU tracing and co-localization studies with neoblast markers, it was shown that these genes are actually markers for lineages of differentiating neoblasts.[90]

The ability to sort, label, and trace neoblasts and their division progeny made possible an impressive series of experiments that have put the debate about neoblasts' contribution to regeneration to rest. A single transplanted neoblast—termed a clonogenic neoblast (cNeoblast) for its ability to generate colonies of cells—has been shown sufficient to rescue a lethally irradiated planarian. cNeoblasts display extensive pluripotency, and can differentiate into all of the cell types in the animal, except possibly the germline. Furthermore, BrdU labeling and strain-specific SNPs used to discern tissues derived from the transplanted cNeoblast versus those of the host suggest that dedifferentiation is unlikely to contribute significantly to planarian regeneration.[101]

Neoblasts and Their Division Progeny Are a Heterogeneous Population

Historically, neoblasts were characterized by their morphology alone (Figure 7(a)). As a result, all neoblasts seemed roughly equivalent, with the exception that slight differences in cell shape could be observed.[208] However, modern studies demonstrate significant molecular heterogeneity amongst neoblasts and neoblast progeny. This suggests that while some neoblasts may be pluripotent, there could be subsets of neoblasts that are lineage-restricted and able to differentiate into only certain cell types.

Dj/Smed-nanos provided the first molecular hints of neoblast heterogeneity. In asexual planarians, it is expressed in only a subset of neoblasts.[62–64,100,209] Subsequently, single-cell PCR, immuno-EM, and *in situ* hybridization studies showed that neoblasts express various combinations of the canonical neoblast markers, in addition to genes normally associated with tissue-specific differentiation.[59,100,101,163,209,210] Even a number of the neoblast progeny markers identified by microarray do not colocalize extensively, suggesting a diversity of progeny lineages.[101]

Finally, the classical observation that neoblasts display subtle diversity in morphology has been confirmed with improved means of isolating these cells.[211] Recent functional data suggest that neoblast morphology might indeed be indicative of heterogeneity in the population. Single cNeoblasts possessing a distinct membrane protrusion produced 75% of all rescue events when transplanted to an irradiated host. However, since the rate of rescue was quite low, with only 7 out of 130 injections successfully grafting, it is possible that many cells expressing the pan-neoblast marker *Smed-piwi-1* might actually be a diverse population of multipotent cells.[101] It is currently unclear whether this molecular and morphological heterogeneity simply results from lineage restriction as pluripotent neoblasts differentiate, or whether there are permanent subpopulations of neoblasts that are restricted in potential.

Neoblasts Cycle Rapidly, Migrate, and Proliferate in Response to Injury

Classical descriptions of the behavior of neoblasts as a cell population are being re-examined with improved resolution and accuracy. For many decades, analysis of cell division in planarians was based on scoring mitotic figures in serial histological sections.[212] With the demonstration that BrdU could be incorporated by neoblasts in 2000,[102] the door opened for detailed analyses of the planarian cell cycle. We now know from continuous labeling with BrdU that around 20% of all planarian cells are cycling neoblasts, coming in at the lower end of classical estimates based on cell macerations.[99,213] Nearly all neoblasts enter S-phase and can be labeled with BrdU within 2–3 days of continuous exposure, suggesting that a large population of slow-cycling or G2-arrested neoblasts is unlikely to exist, as originally proposed.[99,102,214] The length of G2 has been estimated at approximately 6 h and the average cell cycle length is around 21 h.[99,102] In addition, changes in proliferation due to nutritional state were described classically, and it has been confirmed

that a large proliferative burst 12–72 h after feeding indeed corresponds with animal growth.[99,214,215] The basis of degrowth during starvation, however, is still debated. It may result from a decrease in neoblast proliferation, an increase in cell death of neoblast division progeny, or some combination of the two.[215–217]

