# Conserved processes of metazoan whole-body regeneration identified in sea star larvae

Gregory Cary, Veronica Hinman

Short title: comparative regeneration of larval sea stars

Department of Biological Sciences, Carnegie Mellon University, Mellon Institute, 4400 Fifth Ave. Pittsburgh. PA. 15213

Corresponding author:
Veronica Hinman
412 268 9348
veronica@cmu.edu

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**ABSTRACT**

Regeneration is pervasive among the metazoa, but to vastly varying degrees. Platyhelminthes, Cnidaria, and Echinodermata are phyla whose members are capable of whole-body regeneration (WBR). While planaria and hydra have been exemplary models of this phenomenon, the details of echinoderm WBR are less established. We characterize regeneration in the larval sea star (*Patiria miniata*). Normal cell proliferation is reduced following bisection and prior to the onset of wound-proximal proliferation. This blastema-like proliferation is preceded by the restoration of axial patterning. Transcriptome responses highlight functions common to regeneration, such as wound healing and proliferation. Finally, we find evidence for conserved temporal expression of orthologs from published Platyhelminth and Cnidarian regeneration datasets. These analyses show that sea star larvae undergo regeneration through a trajectory including wound response, axis respecification, and blastemal proliferation. Commonalities between this Deuterostome model and other WBR models suggest a deep conservation of whole-body regeneration among the metazoa.

## INTRODUCTION

Questions of how regenerative processes evolve have stimulated researchers for centuries. Examples of species with a capacity for restorative regeneration are distributed throughout the metazoan tree of life (Figure 1A), however the extent to which any animal is capable of regenerating is quite varied. It is only vaguely understood which features of regeneration are common to species possessing robust regenerative abilities and in what ways these features are limited in species with more restricted regenerative capacities. While many attempts have been made to synthesize regenerative phenomena in disparate taxa (Bely and Nyberg, 2010; Sánchez Alvarado and Tsonis, 2006), or to provide an evolutionary context to genes utilized during regeneration within a model (Kao et al., 2013; Petersen et al., 2015), few studies have made direct comparisons between highly regenerative organisms broadly across the metazoa. This is in part because we are as yet missing detailed descriptions of regeneration from key taxa. A goal of making such comparisons is to address to what degree conserved mechanisms underlie regenerative abilities, which has significant implications for whether and how regeneration can be induced in organisms with more limited potential.

Some of the best known models for understanding the mechanisms of regeneration are species of Cnidaria (e.g. *Hydra vulgaris*) and Planaria (e.g. *Schmidtea mediterranea*), which are able to regrow all body parts following amputation, in what has been termed whole-body regeneration (WBR) (Bely and Nyberg, 2010). From extensive studies of regeneration in these organisms, it seems that WBR in these contexts involves transitions through wound healing, immune signaling, axis/organizer specification (especially via WNT signaling), and differentiation of new cells to replace missing cells and tissues (Elliott and Sánchez Alvarado, 2013; Galliot, 2012; Reddien and Sánchez Alvarado, 2004; Wenemoser et al., 2012; Wenger et al., 2014). A key distinction between these models lies in the source of new cells to be differentiated. While planaria utilizes a pool of somatic stem cells (neoblasts) to generate a proliferative blastema that is essential for successful regeneration, hydroids generally do not require cell proliferation for successful regeneration but rather rely on transdifferentiation of existing cells to replace those lost by injury. However, hydra do possess a pool of somatic stem cells (interstitial cells or i-cells) that are both undifferentiated precursors of various hydra cell types (David, 2012) and found to proliferate following injury (Chera et al., 2009). One particularly important question is whether regeneration recapitulates developmental gene regulatory networks (GRNs), and, to date, the embryonic developmental of these animals is poorly described and hence this questions remains unresolved. Species of *Hydra* belong to the Phylum Cnidaria, the radial animals, and planaria are bilateria protostomes. A similarly well studied organism from within the other major grouping of animals (i.e. Deuterostomes) has yet to emerge. This greatly limits our ability to identify mechanisms common to all metazoan regeneration.

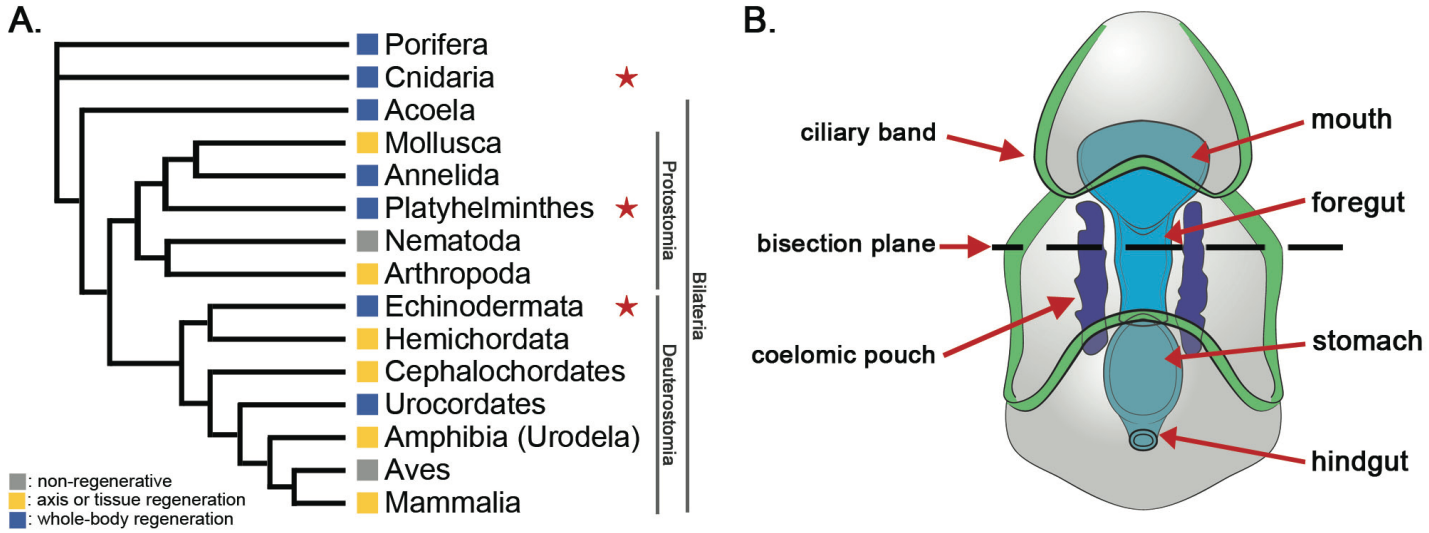


**Figure 1.** Models of whole-body regeneration. (A) Phylogeny depicting regeneration capacity of various taxa, after (Bely and Nyberg, 2010; Somorjai et al., 2012). Species from the three taxa marked with a star were considered in this study. (B) Schematic of a sea star bipinnaria larva indicating the bisection plane (dashed line) and relevant anatomical features including the ciliary band epithelium (green), coelomic pouch epithelium (purple), and enteric organs (blue).

