Regeneration

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REVIEW

Go ahead, grow a head! A planarian's guide to anterior regeneration

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Received: 27 January 2016; Revised: 8 April 2016; Accepted: 15 April 2016

doi: 10.1002/reg2.56

Abstract

The unique ability of some planarian species to regenerate a head de novo, including a functional brain, provides an experimentally accessible system in which to study the mechanisms underlying regeneration. Here, we summarize the current knowledge on the key steps of planarian head regeneration (head-versus-tail decision, anterior pole formation and head patterning) and their molecular and cellular basis. Moreover, instructive properties of the anterior pole as a putative organizer and in coordinating anterior midline formation are discussed. Finally, we highlight that regeneration initiation occurs in a two-step manner and hypothesize that wound-induced and existing positional cues interact to detect tissue loss and together determine the appropriate regenerative outcomes.

Keywords

Organizer, patterning, planaria, polarity, regeneration, Wnt, wound

Introduction

Tissue loss after injury is a fundamental threat for all multicellular organisms. While wound healing is a general first response to prevent further damage and invasion of pathogens, the ability to induce complete and scar-less restoration of lost body parts is less common. Yet, regenerative abilities occur in species throughout the animal kingdom, ranging from restoration of the spinal cord, lens and heart as well as regeneration of whole fins or limbs in teleost fish and urodele amphibians, to whole body and bidirectional regeneration in cnidarians and flatworms (Sanchez Alvarado 2000; Mathew et al. 2007; Galliot & Ghila 2010; Poss 2010; Nacu & Tanaka 2011; Seifert et al. 2012).

The cells contributing to regeneration and their distribution in the animal are as diverse as the tissues that are restored. For instance, while regeneration requires cell populations with the potential to multiply, differentiate, and replace missing tissues, the cell types that are activated differ among animals and tissues. In some animals, a limited number of highly potent adult stem cells exist under homeostatic conditions; dedifferentiation or transdifferentiation of tissue-specific cell types is therefore required to produce multipotent cells (Tsonis et al. 2004; Kragl et al. 2009; Jopling et al. 2011). Conversely, other regenerative animals have abundant sources of somatic stem cells ready to be utilized during regeneration (Tanaka & Reddien 2011).

Regardless of their origin, these cells typically form a blastema, a mass of undifferentiated cells that later give rise to missing tissues. Since they need instructions on what tissues to reconstruct, regenerative organisms must also have systems to record and convey positional information that presumably detect what tissues are missing and/or remaining after injuries. Blastema specification and the subsequent formation of local signaling centers allow blastema growth and patterning, resulting in the highly organized regeneration of lost body parts (Rentzsch et al. 2007; Lengfeld et al. 2009; Wehner et al. 2014). While the cellular source of regeneration differs between species, some instructive signals and concepts may be conserved and applicable to all regenerative animals. Consistent with this, a number of conserved signaling pathways such as retinoic acid, fibroblast growth factor (FGF), Wnt, and transforming growth factor β pathways are required for regeneration throughout the animal kingdom; yet the molecular details of their function and their interactions are not well understood (Gurley et al. 2008; Lengfeld

et al. 2009; Poss 2010; Henry et al. 2013; Kato et al. 2013; Wehner et al. 2014).

Interestingly, tissue loss in closely related species can elicit different restorative outcomes. For instance, the African spiny mouse regenerates full-thickness skin and cartilage, while the common laboratory mouse heals with scarring (Seifert et al. 2012). Furthermore, some planarian species can easily regenerate their head after immeasurable permutations of amputations, while others have more restricted regenerative capacities. Strikingly, inhibition of a single patterning factor is sufficient to enhance the inherent limited ability and rescue this deficiency in three species (Liu et al. 2013; Sikes & Newmark 2013; Umesono et al. 2013). This suggests that conserved regenerative networks exist in which specific control nodes determine whether missing tissue is regenerated after an injury. Comparative approaches using experimental paradigms such as the regenerating head in model organisms like planarians will be crucial for identifying these nodes and their underlying cellular and molecular networks.

In this review, we summarize the recent advances in understanding planarian head regeneration and discuss some of the most fascinating unanswered questions related to this topic. What is the role of the anterior pole? How is regeneration initiated? Understanding the basic principles of regeneration in naturally regenerating species may reveal avenues for inducing restorative programs in non-regenerative organisms.

Essentials of planarian regeneration

Freshwater planarians are a classical model for studying regeneration that has re-emerged in the past decade (Newmark & Sanchez Alvarado 2002; Reddien & Sanchez Alvarado 2004). They are triploblastic bilaterians that belong to the order Tricladida, meaning their digestive cavities consist of three main gut branches. Planarians possess a centralized nervous system, a protonephridial excretory system, and reproduce either sexually as hermaphrodites or asexually by transverse fission (Salo 2006). Regenerative abilities vary among planarian species (Morgan 1904; Brøndsted 1969), but *Schmidtea mediterranea* and *Dugesia japonica* possess impressive capacities to reliably regenerate all body parts within days and have developed into the most prominent planarian model species currently studied (Newmark & Sanchez Alvarado 2002; Reddien & Sanchez Alvarado 2004; Salo et al. 2009; Elliott & Sanchez Alvarado 2013).

Production of competent progenitors is conceivably not a limiting factor during regeneration in *S. mediterranea*, since proliferative cells, called neoblasts, account for approximately 20%–30% of all cells (Baguñá & Romero 1981; Baguñá et al. 1989). Recent studies have uncovered a high level of heterogeneity among neoblasts (Scimone et al. 2014a; van Wolfswinkel et al. 2014), including a pluripotent sub-population called cNeoblasts (Wagner et al. 2011). Neoblasts must be instructed to proliferate, migrate, and differentiate into the required cell types at the right time and place (Wenemoser & Reddien 2010; van Wolfswinkel et al. 2014). Understanding these instructions is critical for understanding planarian regeneration. Recent technological advances have facilitated the generation of omics data to elucidate tissue- and cell-specific gene expression programs (Lapan & Reddien 2012; Wenemoser et al. 2012; Boser et al. 2013; van Wolfswinkel et al. 2014; Wurtzel et al. 2015). For example, global gene expression analysis enabled the identification and classification of genes activated during the first hours after tissue amputation (Sandmann et al. 2011; Kao et al. 2013; Wurtzel et al. 2015; Brandl et al. 2016). Some of these genes are transiently activated in response to any wound, including stress-response genes, which are rapidly turned on and off in many cell types (Wurtzel et al. 2015). In contrast, the wound-induced expression of some patterning factors is maintained throughout the regeneration process only at wounds that involve tissue loss (Gavino et al., 2013; Petersen & Reddien 2009, 2011; Wurtzel et al. 2015). These patterning genes, also known as position control genes, are mainly expressed in subepidermal muscle cells that have been proposed to provide a positional coordinate system for regeneration and homeostatic tissue turnover (Witchley et al. 2013). Amongst them are components of conserved regulatory networks that facilitate embryonic development, including Wingless/int-1 (Wnt) and Hedgehog (Hh) signaling pathways (Yamaguchi 2001; Varjosalo & Taipale 2008), which have been implicated in the re-establishment and maintenance of the anterior–posterior (AP) axis during planarian regeneration (Gurley et al. 2008; Iglesias et al. 2008; Petersen & Reddien 2008, 2009, 2011; Adell et al. 2009; Rink et al. 2009). Similarly, bone morphogenetic protein (BMP) signaling was demonstrated to regulate dorsal−ventral (DV) patterning during regeneration and homeostasis (Ogawa et al. 2002; Molina et al. 2007; Orii & Watanabe 2007; Reddien et al. 2007; Gavino & Reddien 2011; Molina et al. 2011), while *slit* and *wnt5* were shown to be required for proper mediolateral patterning (Cebria et al. 2007; Adell et al. 2009; Gurley et al. 2010). Notably, biophysical factors also regulate regeneration in planarians (Nogi & Levin 2005; Nogi et al. 2009; Oviedo et al. 2010; Beane et al. 2011, 2013; Zhang et al. 2011), consistent with the roles of mechanical forces and bioelectric signals in development, repair, and regeneration (Hotary et al. 1992; Nuccitelli 2003; Davidson 2012; Levin 2014) (Table 1). Hence, while neoblasts constitute the construction material, genetic and biophysical signals originating from differentiated tissues, such as muscles, may provide the construction plan for tissue regeneration in planarians.