Recent work has confirmed and expanded upon older descriptions of the temporospatial dynamics of neoblast proliferation.[218] It now seems that the regenerative response can be divided into two distinct mitotic phases. During the first phase, neoblasts initiate a global burst in proliferation within 6 h of any type of amputation or wound. The second burst requires the removal of tissue and is concentrated near the blastema, peaking around 48–72 hours post amputation (hpa)[219] (Figure 7(f)). Neoblasts can migrate as they differentiate and, in accordance with classical observations, they stop dividing before entering the blastema.[90,102,219,220] Finally, there is evidence supporting classical hypotheses that a signal emanating from the wound may trigger proliferation, as the initial mitotic increase seems to progress away from the wound in a wave-like fashion.[219,221]

With this expanding toolkit and an improved understanding of the dynamics of neoblasts, we can now begin to identify the genes that regulate neoblast self-renewal and differentiation. While it is possible that conserved cell cycle regulators and pluripotency genes play similar roles in neoblasts as they do in other stem cell systems, it is equally likely that novel mechanisms for regulation of neoblasts may be discovered. Already, we are learning that even the most fundamental aspects of planarian cell division are surprisingly unique. Planarians, for instance, are the first animals identified that do not seem to require a centrosome for cell division at any point in their life history.[222] It is possible that such fundamental differences in regulation of cell division might be key to understanding why planarians have exceptionally robust regenerative abilities.

Many Genes Required for Neoblast Function Have Been Identified

RNAi of genes from microarray experiments, expression profiling, EST libraries, and candidate ortholog searches of the planarian genome have already identified close to 200 genes whose phenotypes suggest defects in neoblast self-renewal and/or differentiation (see Table 2 for references). These phenotypes include reduced or absent blastemas, ventral curling, tissue regression, and lysis. A subset of these genes have been characterized in detail (Table 2).

While most of the phenotypes examined ultimately abolish proliferation and deplete neoblasts, an examination of the earlier stages of the phenotype progression reveals that neoblasts can be perturbed in numerous ways. First, neoblast self-renewal can be abrogated, as evidenced by a variety of phenotypes that display a decrease in proliferation and number of neoblasts soon after RNAi administration. Second, neoblasts can be disrupted at the level of differentiation. For instance, *Smed-piwi-2*(*RNAi*) does not affect neoblast numbers, their migratory ability, or their proliferative response after injury. Instead, differentiation is perturbed, as evidenced by the lack of regenerated tissues and the abnormal morphology of neoblast progeny that incorporate into the epithelium during homeostasis.[96,223] Additional examples of differentiation defects include *Smed-p53*(*RNAi*), *Smed-CHD4*(*RNAi*), and *Smed-PTEN-1/2*(*RNAi*). Among other abnormalities, RNAi of these genes seems to stall differentiation, causing an accumulation of neoblasts at the expense of postmitotic progeny.[224,227,229] Lastly, the spatial distribution of proliferating neoblasts can be disrupted, as it is after RNAi of *Smed-egfr-3* or administration of a putative ERK inhibitor.[232,234]

THE EMERGING ROLE OF DIFFERENTIATED TISSUES IN REGENERATION

The regeneration field has focused much of its efforts on the study of stem cells proper. The role of differentiated tissues has been appreciated mostly within the context of a cellular microenvironment known as a niche, which protects and maintains stem cells.[243] Considering that planarian regeneration requires not only local restoration of missing tissues, but also a simultaneous reproportioning of the entire body plan, it stands to reason that differentiated tissues may play important roles in regeneration on scales larger than what has been previously described for a stem cell niche.