Among the Deuterostomes, regenerative ability in vertebrate animals is frequently restricted to specific developmental stages or only found in certain tissues and organs (Bely and Nyberg, 2010), and therefore these species are not exemplary models of WBR. By contrast, many invertebrate deuterostomes are capable of extensive regeneration of all tissues at multiple developmental stages. There has been an increasing interest in understanding the molecular and cellular features of regeneration in these organisms. Colonial ascidians, in particular (e.g. *Botryllus schlosseri*), are capable of WBR (Kawamura and Sunanaga, 2011; Laird and Weissman, 2004), while other solitary species are capable of partial regeneration (e.g. adult siphons in *Ciona intestinalis*) (Hamada et al., 2015; Jeffery, 2015). Hemichordates (e.g. *Ptychodera flava*) are also capable of extensive regeneration, and can regenerate the adult head when bissected from the body (Luttrell et al., 2016; Rychel and Swalla, 2008).

Echinoderms are a phylum of deuterostome animals that are perhaps best known for their remarkably robust regenerative capabilities that extend throughout all life stages. Adult life stages have been a particular focus of study concerning regeneration mechanisms, and these studies have primarily examined the regeneration of specific adult structures (e.g. spines, tube feet, nerve cord, and gut) (Bannister et al., 2008, 2005; Ben Khadra et al., 2014; Burns et al., 2013; Czarkwiani et al., 2013; Hernroth et al., 2010; Mashanov et al., 2010, 2015, 2014, 2013, 2012; Ortiz-Pineda et al., 2009; Reinardy et al., 2015; Sun et al., 2013; Thorndyke et al., 2001). Cellular proliferation is observed during regeneration in many of these models, as is the modulation of several signaling pathways of developmental importance (e.g. WNT, TGFβ, and Delta/Notch). Regeneration has also been observed in larvae from all echinoderm classes examined (M. C. Vickery et al., 2001). Larval regeneration is more similar to the WBR observed in planaria and hydra, for example, as it requires the complete regeneration of all tissues and organ systems in concert. Studies of larval sea star regeneration have identified several regeneration-specific expression changes including the sea star regeneration associated protease (SRAP) (M. C. L. Vickery et al., 2001)), *vasa*, *nodal*, *dysferlin*, and vitellogenins (*vtg1* and *vtg2*) (Oulhen et al., 2016). However, to date no comprehensive survey of gene expression changes during larval echinoderm regeneration have been reported. The potential impact of studying this process in larval echinoderms is great given that this is one of the few deuterostome taxa with species capable of WBR along with the availability of detailed developmental gene regulatory network information for these species (Davidson et al., 2002; Hinman and Davidson, 2004).

Here, we provide a characterization of the molecular and cellular series of events that occur during larval echinoderm regeneration and then assess the expression patterns of orthologous genes in other WBR models. We employ regenerating larval asteroid *Patiria miniata*. We first characterize the regeneration time course including the landmark events of increased cellular proliferation and reestablishment of gene expression patterns associated with axis polarity specification. Then, sampling RNA from regenerating larval segments and surveying gene expression changes by RNA-seq, we find broad classes of genes similarly

expressed in both anterior and posterior regenerating halves. Finally, through identification of orthologous genes between *P. miniata* and published datasets of regenerating hydra and planarian models (Figure 1A), we find sets of genes that have similar temporal expression profiles in these different regenerating organisms. These results highlight similarities in the regeneration programs of a bilaterian deuterostome, a lophotrochozoan, as well as a basal eumetazoan. Such similarities suggest regeneration features common to the base of the metazoans.

## RESULTS & DISCUSSION

### Respecification of Anterior-Posterior axis precedes onset of blastemal proliferation in the sea star

To make an informed comparison to other regenerative models, we first characterized the broad stages of larval regeneration in *P. miniata*. 7 day old bipinnaria larvae were bisected midway along the transverse Anterior-Posterior (AP) axis (Figure 1B). Both resulting larval segments were completely regenerative, restoring all lost tissues and organs over the course of two weeks, similar to previous reports of larval asteroid regeneration (Oulhen et al., 2016; Vickery and McClintock, 1998). Anterior segments were delayed in their regeneration rate compared to their posterior counterparts.

Two aspects of the regenerative process were assessed for the presence and timing of potential landmark events during regeneration. First, the pattern of cell proliferation was observed using 6 hour pulses of EdU to mark actively cycling cells. The normal pattern of EdU incorporation is widely distributed, but notably concentrated in the ciliary bands, mouth, stomach and coeloms (Figure 2A). We infer from this result that the larvae are actively growing in these regions. Upon dissection, the number of $EdU^+$ cells decreased by 3 days post bisection (dpb), but were otherwise similarly distributed. Beginning at 6 dpb there is a marked change with almost no $EdU^+$ cells localized in either the ciliary band or the gut and instead a dramatic localization of $EdU^+$ cells proximal to the wound site in both posterior and anterior regenerating segments (Figure 2A). This population of $EdU^+$ cells is localized at the regenerating edge, corresponding to the area where tissues form during later stages of regeneration. We therefore infer that the larva has stopped normal growth and has concentrated proliferation to the site of regeneration. Regeneration blastemas in other models are typically described as mixtures of undifferentiated epidermal and mesenchymal cells that proliferate proximal to the wound site in order to produce cells that will become differentiated and replace those lost by injury. Therefore, we consider this region the regeneration blastema, although we have yet to define the cell types or differentiation state of these proliferative cells.