Table 1. Summary of factors that affect the three stages of head regeneration.

Gene/biophysical process	Function	Reference
Factors affecting head-vs-tail decisions		
wnt1	Promote tail decisions, likely by stabilizing *β-catenin-1*	Adell et al. 2009; Petersen & Reddien 2009
notum	Promote head decisions, likely by inhibiting Wnt1 function	Petersen & Reddien 2011
evi/wls	Promote tail decisions, likely by enabling Wnt1 secretion	Adell et al. 2009
β-catenin-1	Promote tail decisions, maintain posterior identity; suppress anterior identity	Gurley et al. 2008; Iglesias et al. 2008; Petersen & Reddien 2008
tsh	Promote tail decisions, maintain posterior identity; suppress anterior identity	Owen et al 2015; Reuter et al. 2015
APC	Promote head decisions, inhibit posterior identity, likely by promoting β-catenin-1 degradation	Gurley et al. 2008
hh, *gli-1*, *smo*	Promote tail decisions by increasing early *wnt1* expression	Rink et al. 2009; Yazawa et al. 2009
ptc, *sufu*	Promote head decisions by inhibiting early *wnt1* expression	
Membrane voltage	Membrane depolarization promotes head decisions	Beane et al. 2011
Gap junction communication	Inhibition of gap junction communication by octanol or triple RNAi of *dj-inx-5*, *−13*, and *−12* promotes head decisions	Oviedo et al. 2010
Factors affecting anterior pole formation and function		
foxD	Differentiation, maintenance and midline placement of anterior pole cells	Scimone et al. 2014b; Vogg et al. 2014
zic1	Differentiation and maintenance of anterior pole cells	Vasquez-Doorman & Petersen 2014; Vogg et al. 2014
pbx	Anterior pole formation and maintenance	Chen et al. 2013
notum, *fst*	Likely required for anterior pole function	Petersen & Reddien 2011; Roberts-Galbraith & Newmark 2013
pitx, *islet-1*	Required for anterior pole and midline formation	Currie and Pearson 2013; Marz et al. 2013
Factors affecting head patterning		
pbx, *prep*	Define the anterior domain	Felix & Aboobaker 2010; Blassberg et al. 2013; Chen et al. 2013
ndk, *wnt11-6*, *notum*, *fz5/8-4*, *ndl-2*, *ndl-3*, *ndl-4*, *ndl-5*	Regulation of brain patterning along the AP axis	Crebria et al. 2002; Kobayashi et al. 2007; Adell et al. 2009; Hill & Petersen 2015; Scimone et al. 2016
slit	Prevent collapse of tissues towards the midline	Cebria et al. 2007
wnt5	Prevent excessive lateral expansion of tissues	Adell et al. 2009; Gurley et al. 2010
bmp/bmp4, *smad-1*, *nlg8*, *admp*, *nog1*, *smad4*	Regulate dorsal–ventral decisions and patterning during regeneration and homeostasis	Molina et al. 2007; Orii & Watanabe 2007; Reddien et al. 2007; Gavino & Reddien 2011; Molina et al. 2011

Three stages of planarian head regeneration

One of the most awe-inspiring regenerative abilities is head regeneration, perhaps because of the full morphological and functional recovery of a body part that is vital to most animals. Although planarians have a relatively primitive brain and visual system, restoration of the head involves regenerating and functionally integrating various neuronal subtypes that are arranged in specific molecular and functional domains (Umesono et al. 1999; Nishimura et al. 2007a, 2007b, 2008a, 2008b, 2010; Fraguas et al. 2012; Currie &

Pearson 2013; Marz et al. 2013; Cowles et al. 2014). This has to be orchestrated with regeneration of other organs and cell types, such as those making up the digestive, excretory, and muscular systems. For example, planarian anterior regeneration involves gut remodeling to generate or extend an anterior gut branch (Forsthoefel et al. 2011, 2012). The excretory system, consisting of units called protonephridia, must be restored in order to resume metabolic waste removal and osmoregulation (Rink et al. 2011; Scimone et al. 2011; Thi-Kim Vu et al. 2015). Additionally, the network of muscle fibers lining the body wall, mouth, intestine, and eyes, as well as inner longitudinal and intermediate diagonal muscle fibers that run through the mesenchyme, must be re-established (Orii et al. 2002; Cebria 2016).

Following decapitation, a planarian reaches three main milestones to restore its head. First, it must determine that a substantial amount of tissue is missing and that a head, rather than a tail, should be generated at the wound site. Second, the anterior pole, a signaling center with putative instructive properties, must be formed at the anterior tip. Third, highly patterned tissues need to be reconstructed, which requires tight control of numbers, types, and relative positions of cells.

Head versus tail decision

Planarian regeneration is extremely robust, and even small fragments regenerate to form complete and properly patterned animals (Randolph 1897; Morgan 1898). Given that the same cells can give rise to both anterior and posterior tissues depending on the amputation site, it is likely that they are informed about their relative positions by a tissue-intrinsic polarity. Distal signaling centers that guide either anterior or posterior regeneration have to be re-established in response to an unpredicted injury and therefore an unpredicted starting point. Hence, the regenerative response needs to be flexible in order to reliably re-establish these distal poles de novo and ensure consistent morphological and physiological restoration of the previous state.