Historically, the function of differentiated tissues during planarian regeneration has been largely dismissed as secondary to the action of neoblasts. Brøndsted, for instance, argued that neoblasts and the blastema they generate provide inductive cues to establish axial polarity in the rest of the preexisting tissues.[244] Likewise, Betchaku's cell culture experiments led him to view the fixed parenchymal cells as merely a vehicle for transporting neoblasts to the wound site so they can mount a regenerative response.[245] In the 1980s, the importance of differentiated tissues in directing neoblast differentiation was proposed.[197] However, only recently have the molecular tools become available to test this idea in

TABLE 2 | Summary of Neoblast Dysfunction Phenotypes

Species and gene	# OF STEM CELLS				# OF PROLIFERATIVE CELLS (H3P+)					DIFFERENTIATION (EARLY AND LATE PROGENY EXPRESSION)								CLONOGENIC EXPANSION ASSAY			Notes	Citation	Species and gene
	Normal	Decreased	Increased	Not examined	Normal	Decreased	Increased	Ectopic	Not examined	Early & late progeny normal	Early and/or late progeny lost before neoblasts	Early and/or late progeny lost concurrently with or shortly after neoblasts	Early and/or late progeny ectopically distributed	Excess numbers of early and/or late progeny	Normal	Not examined	Failed colony expansion after sublethal irradiation	Failed colony differentiation after sublethal irradiation	Not examined			
Schmitta mediterranea:																						*Schmitta mediterranea:*
Smed-ego-2		X				X				X									X	Form small blastemas that subsequently regress; expression of several mRNAs is reduced	235	*Smed-ego-2*
Smed-bruli		X				X										X	X			Form small blastemas that subsequently regress	205, 240	*Smed-bruli*
Smed-CHD4			X			X					X							X		Faster loss of progeny markers and lethality after irradiation	229, 240	*Smed-CHD4*
Smed-clp29		X							X							X	X			Reduced blastema and impaired differentiation of photoreceptors	240	*Smed-clp29*
Smed-eed-1		X							X							X	X			Reduced blastema and impaired differentiation of photoreceptors	240	*Smed-eed-1*
Smed-egfr-1			X				X						X						X	Outgrowths develop that express various tissue markers; degrowth; overexpress p53	234	*Smed-egfr-1*
Smed-egfr-3	X				X			X		X									X	Loss of some markers of differentiated cell types	234	*Smed-egfr-3*
Smed-ezh		X							X							X	X			Reduced blastema and impaired differentiation of photoreceptors	240	*Smed-ezh*
Smed-fgfr-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-fgfr-1*
Smed-fhl-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-fhl-1*
Smed-H2B		X				X								X					X		87	*Smed-H2B*
Smed-Hsp40				X					X							X			X		84	*Smed-Hsp40*
Smed-Hsp60				X					X							X			X		84	*Smed-Hsp60*
Smed-Hsp70				X					X							X			X		84	*Smed-Hsp70*
Smed-lin-1	X					X										X			X	Degrowth; reduced testes lobes and number of mature spermatids	217	*Smed-lin-1*
Smed-innexin-11		X				X										X			X		57	*Smed-innexin-11*
Smed-ier-1	X					X										X			X	Degrowth; reduced testes lobes and number of mature spermatids	217	*Smed-ier-1*
Smed-jun1-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-jun1-1*
Smed-khd-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-khd-1*
Smed-lis-1		X					X									X				Mitotic spindles are misorganized, and many neoblasts may be stuck in metaphase/anaphase	236	*Smed-lis-1*
Smed-lst8				X					X							X			X		39	*Smed-lst8*
Smed-mcg-1									X							X	X			Regeneration and homeostasis appear normal	240	*Smed-mcg-1*
Smed-nudC	X								X							X					236	*Smed-nudC*
Smed-nudE		X					X									X				Mitotic spindles are misorganized, and many neoblasts may be stuck in metaphase/anaphase	236	*Smed-nudE*
Smed-p53			X				X				X							X		Outgrowths develop that express various tissue markers	227, 240	*Smed-p53*
Smed-piwi-2			X		X (regenerate)		X (intact)									X	X			Neoblast progeny in epithelium have abnormal morphology; decrease in putative piRNA expression	96, 223, 240	*Smed-piwi-2*
Smed-piwi-3		X							X							X	X			Decrease in putative piRNA expression	223, 240	*Smed-piwi-3*
Smed-PrkC				X					X							X			X		84	*Smed-PrkC*