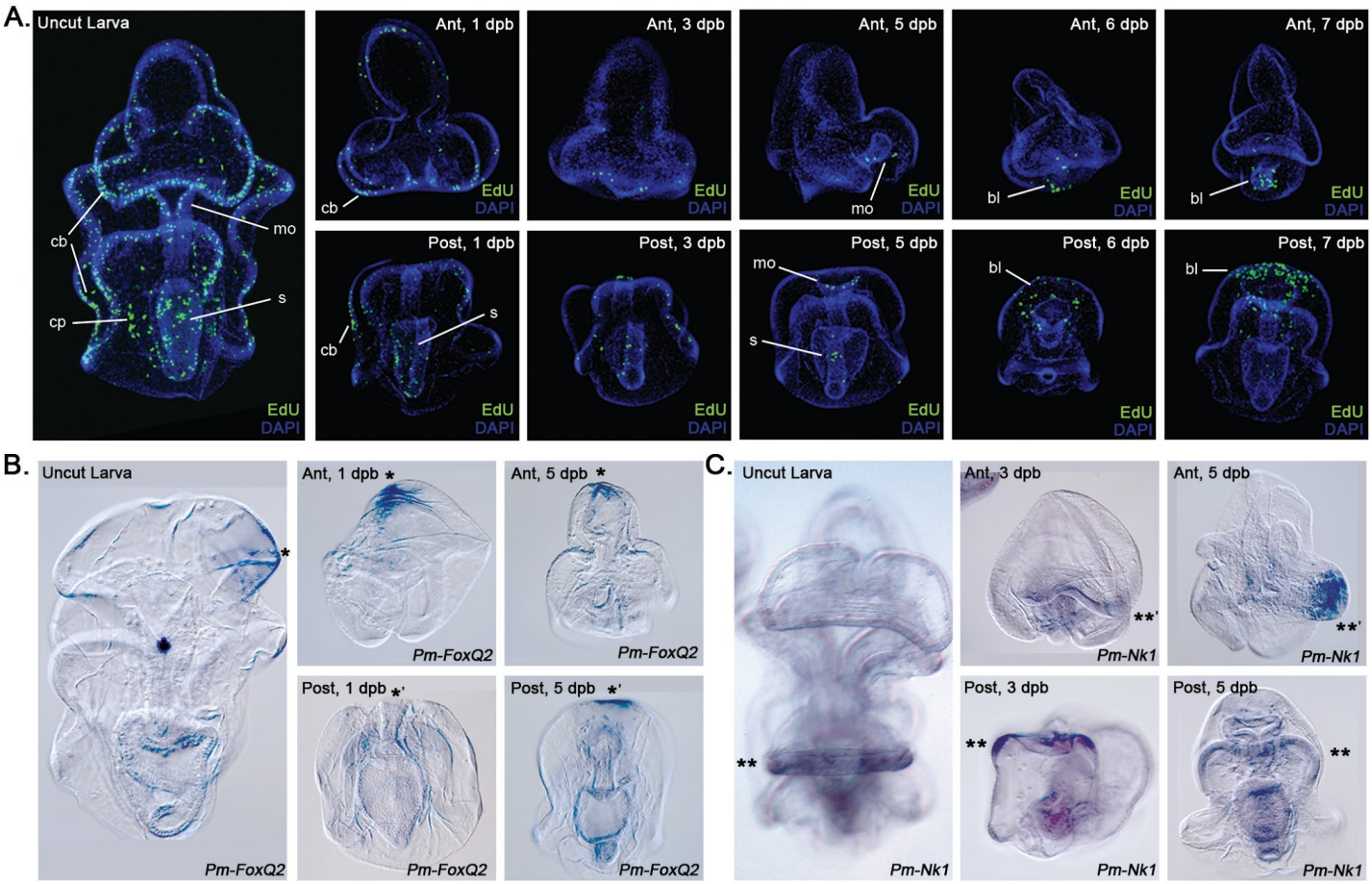


**Figure 2.** Recovery of anterior-posterior axis gene expression precedes blastemal proliferation. (A) EdU staining of S-phase cells in intact and regenerating sea star larvae. Cell proliferation in uncut larvae is concentrated in ciliary band epithelium (cb), mouth (mo), stomach (s), and coelomic pouches (cp). Regenerating anterior segments (top row) and posterior segments (bottom row) demonstrate similar initial distributions of proliferation, although the number of $EdU^+$ cells decreased by 3 dpb. Beginning at 6 dpb, $EdU^+$ cells were concentrated near the wound site in both anterior and posterior regenerating segment in a putative regeneration blastema (bl). (B) WMISH staining shows that *PmFoxQ2* is expressed in the apical domain (*) of intact larvae and regenerating anterior segments (top row). Posterior segments (bottom row) were negative for *PmFoxQ2* staining early, but were stained at the anterior pole of regenerating posterior segments (*') by 5 dpb. (C) *PmNk1* WMISH staining reveals staining in the post-oral ciliary band (**) in both intact larvae as well as regenerating posterior segments (bottom row). Anterior segments (top row) were not stained for *PmNk1* early in the regeneration timecourse, but by 5 dpb there is staining on the posterior aspect of the anterior segment.

We next wanted to understand how genes with known roles in establishing the embryonic axis were expressed during the restoration of these axes during regeneration. A great deal of work has established the GRN for the specification of cell types along the *P. miniata* embryonic axis. *PmFoxQ2* is expressed embryonically in the animal pole domain, where it is involved with establishing an anterior neurogenic GRN (Cheatle Jarvela et al., 2016). This serves as the earliest known marker for the formation of a distinct anterior domain. Uncut larvae and anterior regenerating segments express *FoxQ2* in the anterior domain, while regenerating posterior segments were completely absent any staining immediately following bisection (Figure 2B). *PmFoxQ2* expression was first observed in regenerating posterior segments at 5 dpb. *PmNk1*, by contrast normally expressed in the posterior post-oral ciliary band (Yankura et al., 2010), was observed in uncut larvae as well as posterior regenerating segments but not in anterior segments during early regeneration (Figure 2C). *Nk1* was first expressed in regenerating anterior segments at 5 dpb. This shows that as early as 5 dpb both regenerating anterior and posterior segments have, at least in part, re-established gene expression patterns corresponding to normal AP axis polarity.

These observations of gross cellular and molecular changes during larval asteroid regeneration indicate landmark features of the regenerative process including onset of blastema-like proliferation and re-specification of axes. Moreover, we observe that the restoration of developmental axes precedes the initiation of blastemal proliferation. This pattern is evocative of planarian regeneration where blastema formation and regeneration cannot proceed when axis specification is perturbed (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). Although hydra regeneration does not require blastemal proliferation per se, interstitial cells do proliferate following wounding and this proliferation is initiated by a transient release of Wnt3, a protein implicated in head organizer function (Chera et al., 2009). Thus, across the metazoa, localized proliferative responses during regeneration may require positional signals prior to initiation.