This flexibility is reflected in the rapid injury-induced changes in expression of positional control genes in muscle cells (Witchley et al. 2013), including components of the Hh and Wnt signaling pathways (Table 1). Knockdown of positive regulators of Hh signaling (*hedgehog* (*hh*), *smoothened*, *gli-1*) resulted in severe AP patterning defects, manifesting in the failure to regenerate tails or ectopic head regeneration at posterior-facing wounds (Rink et al. 2009; Yazawa et al. 2009). Conversely, depletion of negative regulators of Hh signaling (*patched* (*ptc*), *suppressor of fused*) produced animals that displayed defects in anterior regeneration, completely failed to regenerate heads, or ectopically regenerated tails at anterior-facing wounds (Rink et al. 2009; Yazawa et al. 2009).

Wnt signaling is also essential for polarity re-establishment during regeneration in planarians. Inhibiting Wnt signaling via knockdown of *β-catenin-1* resulted in animals that regenerated heads at all blastemas, while *APC* RNAi animals, with constitutively active Wnt signaling, regenerated tails (Gurley et al. 2008; Iglesias et al. 2008; Petersen & Reddien 2008). More recently, *teashirt* (*tsh*), a positive regulator of Wnt signaling during *Xenopus* axial determination (Onai et al. 2007), was also found to be crucial in promoting posterior identities at posterior-facing blastemas during planarian regeneration (Owen et al. 2015; Reuter et al. 2015). Similarly, RNAi against the planarian homolog of Evi/Wls (also known as GPR177 and Sprinter), which is required for secretion of Wnt ligands in vertebrates and invertebrates (Banziger et al. 2006; Bartscherer et al. 2006; Goodman et al. 2006; Fu et al. 2009; Augustin et al. 2012), resulted in regeneration of an ectopic posterior head, among other phenotypes (Adell et al. 2009). In planarians, the Wnt ligand most likely responsible for regulating head-versus-tail decisions is Wnt1 (also known as WntP-1). Consistent with the *β-catenin-1* RNAi phenotype, *wnt1*-depleted planarians failed to regenerate tails or ectopically regenerate heads at posterior-facing blastemas (Adell et al. 2009; Petersen & Reddien 2009; Hayashi et al. 2011). Notum, a conserved feedback inhibitor of Wnt signaling (Kakugawa et al. 2015), plays an opposing role in head-versus-tail decisions, with RNAi resulting in regeneration of headless or two-tailed animals (Petersen & Reddien 2011). Importantly, knocking down *hh* or *ptc* led to decrease or increase in early *wnt1* expression, respectively, implicating Hh signaling in the regulation of the Wnt pathway during the first day of regeneration (Rink et al. 2009; Yazawa et al. 2009). While it is yet unclear how Hh signaling influences *wnt1* levels during regeneration, neither *hh* nor *ptc* depletion affected *wnt1* expression during homeostasis (Rink et al. 2009). Together, the evidence indicates that high levels of Wnt signaling promote posterior identity of a regeneration blastema, while low levels are required for anterior identity.

What may be the role of Wnt signaling in determining blastema identity? In uninjured animals, *wnt1* is expressed in a few cells at the posterior tip of the midline, collectively called the posterior pole (Adell et al. 2009; Petersen & Reddien 2009; Hayashi et al. 2011), while *notum* is expressed at the anterior pole (Petersen & Reddien 2011). During regeneration, *wnt1* and *notum* display two distinct phases in gene expression, with clear parallels in their dynamic expression profile (Fig. 1). Expression of both *wnt1* and *notum* is detected across the wound site in a 'salt and pepper' pattern within 6 h post amputation (hpa) (Petersen & Reddien 2009, 2011; Witchley et al. 2013; Wurtzel et al. 2015). In this 'early phase', *wnt1* and *notum* are expressed in muscle cells at all wounds in a stem-cell-independent manner, with higher *notum* expression at anterior-facing wounds (Petersen

& Reddien 2009, 2011). As regeneration continues, early *wnt1* expression persists exclusively in the posterior while *notum* expression is limited to the anterior. By 48 hpa, *wnt1* and *notum* transcripts are primarily detected at the posterior and anterior poles, respectively. This 'late phase' activation of *wnt1* and *notum* is stem cell dependent and re-establishes their homeostatic expression pattern (Petersen & Reddien 2009, 2011).

Such distinct spatio-temporal expression characteristics strongly suggest the existence of two Wnt-signaling-dependent phases of regeneration with potentially distinct functions. Indeed, it was shown that early *wnt1* expression is necessary for establishing positional identities in blastemas that correspond to existing tissue polarity (Petersen & Reddien 2009). For example, early but not late stage *wnt1* expression was detected at lateral wounds, making these injuries useful for separating the two phases of *wnt1*. Strikingly, *wnt1* RNAi led to the generation of ectopic head tissues at these lateral wounds, indicating that early Wnt1 is involved in polarity decisions (Petersen & Reddien 2009). Additionally, *wnt11-5* (also known as *wntP-2*), a Wnt1 target expressed at posterior-facing wounds, was induced in stem-cell-deficient planarians that cannot induce late phase *wnt1*, indicating that late phase *wnt1* is not needed for the initial head-versus-tail decision (Petersen & Reddien 2009; Gurley et al. 2010). Early wound-induced *wnt1* might therefore be involved in interpreting existing tissue polarity and establishing corresponding blastema identities, paving the way for the subsequent formation of either an anterior or a posterior regeneration pole.

Surprisingly, *notum* was the only gene detected as differentially expressed between 6 and 12 h after tissue amputation when whole transcriptomes of anterior- and posterior-facing wounds were compared (Wurtzel et al. 2015). Asymmetric expression of early *notum* suggests that the head-versus-tail decision process starts within the first 6 hpa. Perhaps formation of the poles marks when this decision is complete and anterior blastemas are no longer pliable to make posterior tissues and vice versa. Interestingly, perturbation of Hh signaling affects the early phase of *wnt1* expression and results in changes in head-versus-tail decisions, without affecting early *notum* expression (Rink et al. 2009; Yazawa et al. 2009; Petersen & Reddien 2011). Conversely, *β-catenin-1* and *APC* RNAi affects levels of early *notum* expression, but not *wnt1* (Rink et al. 2009; Petersen & Reddien 2011; Scimone et al. 2014b). Since Notum is a Wnt inhibitor, this suggests that antagonistic activities of Wnt1 and Notum, rather than absolute expression levels, determine polarity outcomes.

Notably, one factor that is often overlooked is the influence of biophysical properties on regenerative outcomes, despite their key role during embryogenesis and regeneration in many organisms (Levin 2007, 2014). For example, inhibition of gap junction communication by organic compounds or triple knockdown of *innexin-5*, *-13* and *-12* induced ectopic generation of heads at posterior-facing blastemas (Nogi & Levin 2005). Similarly, increase in intracellular calcium levels by treatment with praziquantel resulted in two-headed animals, which was rescued by knockdown of voltage-operated calcium channel subunits (Nogi et al. 2009). Supporting this, H^+,K^+-ATPase-mediated membrane depolarization was required to increase intracellular calcium levels at anterior-facing blastemas at 24 hpa, and inhibition of membrane depolarization blocked anterior regeneration (Beane et al. 2011). However, little is known about how bioelectrical cues interact with and fit into the network of key genetic regulators of head regeneration. For example, is early *wnt1* or *notum* expression altered by ectopic increases in calcium signaling? Alternatively, does modification of Hh or Wnt signaling regulate intracellular levels of calcium? Synergistic integration of the various genetic and biophysical factors known to regulate regeneration, as well as modeling these interactions to predict various system features that can be validated experimentally, represents a new aspect of a young but growing field (Lobo & Levin 2015; Werner et al. 2015).