Smed-PRMT5		X							X							X			X	Degrowth; fewer chromatoid bodies; increased transcription of transposons and repetitive DNA; disorganized blastema; may methylate SMD3/SM1-3	238	*Smed-PRMT5*
Smed-PTEN-1/PTEN-2			X				X									X			X	Tissue hyperplasia and disorganization; reduction in some differentiated cell types	224	*Smed-PTEN-1/PTEN-2*
Smed-Rab-11B				X					X							X			X		84	*Smed-Rab-11B*
Smed-Rac-1				X					X							X			X		84	*Smed-Rac-1*
Smed-raptor	X[39,41]	X[40]				X[39,40]					X[39]	X?[40]			X[41]				X	Regeneration of new anatomy in old tissue (39,40)	39, 40, 41	*Smed-raptor*
Smed-RbAP48	X					X										X			X		228	*Smed-RbAP48*
Smed-RRM2				X					X							X			X		90	*Smed-RRM2*
Smed-rtel-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-rtel-1*
Smed-setd8-1									X							X	X			Regeneration and homeostasis appear normal	240	*Smed-setd8-1*
Smed-Smd		X (regenerate)	X (intact)						X							X			X	Chromatoid bodies are lost; impaired splicing of cyclinB	84, 230	*Smed-Smd*
Smed-smg-1	X (intact)		X (regenerate)			X (fed)	X (regenerate & intact)	X					X	X					X	Regeneration of new anatomy in old tissue; outgrowths; epidermal hypertrophy; phenotype rescued by rapamycin	39	*Smed-smg-1*
Smed-soxP-1		X							X			X?					X				240	*Smed-soxP-1*
Smed-sz12-1		X							X							X	X			Reduced blastema and impaired differentiation of photoreceptors	240	*Smed-sz12-1*
Smed-TOR	X[39,41]	X[40]			X (intact[42]) (fed[40])	X (regenerates[39,40,41])(intact[40])				X[39,41]		X?[40]							X	Regeneration of new anatomy in old tissue (39,40); increased TUNEL+ nuclei in intact (40,41) and regenerates (40); some anatomical remodeling occurs (41); decrease in cell size (40); faster degrowth during starvation (41); failure to grow when fed (40)	39, 40, 41	*Smed-TOR*
Smed-vasa-1		X							X			X?						X			240	*Smed-vasa-1*
Smed-vasa-2									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-vasa-2*
Smed-zf207-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-zf207-1*
Smed-zfp-1		X							X		X?							X			240	*Smed-zfp-1*
Smed-smyn-1									X							X	X			Regeneration and tissue homeostasis appear normal	240	*Smed-smyn-1*
~140 additional genes from first planarian RNAi screen				X	SOME	SOME	SOME									X	SOME			All show loss or reduction in blastemas, curling, and/or lysis; awaiting characterization	84, 240	~140 additional genes from first planarian RNAi screen
Dugesia japonica:																						*Dugesia japonica:*
Djsgo-2		X				X										X			X		231	*Djsgo-2*
Djbruli		X				X										X			X		231	*Djbruli*
Djcbc-1	X						X									X			X		209, 231	*Djcbc-1*
Djdicer-1				X					X							X			X		231	*Djdicer-1*
Djedc-4	X				X											X			X		231	*Djedc-4*
DjeIF-2A	X					X										X			X		231	*DjeIF-2A*
DjeIF-3A	X					X										X			X		231	*DjeIF-3A*
DjeIF-4a1				X					X							X			X		231	*DjeIF-4a1*
DjeIF-4a3				X					X							X			X		231	*DjeIF-4a3*
DjerK4 / MEK inhibitor U0126	X				X			X								X			X	Reduction in differentiated cells labeled after 5 days of BrdU administration	232, 233	*DjerK4 / MEK inhibitor U0126*
Djgaminin-5				X					X							X			X		231	*Djgaminin-5*
Djhsp60		X							X							X			X		237, 242	*Djhsp60*
Djhsp90				X					X							X			X	Degrowth	237, 241	*Djhsp90*
Djmot		X				X										X			X		226, 242	*Djmot*
DjP2XA				X		X (starved)	X (fed)									X			X	Increased frequency of fissioning, animals regenerate normally	239	*DjP2XA*
Djpolpc-1				X					X							X			X		231	*Djpolpc-1*
Djpumilio		X				X										X			X		203	*Djpumilio*
Djsalf-1	X						X									X			X		231	*Dj-salf-1*
Djsalf-2				X					X							X			X		231	*Dj-salf-2*
Djves-1	X						X									X			X		231	*Djves-1*
Djsrn-1	X						X									X			X		231	*Djsrn-1*
Djsrn-2	X				X											X			X		231	*Djsrn-2*
JNK inhibitors SP600125 & AS601245	X					X										X			X	Loss of expression of the early wound-response gene *Djnjlk* and brain marker *Djsoh*	233	JNK inhibitors SP600125 & AS601245
Schmitta polychroa:																						*Schmitta polychroa:*
Spolhsd-1	X				X											X			X	Neoblasts function normally without SPOLTUD-1; for ~5 weeks, then neoblasts are lost	225	*Spolhsd-1*