**Gene expression changes delineate phases of larval regeneration: wound healing, axis respecification, and blastemal proliferation**

To better characterize the molecular series of events operating during larval sea star regeneration, and to establish a dataset amenable to inter-species comparison, we surveyed gene expression changes across a time course of larval regeneration. Pools of regenerating posterior segments, anterior segments, and non-bisected sibling control larvae were collected at three points following bisection: one early (approximately 3 hours post bisection, hereafter 0 dpb), one intermediate timepoint (3 dpb), and one timepoint at the initiation of blastemal proliferation (6 dpb). Separately sampling RNA from each regenerating segment allowed for examination of gene expression changes that behave similarly in the anterior and posterior segments as well as those that are specific to the regeneration of each segment. Using RNA from non-bisected, age-matched, sibling larvae as a control diminished the import of expression changes due to continuing larval development

or genetic differences between cultures. Genes significantly differentially expressed between each regenerating segment and the control larvae were identified for each sampled timepoint. In total we identified 9,211 differentially expressed genes (DEG) across all assessed regeneration vs control contrasts.

We implemented a hierarchical clustering approach to distinguish segment-specific expression patterns from those expression changes that are shared in both regenerating segments (Figure 3A). In total, five separate expression clusters were identified: (I) genes upregulated early in both anterior and posterior segments, (II) genes downregulated early in both segments, (III) genes up in the anterior, down in posterior, (IV) genes up in the posterior, down in anterior, and (V) genes up-regulated later (i.e. by 6 dpb) in both segments (Figure 3A). Thus, we have identified three subsets of DEG that have similar expression profiles across our time course independent of which segment is measured (i.e. Clusters I, II, and V) and two subsets that are strongly segment-specific (i.e. Clusters III and IV). To provide further insight into the shared functions of genes assigned to each cluster, we examined the enrichment of Gene Ontology (GO) terms (Figures 3B and S2).

Genes in Clusters I and II, those genes that are either up- or down-regulated early, respectively, in both regenerating segments, are enriched for GO terms that indicate a robust wound response. Up-regulated genes are enriched for terms that include cell signaling pathways (e.g. “MAPK cascade” and “calcium channel activity”), “response to wounding”, and “immune system process” (Figures 3B and S2). Furthermore the enrichments for terms such as “neuron projection development” and “motile cilium” among up-regulated genes indicate the early involvement of innervation and ciliogenesis, which are common in other models of regeneration (Hellman et al., 2010; Joiner et al., 2015; Kumar and Brockes, 2012; Schaffer et al., 2016), as we discuss later. Among down-regulated genes, term enrichments point to a shut-down of anabolic processes such as “ribosome biogenesis”, “gene expression”, as well as primary metabolism more broadly (e.g. “mitochondrion” and “metabolic process”). These term enrichments are consistent with an early response to the bisection insult that involves down-regulation of highly energetic cellular processes and up-regulation of functions that are specific to the injury response (e.g. wound healing and immune signaling).

Clusters III and IV are composed of genes whose profiles are highly segment specific; these are genes specifically down-regulated in one segment relative to the control larvae and unaffected or up-regulated in the other. As we describe in more detail below, many of these genes are asymmetrically expressed along the AP axis, therefore bisection results in the loss of posterior-specific gene expression from anterior segments and vice versa. This is evidenced by the GO term enrichments for these clusters. Cluster III is