Anterior pole formation

Wnt and Hh pathway components are often referred to as 'polarity genes', as RNAi causes alterations of blastema identity and the formation of ectopic heads or tails. In contrast, RNAi against a number of anteriorly expressed genes, such as *foxD*, *zic1* (also known as *zicA*), *follistatin* (*fst*), and *pbx*, resulted in the failure to regenerate a head but without the induction of ectopic posterior markers at anterior-facing blastemas (Table 1) (Felix & Aboobaker 2010; Blassberg et al. 2013; Chen et al. 2013; Roberts-Galbraith & Newmark 2013; Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). This inability to regenerate a head comprises a variety of defective processes, ranging from defects in differentiation and patterning of anterior structures to severe blastema formation defects. Consistent with the two Wnt-dependent phases controlling regeneration, RNAi against these genes did not affect early *wnt1* or *notum* induction, but impaired the later expression of *notum* at the anterior pole (Chen et al. 2013; Roberts-Galbraith & Newmark 2013; Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). This supports the idea that head-versus-tail decisions are made during the early phase of *wnt1*/*notum* expression, while the later phase is required further downstream in the head formation process.

Formation of the anterior regeneration pole constitutes a hallmark of head regeneration. The pole is formed by a cluster of *collagen+* cells, most likely muscle cells, at the midline of anterior blastemas, which is characterized by the co-expression of *notum* and the Activin inhibitor gene *fst*, and the transcription factor genes *foxD* and *zic1* (Scimone

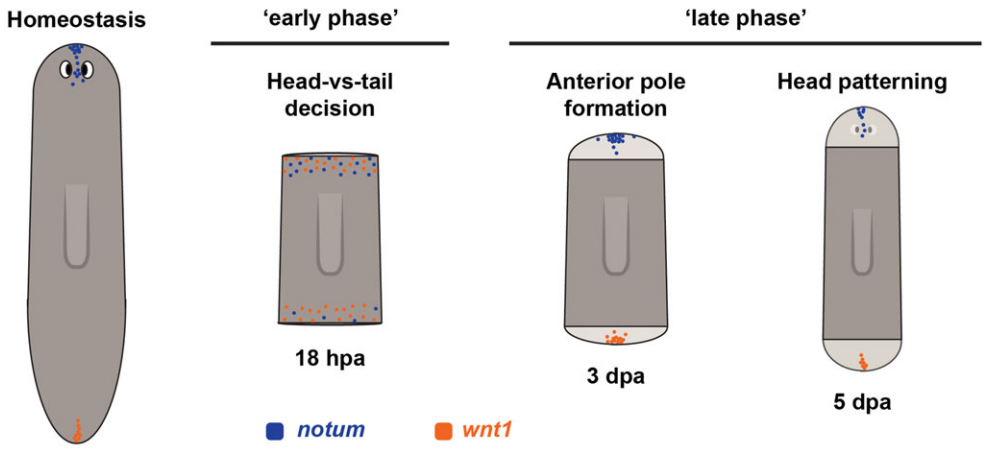

Figure 1. Two phases of *wnt1/notum* expression and function. In uninjured animals, *notum* is expressed at the anterior pole and anterior commissure in the brain, while *wnt1* is expressed at the posterior pole. During regeneration, both genes are induced at all wounds in a dispersed 'salt and pepper' pattern in the early phase, although *notum* expression is higher in anterior-facing blastemas. In the late phase, *notum* and *wnt1* expression clusters at the anterior-most and posterior-most tips of the fragment, respectively, the regeneration poles. As regeneration continues, *notum* and *wnt1* expression domains elongate until the homeostatic pattern is restored.

et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). Interestingly, like *wnt1* and *notum*, *foxD* is activated in two distinct phases. In addition to its expression at the anterior pole, *foxD* expression was detected in subepidermal cells at the ventral midline at anterior- and posterior-facing wounds in a wound-induced and neoblast-independent manner by 6 h after injury (Scimone et al. 2014b). Neither manipulating Wnt nor Hh pathways affected this early *foxD* expression, suggesting that it is activated independently of the head-versus-tail decision as a generic wound-induced gene.

What might be the function of *foxD* at the pole? Given the lack of late *notum* and *fst* expression after *foxD* RNAi, FoxD as a transcription factor may transcriptionally control the expression of genes required for pole formation and/or function (Scimone et al. 2014b; Vogg et al. 2014). Since knockdown of *notum*, *fst*, *foxD,* or *zic1* all interfere with formation of the anterior pole, it is difficult to draw concrete conclusions about their epistatic relationships during regeneration. However, experiments in homeostatic animals show that *foxD* and *zic1* are required for maintaining the other's expression at the anterior pole as well as for the expression of *notum* and *fst*, while *notum* and *fst* are dispensable for the maintained expression of all pole genes tested (Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). This suggests that even though *foxD* and *zic1* are downstream of *notum*-dependent head-versus-tail decisions, they work upstream of *notum* and *fst* at the anterior regeneration pole (Vasquez-Doorman & Petersen 2014; Vogg et al. 2014).

In fact, both *foxD* and *zic1* are induced in *smedwi+* neoblasts within 24 hpa, possibly marking the onset of pole formation, and are later found to be co-expressed with *notum* and *fst* in SMEDWI-1+ neoblast progeny as well as differentiated SMEDWI-1-/*collagen*+ pole cells (Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). Notably, cells appear more differentiated towards the anterior tip, judging from decreasing levels of *smedwi-1* and SMEDWI-1 (Vogg et al. 2014). RNAi phenotypes during regeneration and homeostasis as well as the order and cell populations that these genes are expressed in suggest that *foxD* and *zic1* are required for the differentiation of neoblasts into *notum+/fst+* anterior pole cells (Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). Interestingly, a similar mechanism might give rise to the posterior pole during tail regeneration, where the transcription factor genes *pitx* and *islet1* are required for late phase *wnt1* expression and possibly for pole formation (Hayashi et al. 2011; Currie & Pearson 2013; Marz et al. 2013).

Head formation and patterning

Once the anterior pole is re-established, other genes continue to orchestrate downstream patterning events and ensure commitment of stem cells and progenitors to the correct lineages and appropriate tissues (Table 1). For instance, RNAi against two TALE class homeobox transcription factor genes, *pbx* (Blassberg et al. 2013; Chen et al. 2013) and *prep* (Felix & Aboobaker 2010), blocked head regeneration. Both genes are unlikely to affect head-versus-tail decisions but rather act downstream in stem cell differentiation and/or in the determination of anterior position control genes.