All phenotypes characterized that cause a loss or reduction in blastema formation (not associated with a known polarity defect) and/or ventral curling and lysis are listed in alphabetical order for each species.[39–41,57,84,87,90,94,96,203,205,209,217,223–242] Reported data regarding numbers of neoblasts, cell proliferation based on Phospho-Histone H3 (Ser10) staining, expression of early and late neoblast progeny markers,[90] results from clonogenic expansion assays,[240] and miscellaneous phenotype data are included.

a background completely devoid of neoblasts. These experiments have revealed a striking plasticity of the differentiated tissues during regeneration that occurs on a body-wide scale.

After irradiation ablates neoblasts and depletes their recent division progeny, the animals cannot regenerate new tissues. However, they can still undergo normal transcriptional responses after amputation. For example, in the complete absence of neoblasts, the differentiated tissues upregulate expression of early wound-response genes, in addition to re-specifying the A/P axis within 1 day post amputation (dpa).[73,134,152,232,246] Further examination of cell death dynamics reveals that two distinct waves of apoptosis occur within 4 hpa and 3 dpa. Surprisingly, the amount of apoptosis measured by TUNEL positive nuclei is normal in the absence of neoblasts, meaning that the cell death required for proper tissue remodeling occurs independently of stem cells.[247] Finally, it has been shown that the differentiated tissues in irradiated animals can dynamically modify body-wide transcriptional output for at least four days after an amputation, as evidenced by the oscillation of *Smed-wnt11-5* expression across the A/P axis in irradiated tail fragments. It is only after 4 dpa that obvious defects in the expression of this gene become apparent, suggesting that the differentiated tissues may eventually need to integrate their new positional identity with the regenerated anatomy or neoblasts after a certain point in time.[73,134] While it is still unknown whether a niche for neoblasts exists, neoblasts and their local microenvironment are likely not the only elements required to understand planarian regeneration. Something about the nature of the pre-existing differentiated tissues as a whole could be an important factor in determining to what extent an animal—whether it be a planarian or a human—can regenerate.