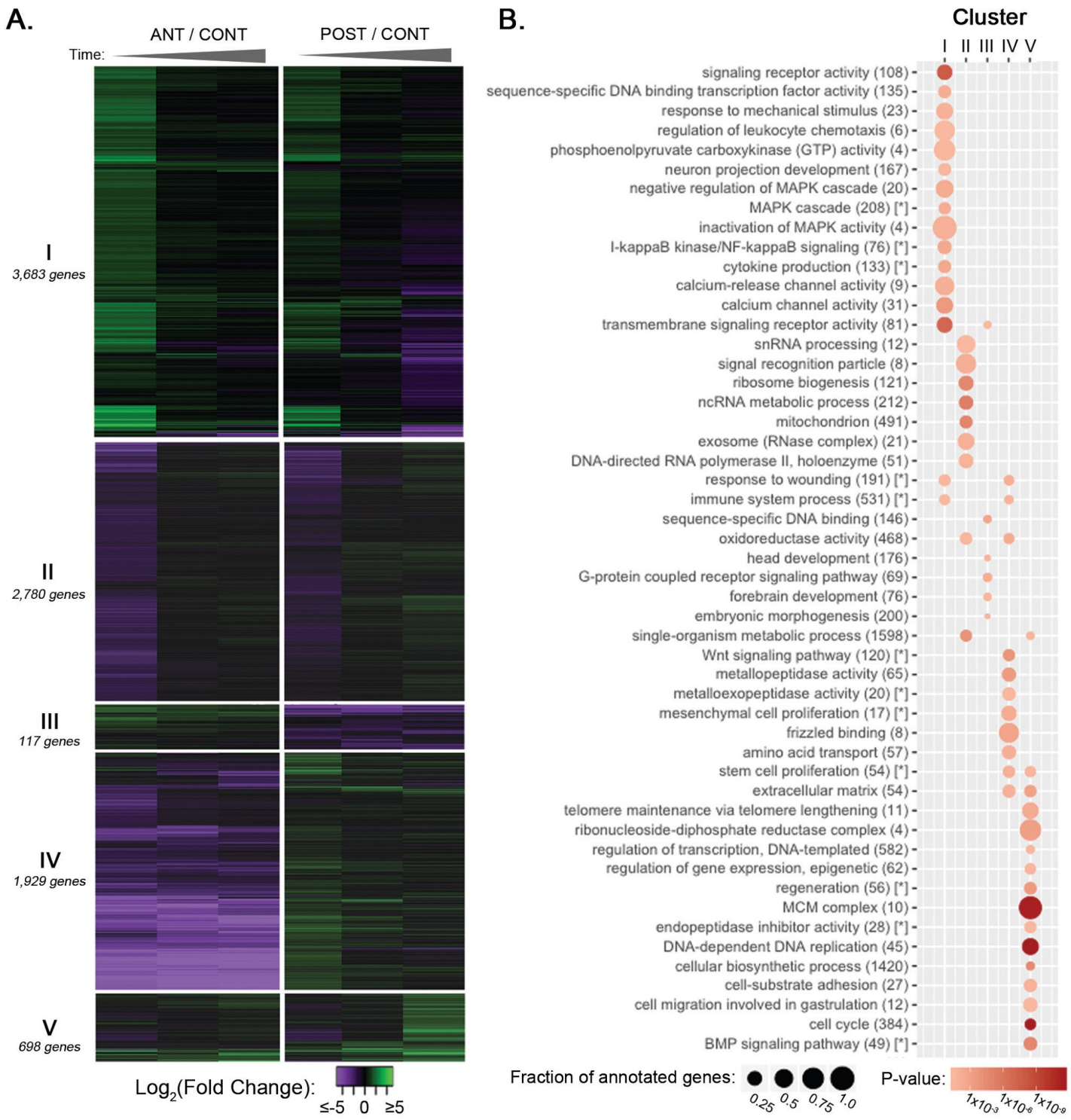


**Figure 3.** Cluster analysis indicates genes involved in regenerative functions. (A) The heatmap depicts log fold change values for genes (rows) in anterior (ANT) and posterior (POST) regenerating segments compared with sibling uncut control larvae (CONT) over the sampled regeneration time points (columns). (B) Gene ontology (GO) term enrichments for each of the five clusters. The enrichment of each GO term is indicated by a circle where the area corresponds to the fraction of genes annotated with that term are present in the cluster, and the color of the circle corresponds to the corrected hypergeometric p-value of term enrichment. Terms marked with a [*] are from the annotation set generated by mouse gene ortholog prediction (Figure S2).

enriched for genes annotated with functions specific to anterior larval segments, such as “head development” and “G-protein coupled receptor signaling pathway” (Burke et al., 2006), while Cluster IV is enriched for genes associated with posterior fates, such as “peptidase activity” and “Wnt signalling pathway” (McCauley et al., 2013). Restoration of these gene expression patterns is a demonstration of functional regeneration and, as we discuss later, we find that several genes within these clusters recover normal expression levels over this regeneration time course (Figure 4).

Although Cluster V is comprised of a relatively small number of genes, it is perhaps the most functionally coherent cluster as the GO term enrichments are among the most statistically significant and most reproducible across the three sources of functional annotations tested (Figure 3B and Figure S2). Genes assigned to Cluster V are enriched for terms related to the cell cycle, DNA replication, and ECM remodeling, among others. Thus Cluster V genes, up-regulated by 6 dpb in both regenerating segments, are likely associated with the onset of localized cellular proliferation observed during this same period of regeneration (Figure 2A). Importantly, these genes are up-regulated in both regenerating segments over control larvae that also clearly contain many cycling cells. Thus, genes assigned to Cluster V may represent a regeneration-specific increase in expression of proliferation associated genes that is distinct from the normal, growth-associated proliferation. Finally, the transcriptome-based observations of AP axis polarity gene expression restoration (i.e. Figure 4) prior to the up-regulation of cell cycle associated genes reinforces our whole-mount staining results (i.e. Figure 2).

Several other trends are suggested by the analysis of GO term enrichments among these clusters. First, we see enrichment of processes that are common to other models of regeneration such as “response to wounding”, tissue homeostasis (e.g. “cell adhesion”, “extracellular matrix”, “metalloproteinases”), cell proliferation, and even “regeneration”. Furthermore, there is enrichment of several specific cell signaling pathways that are implicated in other regenerative contexts, including intracellular $Ca^{2+}$ signaling in wounding, MAP kinase, WNT, and TGFβ/BMP signaling. Notably, several of these pathways are enriched in distinct clusters, suggesting a differential employment of these pathways during specific phases of larval regeneration. For example, $Ca^{2+}$ and MAPK signaling are enriched in Cluster I (i.e. up-regulated early) while TGFβ/BMP signalling is enriched in Cluster V (i.e. up-regulated late). In concert, this clustering analysis highlights sets of genes commonly regulated in both larval segments and underscores broad functional commonalities with other regenerative models.

**Identification of orthologous genes with shared expression dynamics in other WBR models**

Having identified gross phases of larval sea star regeneration (i.e. wound response, axis respecification, and blastemal proliferation), we sought to determine if orthologous genes with similar temporal expression exist in

other models of WBR. Such homology could indicate underlying commonalities of the molecular mechanisms controlling this developmental process. To address this question, we used published transcriptome data from regenerating planaria (*S. mediterranea*) (Kao et al., 2013) and hydra (*H. magnipapillata*) (Petersen et al., 2015) as bases for comparison. The Kao *et al.* dataset was selected because it consolidated several prior planarian transcriptome assemblies, resulting in a more complete gene set, and also independently sampled both regenerating anterior and posterior worms, which is analogous to our own study design. Furthermore, the time points sampled range from 0 hours post amputation (hpa) to 72 hpa, at which point planarian blastemal proliferation is reaching its peak (Elliott and Sánchez Alvarado, 2013), which roughly corresponds to the phases of regeneration considered in our study of larval sea stars. The Petersen *et al.* dataset, on the other hand, represents the only reported transcriptome study of a regeneration time-course in this species. Here, RNA was sampled only from the distal tip of regenerating aboral tissues during the 48 hours it takes to achieve complete head regeneration. As blastemal proliferation is not a feature of hydra regeneration, this characteristic cannot be used to consider synchronization of the regenerative phases in this study to the other datasets. Nonetheless, these published datasets provide a reasonable basis for comparison to our sea star dataset.

We identified orthologous genes between the sea star and each of the other datasets. In order to identify which orthologs share similar temporal dynamics, the reported expression values from each dataset was clustered. For each dataset we sought very coarse cluster classifications such that assigned genes were either up-regulated early and down later or vice versa in their respective time course. The result is three clusters each for the *S. mediterranea* and *H. magnipapillata* datasets (Figure S3 and S4). Finally, we identified genes in each sea star cluster with an ortholog in each of the defined planaria and hydra clusters. Using this approach, we find statistically significant overlaps between genes differentially expressed early in all three datasets as well as genes in the posterior-specific sea star cluster with clusters indicating segment specificity in each of the other organisms. In the following sections we describe how this allowed us to identify not only broad groupings of conserved expression patterns but also specific orthologs similarly expressed across regeneration in these metazoans.