Neoblasts have to differentiate along the correct lineages and in accordance with a positional coordinate system

(Witchley et al. 2013). This process is required during regeneration as well as homeostasis, suggesting that the same regulators may be used in both scenarios. Recent studies identified factors that are required for neoblast differentiation in general, such as CHD4, p53, and MEX3 (Pearson & Sanchez Alvarado 2010; Scimone et al. 2010; Zhu et al. 2015), as well as cues that guide lineage choice (Roberts-Galbraith & Newmark 2015). As head regeneration depends on a multitude of processes, including proliferation and differentiation, failure to regenerate heads should be viewed as a convenient but imprecise readout for genes with roles specific to anterior regeneration.

Interestingly, many differentiated-tissue-associated transcription factors are expressed in planarian neoblast populations. These include *FoxA*, *gata4/5/6*, and *myoD*, which are also expressed in differentiated pharynx, gut, and muscle cells (Adler et al. 2014; Scimone et al. 2014a), as well as multiple central-nervous-system-associated transcription factors (Lapan & Reddien 2011, 2012; Wenemoser et al. 2012; Currie & Pearson 2013; Marz et al. 2013; Cowles et al. 2014). Notably, *coe* is required for regeneration and maintenance of multiple neuronal subtypes, while *lhx1/5-1* and *pitx*, *klf*, and *pax3/7* regulate differentiation of *tph*+ serotonergic, *cintillo*+ sensory and *DBH*+ ventral midline neurons, respectively, during regeneration as well as homeostasis (Currie & Pearson 2013; Marz et al. 2013; Cowles et al. 2014; Scimone et al. 2014a). Similarly, *ovo* encodes a transcription factor that is expressed throughout the eye lineage from neoblasts and progenitors to fully differentiated cells (Lapan & Reddien 2012). *ovo*, *sine oculis* and *eyes absent* (*eya*) are required for differentiation of both photoreceptor neurons and pigment cup cells, whereas *epidermal growth factor receptor 1* (*egfr-1*), *tryptophan hydroxylase* (*tph*), *specificity protein 6-9* (*sp6-9*) and *distal-less homeobox* (*dlx*) are specifically required for regeneration of pigment cup cells (Pineda et al. 2000; Mannini et al. 2004; Fraguas et al. 2011; Lapan & Reddien 2011, 2012; Lambrus et al. 2015).

Advances have also been made towards understanding patterning within the head region. For example, an antagonistic interaction between Notum and Wnt11-6 (also known as WntA or Wnt4a) was recently described to control brain size (Hill & Petersen 2015). Notably, in addition to anterior pole cells, *notum* is produced in cells at the anterior brain commissure, predominantly in *chat*+ neurons (Hill & Petersen 2015). In contrast, *wnt11-6* is expressed in neurons at the posterior brain border (Kobayashi et al. 2007; Adell et al. 2009; Hill & Petersen 2015). Correspondingly, knockdown of *wnt11-6* resulted in posterior expansion of the brain (Kobayashi et al. 2007; Adell et al. 2009; Hill & Petersen 2015), while animals injected with *notum* dsRNA at 24 hpa successfully formed anterior poles and heads but displayed compressed brains (Hill & Petersen 2015). This demonstrates a role of *notum* in brain size regulation independent of head-versus-tail decisions.

Knockdown of *notum* or *wnt11-6* led to a reduction or increase in the number of brain cells, respectively, during regeneration as well as homeostasis (Hill & Petersen 2015). Notably, neuron size and density, functional domains within the brain, pharynx to body ratio, and overall body size were all unaffected in both *notum* and *wnt11-6* RNAi animals (Hill & Petersen 2015). Wnt11-6 most likely regulates brain size through a non-canonical Wnt pathway, since *dvl-1/dvl-2* and *evi/wls* RNAi animals, but not *β-catenin-1* RNAi animals, displayed similar brain expansion phenotypes (Adell et al. 2009; Almuedo-Castillo et al. 2011; Hill & Petersen 2015). This suggests that product(s) from a single *notum* gene interact with both canonical and non-canonical Wnts to inhibit their function in a variety of processes during regeneration and homeostasis.

Knockdown of *nou-darake* (*ndk*), a putative FGF antagonist expressed in brain and muscle cells in the head region, and of *frizzled5/8-4* (*fz5/8-4*), a putative Wnt receptor expressed in anterior muscle cells, also caused ectopic posterior expansion of the brain (Cebria et al. 2002; Witchley et al. 2013; Scimone et al. 2016). Interestingly, simultaneous knockdown of *fz5/8-4* with either *ndk* or *wnt11-6* enhanced this phenotype (Scimone et al. 2016). Furthermore, RNAi of other *ndk-like* genes showed a synergistic effect with the *fz5/8-4;ndk* double knockdown, suggesting cross-talk between *FGF receptor-like* genes and Wnt signals (Scimone et al. 2016).

In addition to the formation of new tissues in the blastema, such as brain and eyes, remodeling of pre-existing tissues, such as the gut, is also required during regeneration to adjust to changes in body length and restore the original body plan (Forsthoefel et al. 2011, 2012). Interestingly, remodeling involves increased levels of cell death and proliferation during both regeneration and homeostasis, when planarians dynamically adapt their body size to food availability (Oviedo et al. 2003; Takeda et al. 2009; Forsthoefel et al. 2011; Gonzalez-Estevez et al. 2012; Hill & Petersen 2015). Moreover, numbers of mitotic cells are strongly elevated in both homeostatic *APC* RNAi and *β-catenin-1* RNAi planarians that are under high and low Wnt conditions, respectively (Reuter et al. 2015). This implies that changes in positional cues may increase tissue turnover to modify tissues according to the new positional values in the planarian coordinate system, and suggests that an adaptation of this system to instructive positional cues precedes tissue remodeling. The ability to dynamically adjust to external challenges requires a surveillance system for constant detection and adaptation of various tissues, and this system might therefore be one of the factors that render planarians 'masters of regeneration.'

Instructive properties of the anterior pole

The anterior pole as an organizer

The inductive properties of the Spemann−Mangold organizer, a small group of cells in the amphibian embryo, were discovered in 1924 when a portion of one embryo was grafted into another (De Robertis et al. 2000). This transplantation led to the induction of a new body axis and showed that the identity of some cells can guide the fate of surrounding cells. The Spemann−Mangold organizer then segregates into head, trunk, and tail organizers with corresponding inductive abilities. Head induction in vertebrates requires the inhibition of BMP and Wnt pathways and the head organizer is characterized by the expression of a set of their antagonists, such as Noggin and Fst. These proteins form signaling gradients, which pattern the early embryo (Niehrs 2004). Interestingly, the anterior regeneration pole in planarians has striking molecular and functional similarities to the vertebrate head organizer. One of these similarities is the expression of Wnt and Activin inhibitors in pole cells, and their requirement for head formation. Notably, transcription factors homologous to the planarian anterior pole genes *foxD* and *zic1* are expressed in and/or are required for the development of signaling centers in other organisms (Pohl & Knochel 2001; Steiner et al. 2006; Fujimi et al. 2012), while Notum and Fst are conserved secreted molecules that regulate fate decisions and differentiation in a variety of contexts (Hashimoto et al. 1992; Fainsod et al. 1997; Giraldez et al. 2002; Flowers et al. 2012). It is therefore tempting to speculate that the planarian anterior pole acts like a head organizer, with FoxD and Zic1 controlling pole formation and positioning and Notum and Fst instructing surrounding cells.