LOOKING TO THE FUTURE

After centuries of fascination with planarians, their regenerative abilities have transformed from a curiosity ultimately deemed intractable for detailed study by Morgan to an established animal model of regeneration. If classical biologists could have peered into the future, they would probably have been impressed by the amount of knowledge generated in just the past 15 years. Topping this list of accomplishments, it was confirmed that a pool of pluripotent neoblasts act as stem cells to replenish missing tissues. Numerous genes important for neoblast self-renewal and differentiation have been identified, and the first markers of neoblast lineages have been described. The molecular principles underlying axial polarity and organogenesis are already being teased out. In addition, the surprisingly dynamic behavior of whole tissues devoid of neoblasts is challenging us to reassess a stem cell-centric philosophy of regeneration. These results indicate that instead of acting only as a local niche, differentiated tissues may provide a macroenvironment capable of initiating wound responses, specifying axial polarity, and integrating global positional information that direct the subsequent differentiation of neoblasts. Of course, much still remains to be learned about these fascinating organisms.

From the top down, there are many facets of planarian regeneration biology to be elucidated. At the highest level, the rapid changes in transcriptional output observed after amputation or wounding suggest that the chromatin landscape and access to genomic promoters must be tightly coordinated. Chromatin dynamics will likely play key roles in these global transcriptional responses as the cells of the animal reassess positional information and facilitate the proper differentiation of neoblast progeny. Transcription factors and the targets they regulate in response to injury should also be studied so that gene regulatory networks for regeneration of specific tissues can be made to integrate the large body of functional data that will undoubtedly be generated. Additionally, increased cellular resolution will be required to study the effects of genes that undoubtedly have diverse temporospatial roles during physiological and restorative regeneration. Tools must be developed for indelibly labeling cells *in vivo* for fate mapping and live imaging studies. Single-cell transcriptional profiling, in addition to analysis of post-transcriptional and post-translational modifications occurring shortly after injury will also be key to teasing apart the molecular tapestry underlying regeneration.

By using this knowledge base, we can begin exploring how the mechanisms of regeneration formally compare to planarian embryogenesis. Such a comparison would begin to address the long-standing question of whether regeneration is simply a recapitulation of development or whether it is made possible by independent mechanistic innovations. How, for instance, are embryonic stem cells functionally different from neoblasts? How and when are neoblasts specified during embryogenesis? Is the same genetic toolkit required during embryogenesis to organize the body axes and facilitate organogenesis as it is during regeneration? This comparison may provide vital insights into a particularly curious phenomenon. Specifically, a variety of organisms display an impressive ability to undergo regulative embryonic development. Animals like the mouse, fruit fly, and frog can recover from ablation of numerous blastomeres or substantial

injury to embryonic organs. However, these animals display limited regenerative capacities as adults. Understanding whether regulative development happens in planarian embryos and how it might differ from these other organisms may help us identify key differences crucial to preserving regenerative abilities into adulthood.

Finally, one of the ultimate goals of studying planarian regeneration is to understand why some animals regenerate robustly while others—such as humans—do not. Comparing the mechanisms of adult regeneration in diverse animals that have varying abilities to regenerate may be another way of pinpointing the core requirements for regeneration. Such an approach may also provide insights into the permutations that evolution has enacted upon this biological process over time. The first step in this endeavor should be to compare different planarian species, some of which regenerate robustly, while others display more limited abilities depending upon the plane of amputation. Elegant irradiation and grafting experiments performed on *Procotyla fluviatilis*, which does not regenerate robustly after post-pharyngeal amputation, suggest that variation in regenerative ability may result from the signals provided by the differentiated tissues,[189,248–250] and may not be explained simply by total numbers of neoblasts, as first thought.[208] Revisiting these classical studies with modern tools may help us identify a core set of molecular and physical principles guiding regeneration, which could then be examined in more distantly related animal species.

With a rich history and giant leaps forward in recent years, the future of the planarian field is bright. Our mechanistic view of regeneration will undoubtedly come into much greater focus as more techniques are developed and more investigators pursue questions in this classical model system. It will be exciting to see what biological insights these animals will reveal to us next.

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FURTHER READING

Schmidtea mediterranea Genome Database (SmedGD): http://smedgd.neuro.utah.edu/

Sánchez Alvarado lab website: http://planaria.stowers.org

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