**Axis respecification involves orthologous genes with distinct axial relationships in each model**

Expression of genes in Clusters III and IV, the anterior and posterior-specific clusters respectively, suggest that these clusters are composed of genes asymmetrically expressed along the AP axis. Thus we impute that early down-regulation of anterior genes in posterior segments is a result of the removal of anterior structures, and vice versa. The identities of many of the down-regulated genes support this as, for example, genes normally expressed in anterior domains of the larvae (e.g. *PmFoxQ2*, *PmFrizz5/8*, and others) are initially down-regulated in the posterior segments relative to uncut larvae but are unaffected in anterior segments

(solid lines, Figure 4; Cluster III, Figure 3). Correspondingly, genes normally expressed in the posterior domain (e.g. *PmWnt8*, *PmFrizz9/10*, *PmNk1*, and others) are down-regulated in anterior segments but unaffected in posterior segments (dashed lines, Figure 4; Cluster IV, Figure 3). We noted that many of these genes are components of the previously described developmental GRN and, indeed, we detect a significant enrichment of genes with orthologs in the GRN in the posterior-specific sea star cluster (Cluster IV, hypergeometric $p=9.97x10^{-8}$). Restoration of normal larval gene expression levels along the AP axis is clearly a component of regeneration (e.g. Figure 2B-C). Several genes in each of these clusters do recover normal larval expression levels within the 6 days sampled, although the recovery of many posterior-specific genes is apparently delayed in regenerating anterior segments compared to the recovery of anterior-specific genes in posterior segments (Figure 4). Similar differences have been observed in the rate of planarian regeneration depending on the position of the wound along the AP axis (Reddien and Sánchez Alvarado, 2004).

We examined the genes assigned to these segment-specific clusters to identify genes with similar expression trends in the other datasets. In the planarian data, one cluster is comprised of genes asymmetrically up-regulated in the anterior relative to the posterior segments (i.e. Cluster 2, Figure S3), thus this cluster consists of genes typically expressed in anterior regions of the worm. Paradoxically, a significant number of these genes overlap the posterior-specific sea star cluster (Cluster IV, hypergeometric $p = 1.4 \times 10^{-2}$) (Figure S3). The genes from the sea star posterior-specific set (Cluster IV) found to be overlapping this planarian anterior-specific cluster include *PmFrizz9/10*, *PmWnt7*, and *PmSix1/2*. Sea star *Wnt7* corresponds to the planarian *Wnt2-1, and both SmWnt2-1 (Gurley et al., 2010; Petersen and Reddien, 2008)* and *SmSix1/2* (Lapan and Reddien, 2011) are expressed in anterior domains in planaria. Given that we expect this planarian cluster consists of anterior-specific genes, we also considered the genes that overlap the anterior-specific sea star cluster (Cluster III). One of these overlapping genes is *PmZic*, which is also anteriorly expressed in these worms (Vogg et al., 2014).

Since the hydra dataset reports on RNA sampled from oral regions of the regenerating aboral body stalk, we reason that the signature of late stage up-regulation would reflect oral, or head, gene expression recovery (i.e. Cluster 1, Figure S4). Here as well, there is a significant overlap of posterior-specific sea star genes (Cluster IV) with this set of nominally oral-specific genes in the hydra dataset (hypergeometric $p = 2.7 \times 10^{-3}$) (Figure S4). As the oral-aboral axis of hydra roughly corresponds to the posterior-anterior relationship of bilaterian axes (Guder et al., 2006), oral gene expression in hydra would be expected to be equivalent to posterior gene expression in sea star. The overlapping genes included the sea star genes *PmWnt7*, *PmBra*, *PmWnt5, PmNanos* (XLOC_067690), *PmHox11/13a,* and *PmNotum*. Sea star *Wnt7* is the ortholog of the classical hydra head organizer *Wnt3* (Lengfeld et al., 2009). Hydra *Bra* is also associated with head formation (Technau and Bode, 1999). *HmWnt5* has not been associated with hydra axial patterning, but rather is associated with