In fact, molecules secreted from the anterior regeneration pole may assist in re-establishing signaling gradients between the pole in the blastema and the pre-existing tissues. The intercalary regeneration model, which was proposed to be a general characteristic of regeneration among metazoans (Agata et al. 2007), states that positional markers along the main body axes can be used as 'molecular rulers', or coordinates that help planarians determine what tissues are missing (Fig. 2A). Consistent with this, many genes are expressed in specific regions along the planarian body axes. Recently, division of the planarian body into multiple zones along the AP axis and RNA sequencing identified transcripts enriched in specific regions (Currie et al. 2016; Scimone et al. 2016). These included transcripts encoding Frizzled receptors, FGF receptor-like proteins, as well as HOX transcription factors, conserved regulators of AP patterning (Schilling & Knight 2001; Hueber & Lohmann 2008; Mallo et al. 2010). Some of these are expressed in a graded and Wnt1/β-catenin-1-dependent manner and enriched in the posterior (Gurley et al. 2008; Iglesias et al. 2011; Owen et al. 2015; Reuter et al. 2015; Currie et al. 2016), while others are mainly found in the trunk or the head. Interestingly, ectopic formation of organs along the AP body axis of intact planarians was observed after modulating the expression of *wnt11-5*, *ptk7*, or *FGF receptor-like* genes suggesting that their signaling networks may be part of the 'molecular ruler' (Cebria et al. 2002; Lander & Petersen 2016; Scimone et al. 2016).

According to the intercalary model, juxtaposition of tissues that originate from different positions along these axes, resulting in contact of tissues with conflicting positional cues, induces generation of missing parts of the 'ruler' (Fig. 2A). Consequently, for intercalary regeneration to occur at an anterior-facing wound, the anterior-most identity must first be established at the distal tip of the blastema to juxtapose the positional coordinates encoded in the pre-existing tissues at the amputation site. The pole may act as this required anterior-most positional determinant: Notum produced in pole cells may shift the putative Wnt signaling activity gradient posteriorly along the AP axis resulting in extremely low levels at the pole juxtaposing higher levels in the pre-existing tissue. Genes responsive to this gradient, such as *abdBa*, *sp6*, *tsh*, and other Wnt/β-catenin-dependent genes that are expressed in neoblasts as well as differentiated cells (Owen et al. 2015; Reuter et al. 2015), may then instruct neoblast progeny to fill in between the pole and the pre-existing body part, restoring lost tissues.

Interestingly, experiments that involved transplanting grafts along the AP and DV axes, such as grafting tail tissues into prepharyngeal areas or reversing the orientation of a graft along the DV axis (Kato et al. 1999; Kobayashi et al. 1999), also induced the ectopic generation of tissues that normally lie between the juxtaposed positions (Fig. 2B, C). Regardless of whether the intercalary model truly represents the molecular mechanisms underlying regeneration, this observation complicates the typical definition of organizers as 'cells that influence the fate of surrounding cells' in planarians, since other tissues can also have instructive properties given the right context. However, analyzing the sequence of early head regeneration events in detail using molecular markers for regionalization in the blastema and neoblast differentiation should reveal whether pole formation precedes the induction of new tissues. Moreover, analyzing early markers of regeneration, such as *wnt1* and *notum*, in grafted and surrounding tissues may reveal currently unknown aspects of the intercalary regeneration model.

Role of the anterior pole in anterior midline formation

Erroneous head-versus-tail decisions can lead to extreme phenotypes, such as ectopic regeneration of tail tissues in anterior-facing blastemas, or total failure to regenerate