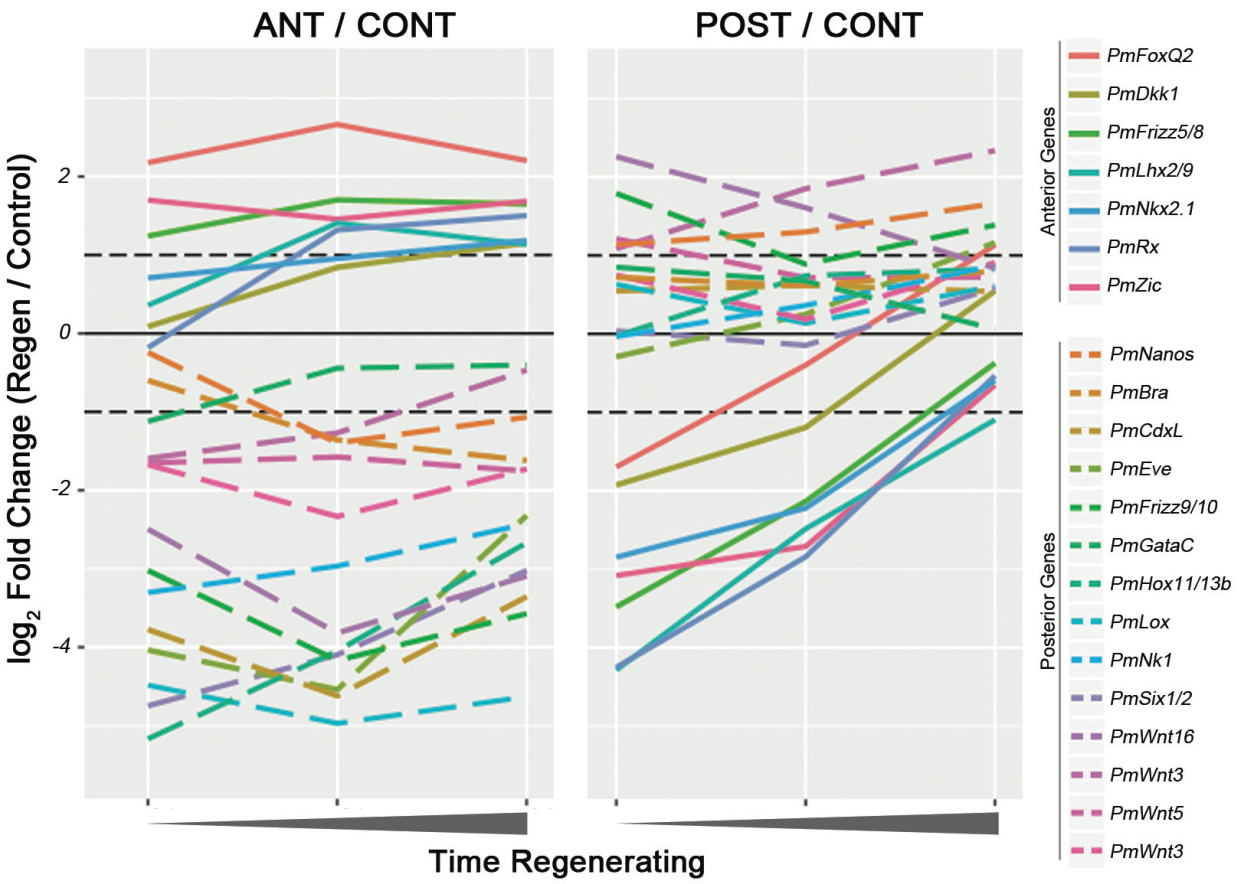


**Figure 4.** Segment-specific recovery of appropriate anterior-posterior gene expression. The expression of genes asymmetrically expressed in either anterior (solid lines, Cluster III) or posterior (dashed lines, Cluster IV) sea star larval territories was examined. The log fold change values for each gene in regenerating anterior or posterior segments compared with non-bisected sibling control larvae is reported for each segment (ANT/CONT and POST/CONT, respectively) over the regenerating time course sampled.

tissue evagination involved in bud and tentacle formation (Philipp et al., 2009), and as such is expected to be expressed in oral regions near the hypostome.

The observed overlap in asymmetrically expressed genes between these datasets suggests a commonality of the regulatory toolkit used for axis respecification, especially WNT ligands and receptors, during regeneration in all of these models. Strikingly however, the orientation of the axes is not conserved, which likely reflects developmental usage.

**Expression of proliferation-associated genes occurs in distinct temporal phases in these models**

The pattern and use of cellular proliferation is one aspect in which these models differ considerably. Both sea star and planaria exhibit concerted wound-proximal, blastemal proliferation that coincides with the final time points sampled here, 6 dpb for sea star larvae and 3 dpb for planaria. There is also a burst of global neoblast proliferation observed early during planarian regeneration (i.e. within 6 hours) (Elliott and Sánchez Alvarado, 2013). No such early increase in proliferation is observed in sea star larvae (Figure 1A). While hydra do not rely on a proliferative blastema to resupply cells for regeneration, interstitial stem cells (i-cells) have been observed to proliferate in the region proximal to the wound within the first 2-4 hours post amputation (Chera et al., 2009). This i-cell proliferation follows the early suppression of mitosis that is observed after wounding (Petersen et al., 2015).

The sea star genes up-regulated at later stages in both anterior and posterior larval segments (i.e. Cluster V) are strongly associated with cellular proliferation and this is correlated with the emergence of blastemal proliferation. We examined these genes for orthologs with similar expression dynamics in the other datasets. None of the planaria or hydra clusters have a significant number of orthologous genes that overlap the genes in this proliferation associated sea star cluster. The planaria and hydra clusters corresponding to up-regulation at later stages in each time course, i.e. planaria Cluster 1 (Figure S3) and hydra Cluster 3 (Figure S4), consist of very few orthologs to the comparable sea star cluster. There is a stronger, though not statistically significant, overlap between the genes up-regulated late in sea star and those up-regulated early in planaria (e.g. Cluster 3, Figure S3) and hydra (e.g. Cluster 1, Figure S4), and many of the overlapping genes in these comparisons are clearly associated with cycling cells (e.g. DNA polymerase subunits, MCM genes, SMC genes, *PmOrc3*, *PmRrm1*, *PmPlk*, and *PmTtk*). Thus, gene expression associated with blastemal proliferation in the sea star are more similar to the genes associated with observed proliferation at early stages in both regenerating planaria and hydra models.

We also examined the expression patterns of genes implicated in blastema regulation in other models and identified genes with expression patterns suggesting a related function in sea star larvae. *Msx* genes are