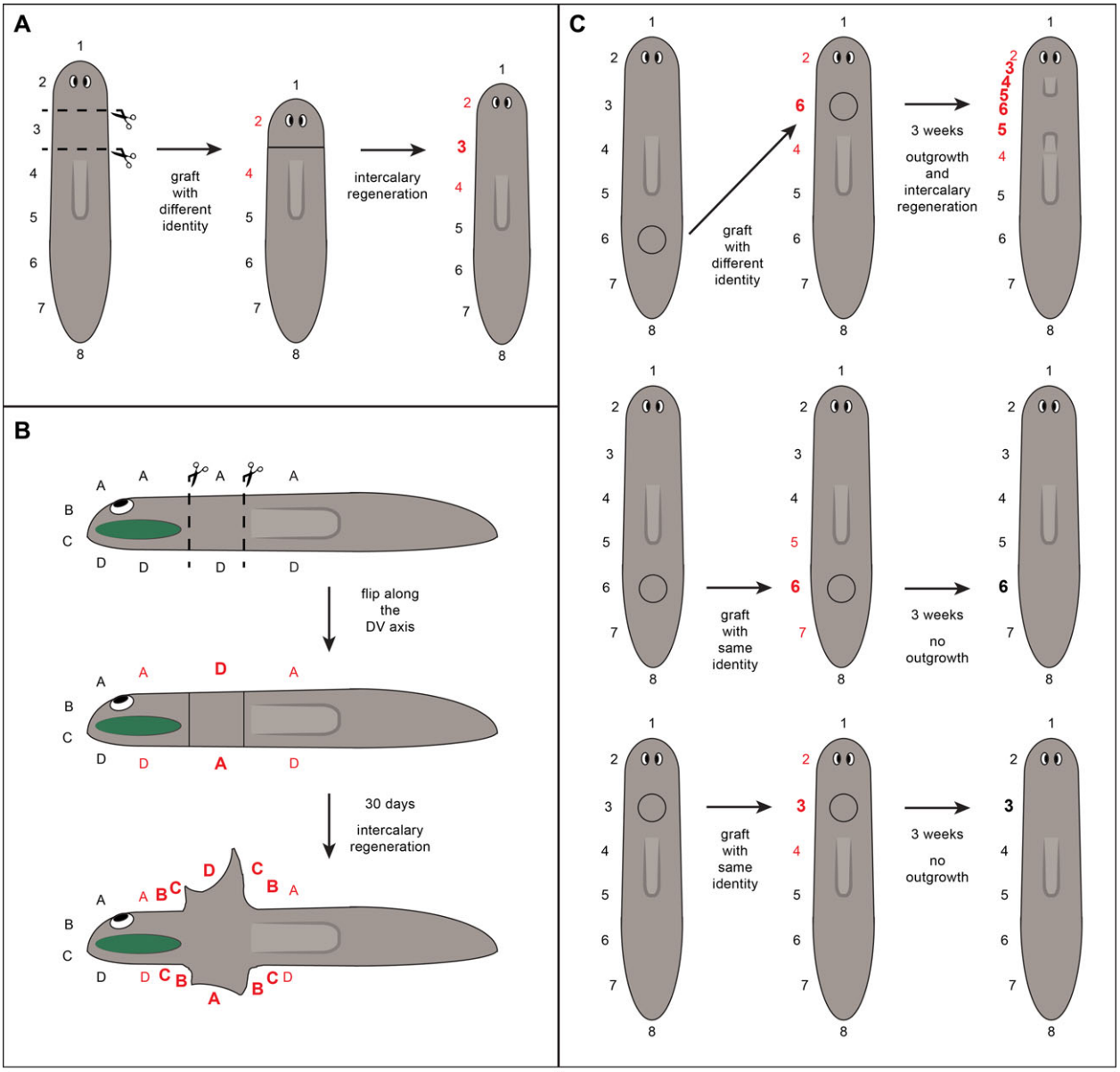

Figure 2. Juxtaposition of tissues with different positional identities induces intercalary regeneration. Differential expression of position control genes, biophysical properties and presence of different tissues manifest positional identities along the AP (1–8; 1 is most anterior, 8 is most posterior) and DV (A–D; A is dorsal, D is ventral) axes. (A) Grafting head fragments onto pharyngeal areas induces intercalary regeneration of the missing anterior areas (Reddien & Sanchez Alvarado 2004). (B) Reversing the orientation of a prepharyngeal graft along the DV axis induces generation of ectopic outgrowths (Kato et al. 1999). (C) Grafting posterior fragments into anterior areas induces development of outgrowths and regeneration of new pharynges on either side of the outgrowth (Kobayashi et al. 1999). In contrast, grafts from posterior tissues into tail areas or anterior tissues into prepharyngeal areas did not induce formation of outgrowths (Kobayashi et al. 1999).

(Gurley et al. 2008; Rink et al. 2009; Roberts-Galbraith & Newmark 2013; Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). In these cases, the animals completely fail to regenerate their anterior poles. However, in milder cases, where anterior pole formation and/or function are merely impaired, anterior regeneration may occur (Currie & Pearson 2013; Marz et al. 2013; Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). In theory, a defective anterior regeneration pole could manifest in different phenotypes, such as correctly positioned but poorly developed brains or completely disorganized masses of differentiated tissues (Adell et al. 2009; Almuedo-Castillo

et al. 2011; Fraguas et al. 2011). Instead, planarians partially depleted of factors required for the formation of the anterior pole, such as *foxD*, *zic1*, *prep*, *pbx*, and *ptc*, frequently displayed fused brain lobes and cyclopic or fused eyes (Rink et al. 2009; Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). In such cases, where tested, expression of midline genes such as *slit*, *admp*, and *ephR1* were defective in anterior but not posterior parts of the animals (Scimone et al. 2014b; Vasquez-Doorman & Petersen 2014; Vogg et al. 2014). This indicates that the observed collapse of anterior organs and positional markers along the midline is not the consequence of a general midline defect.

Notably, the anterior regeneration pole not only marks the anterior tip, but also the medial point of the blastema. Together with the correlation between a dose-dependent midline phenotype and strength of the AP phenotype, this raises the possibility that a properly formed anterior pole is a prerequisite for correct anterior midline formation. Correspondingly, the transcription factor genes *pitx* and *islet1*, which are expressed at both anterior and posterior poles, are required for proper midline formation as well as *slit* expression at both blastemas (Hayashi et al. 2011; Currie & Petersen 2013; Marz et al. 2013).

Recently, it was shown that some regenerating *foxD* RNAi animals were capable of forming an anterior regeneration pole, but failed to form it on the existing midline. This suggests a function of FoxD independent of pole cell specification (Scimone et al. 2014b). Unlike other wound-induced position control genes, such as *wnt1* and *notum*, *foxD* is the only gene known to be wound-induced in *slit*+ cells in the ventral midline and only after injuries that require the replacement or repositioning of the midline (Scimone et al. 2014b). Hence, early expression of wound-induced *foxD* may specify the position where *foxD*-dependent pole progenitors are induced to form the anterior regeneration pole. Taken together, these results suggest that *foxD* is important not only for the formation of a functional regeneration pole but also for positioning the anterior pole relative to the existing midline. This strengthens the hypothesis of a functional interdependence between pole and midline formation that appears to be valid for both head and tail regeneration.

A synergistic role of wound signals and positional information in initiating regeneration: an unproven hypothesis

How animals distinguish between wounds that only require healing and those that involve tissue loss and require regeneration is a long-standing question. In planarians, different injury contexts induce distinct responses. Injuries that involve tissue loss activate distinct proliferative and apoptotic programs that eventually lead to blastema formation and tissue remodeling (Pellettieri et al. 2010; Wenemoser & Reddien 2010). Interestingly, however, recent work has shown that most genes that are induced within the first 12 h in response to amputations (involving tissue loss) are also activated in response to incisions (no tissue loss involved) (Wenemoser et al. 2012; Wurtzel et al. 2015). While the expression profile of an early cluster of genes (including stress-response genes) is similar across all injury types, the wound-induced expression of many other genes (including patterning factors) is maintained for longer at wounds that involve tissue loss than at those that do not (Wurtzel et al. 2015). It is surprising that patterning genes, such as *zic1* and *ndk*, were induced within 4 h post injury, since they are unlikely to have a role in wound healing.

These results suggest that regeneration initiation occurs in a two-step process. First, injury generically triggers the regenerative program: the initial response includes rapid transcription of genes required in various injury contexts (Wurtzel et al. 2015). In the second stage, the tissue context is necessary for evaluating whether the regeneration initiation program should continue and, if so, which regenerative response should be deployed. We hypothesize that wound-induced position control factors transmit the information to the surrounding tissue via molecular interactions, resulting in the selective maintenance of genes that are required for responding to a particular injury (Wurtzel et al. 2015). In fact, expression of the patterning gene *fst*, which is wound induced and exclusively maintained at wounds that involve tissue loss, has been shown to be required for blastema formation, and was suggested to act as a gauge that distinguishes between wounds that involve tissue loss and those that do not (Gavino et al. 2015)

There is also evidence to support that wound-induced *wnt1* interacts with positional cues from existing tissues. *wnt11-5* expression requires both Wnt1 and β-catenin-1, the major downstream effector of canonical Wnts. Since *wnt1* is induced at all wound sites but *wnt11-5* is only expressed at posterior-facing wounds, it is likely that surrounding tissues differentially interpret the effects of wound-induced *wnt1* to create this asymmetric *wnt11-5* induction. Binding of Wnt1 to Frizzled receptors on muscle cells may be an example of molecular interactions between a wound and its surrounding tissue. Recently, putative Wnt−Frizzled pairs were identified through their synergistic RNAi phenotypes affecting AP patterning (Lander & Petersen 2016; Scimone et al. 2016). Further studies will probably reveal other wound-induced factors required to decide whether the regeneration initiation program should continue, and if so which tissues should be replaced.

The graded expression of patterning genes along a body axis explains, in part, the different regenerative outcomes in response to the same stimulus. Since positional information must be reset and can change dramatically during

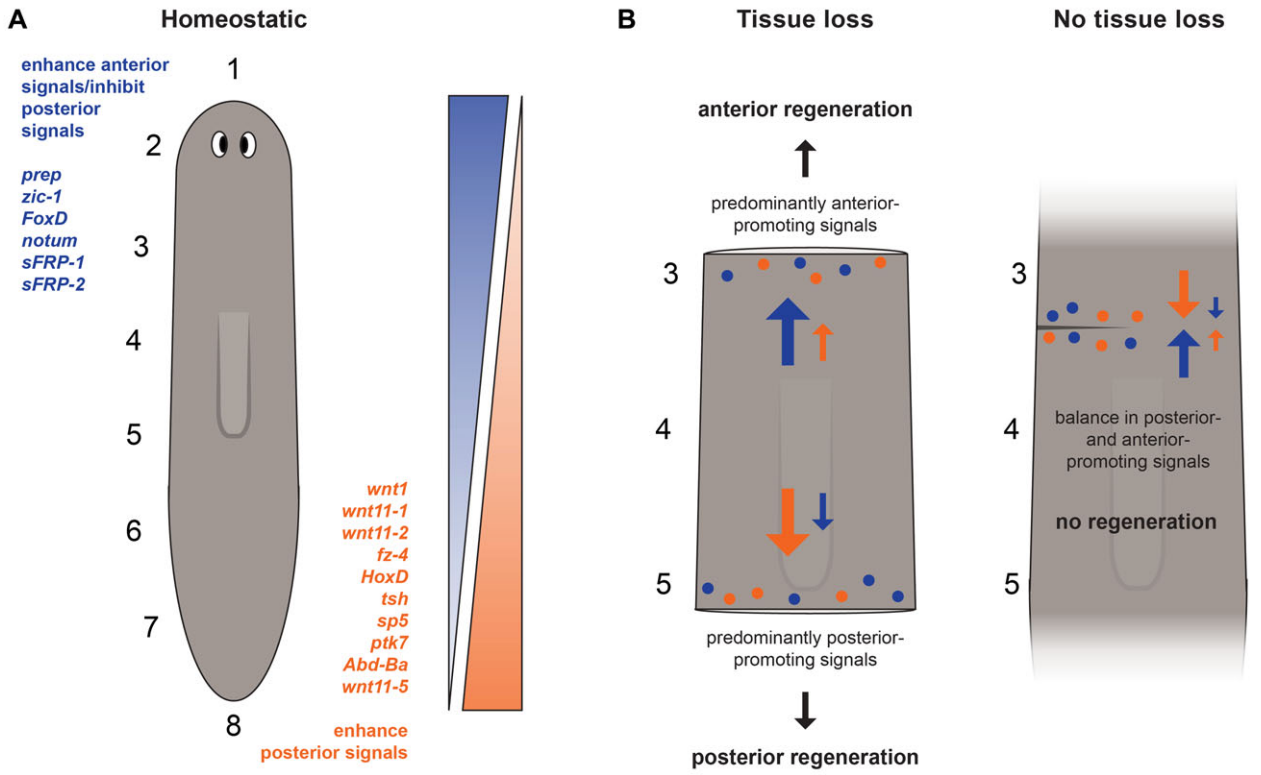

Figure 3. Graded competences along the body axes and their interactions with wound-induced patterning signals may determine the regenerative outcome. (A) Under homeostatic conditions, posteriorizing signals (e.g., Wnt1) are expressed in the tail (orange gradient), while their inhibitors (e.g., Notum) are more highly expressed in the anterior (blue gradient). This creates graded competences along the AP axis, resulting in anterior tissues that are more competent to produce and respond to anterior-promoting signals and less competent to produce and respond to posterior-promoting signals, and vice versa. (B) Anterior- and posterior-promoting patterning genes are wound induced (blue and orange dots) and communicate with surrounding positional information in the existing tissues. Because of the graded competence of cells in this tissue, neighbor-to-neighbor interactions and interactions with wound-induced signals may result in predominantly anterior-promoting signals (blue arrows) at anterior-facing blastemas and posterior-promoting signals (orange arrows) at posterior-facing blastemas of amputated animals. In contrast, anterior-promoting and posterior-promoting signals may be balanced at incisions that do not involve tissue loss, resolving the wound-induced signals and resulting in no regeneration.

regeneration, depending on which tissues remain, animals most likely rely on relative rather than absolute levels of instructive signals. Anterior areas may promote anterior signals and inhibit posterior signals more so than posterior areas. Supporting this, the 'head frequency curve' phenomenon, where anterior-facing wounds induce head regeneration faster when the wounds are placed more anteriorly, suggests that anterior cells are more competent to produce and respond to anterior signals than posterior cells (Child 1911; Sivickis 1931; Evans et al. 2011). Hence, a more anteriorly positioned wound would establish anterior identity more quickly, allowing it to inhibit other wounds from forming additional heads (Meinhardt 2009) (Fig. 3).

In summary, we highlight that various position control genes are first generically wound induced. Following this, they possibly communicate with existing positional information, probably provided by a muscle coordinate system (Witchley et al. 2013). This interaction with wound-induced patterning genes conceivably determines whether regeneration is required and which tissues will be formed. Therefore, the two stages of regeneration initiation would together make up the head-versus-tail phase of head regeneration. It is likely that subepidermal muscle cells possess a positional code manifested in the type and/or number of its cell surface receptors and/or secreted molecules that enable paracrine interactions with neighboring cells and interactions with wound-induced signals. Non-genetic cues, such as reactive oxygen species and calcium, are generically released upon injury in a number of organisms (Love et al. 2013; Razzell et al. 2013); whether and how they may contribute to regeneration initiation through interactions with positional cues remains an open question.

Concluding remarks

Planarians can regenerate after virtually all amputation scenarios. This requires a robust system that instructs stem cells to correctly replace missing tissues. Head regeneration starts with head-versus-tail decisions at amputation sites, which involves temporarily resetting positional information. This is followed by the formation of the regeneration poles, which establishes a new set of positional cues that guide the downstream regeneration and patterning processes.

Interestingly, even at wounds that do not involve tissue loss, many patterning genes are generically induced. These genes, however, are not sustained to the same extent as they are at wounds where tissue is missing. This suggests that a generic wound response in planarians, and possibly in other organisms, is permissive for a variety of regeneration programs, and may also be a critical process in assessing whether and which regenerative responses are required.

Acknowledgments

This work was supported by the Max Planck Society. We thank David Schmidt, Hanna Reuter, Katja Huettner, and three reviewers for critical reading of the manuscript and valuable feedback. The Bartscherer laboratory is part of the Cells in Motion Cluster of Excellence. S.O. is forever grateful to the late Dr. h.c. Songsakdi Owlarn for his consistent and unconditional support. The authors declare that there are no conflicts of interest.

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