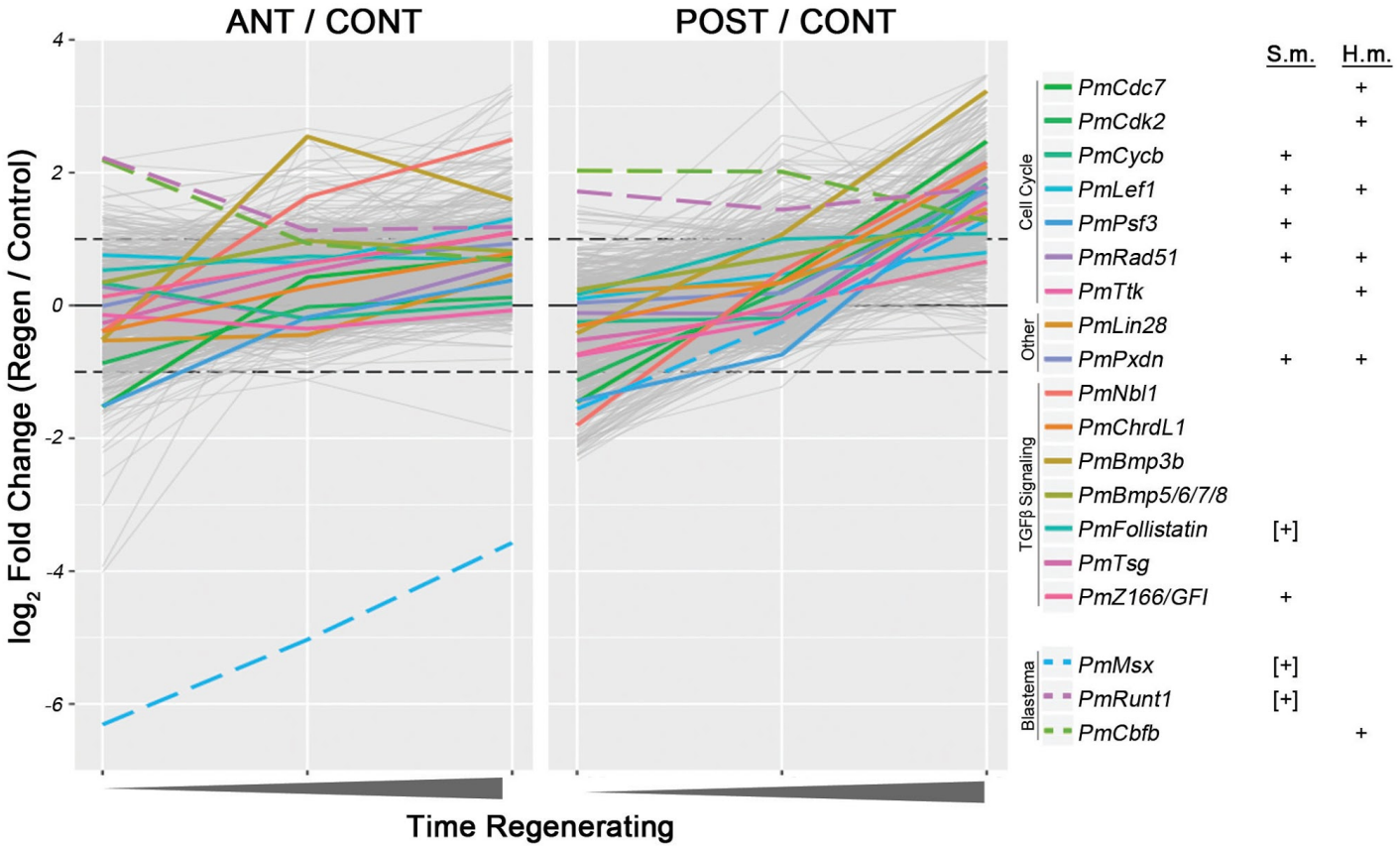


**Figure 5.** Conserved proliferation-associated genes. These data show sea star log fold change values for genes differentially expressed at later stages in regenerating segments compared with non-bisected sibling control larvae (i.e. sea star cluster V). All genes assigned to cluster V are plotted in grey. Several genes, either referenced in the text or representative of functions considered, are indicated with colored lines. The genes plotted with dashed lines are not part of cluster V, but may be associated with blastemal proliferation, as discussed in the text. Next to the key for each gene is an indication (i.e. "+") of whether an ortholog for that gene was found in an analogous cluster in either the planaria (S.m.) or hydra (H.m.) datasets. Indicators in brackets (e.g. "[+]") are those where no overlapping ortholog was identified by our analyses, but genes with the same name were implicated by published datasets.

expressed in regeneration blastema structures in diverse organisms (Akimenko et al., 1995; Koshiba et al., 1998; Somorjai et al., 2012). In planaria, *msx* genes are involved in blastema formation and regulates BMP signaling during regeneration (Mannini et al., 2008). The sea star *msx* gene is initially down-regulated in both regenerating segments, more so in anterior segments than posterior and hence this gene is assigned to the posterior-specific cluster (Cluster IV). However in both segments, *PmMsx* increases expression as regeneration proceeds and is up-regulated in posterior segments by 6 dpb (Figure 5). This corresponds with the late stage up-regulation of a suite of TGFβ signaling genes and modulators, especially in regenerating posterior segments (e.g. *PmBmp2/4*, *PmBmp5/6/7/8*, *PmBmp3b*, *PmTgfbrtII*, *PmFollistatin*, *PmChrdL*, *PmNbl1*, *PmTsg*, *PmVwdf*, and *PmZ166*). Therefore, *PmMsx* may represent a conserved component of blastemal gene regulation in these models.

Finally, the coincident and sustained up-regulation of both *PmRunt1* and *PmCbfb* is particularly compelling (Figure 5). Although *PmRunt1* is up-regulated early (sea star Cluster I), its up-regulation persists into the later stages in both anterior and posterior segments. Runx genes are associated with cell cycle regulation in a variety of contexts (Coffman, 2009; Hoi et al., 2010; Keita et al., 2013). The sea urchin ortholog is mitogenic, regulating Wnt and cyclin gene expression, and functions in concert with *cbfb* in specific contexts (Robertson et al., 2008, 2006). Finally, *runx* genes are both up-regulated early during planarian regeneration, expressed in the neoblasts within the blastema, and required for the regeneration of certain tissues and structures (Wenemoser et al., 2012). We expect that sea star *Runt1* may be involved in larval sea star regeneration in a similar capacity. The assignment of these two genes (i.e. *PmRunt1* and *PmMsx*) to clusters other than Cluster V, in which genes are up-regulated in later stages in both segments and highly enriched for cell cycle functions, indicates that the regulators of blastema formation may not share the same temporal expression profile. Ultimately we find very little evidence for conserved temporal regulation of cell proliferation and blastema formation, underscoring that this is an aspect in which these models may be quite divergent. This discrepancy could reflect inherent differences in stem cell regulation between these models; for example, unlike both hydra and planaria, there may not be a pool of pluripotent progenitors primed for proliferation early following injury in larval sea stars.

**The evolutionarily conserved early regeneration response**

The strongest overlap between these WBR models is among genes differentially expressed early in each dataset. We identified a significant number of orthologs that are up-regulated at early stages in both the sea star and planaria, as well as the sea star and hydra datasets (hypergeometric $p = 4.5 \times 10^{-3}$ and $p = 8.8 \times 10^{-9}$, respectively) (Figure S3 and S4). We also found a significant number of orthologs commonly down-regulated at early stages in the sea star and planaria datasets (hypergeometric $p = 3.3 \times 10^{-4}$). Genes down-regulated early in both sea star and planaria are enriched for GO terms such as “ncRNA processing” and “ribosome”,

indicating a common early repression of the energetically expensive process of ribosome biogenesis. Meanwhile, genes that are up-regulated early across these datasets are enriched for GO terms including "cilium", "calcium transport", and "signaling".

Mobilization of $Ca^{2+}$ and MAPK signaling pathways are both important aspects of wound response in many systems (Aihara et al., 2013; Cheng et al., 2015; Cursons et al., 2015; Leiper et al., 2006; Yoo et al., 2012). Recent proteomic data indicate that calcium signaling is involved in the anterior regeneration in planaria (Geng et al., 2015). MAPK signaling, through both ERK and JNK pathways, is important in the neoblast control and blastema differentiation in planaria (Tasaki et al., 2011a, 2011b). Studies in hydra have demonstrated that wound-responsive MAPK signaling is necessary for early specification of the head organizer, and thus functional regeneration, and that this may be a shared feature of highly regenerative organisms (DuBuc et al., 2014). The observation of similar signatures in the genes up-regulated early in larval sea star regeneration point to these signaling pathways being highly conserved aspects of the response to wounding in these models of regeneration.

The enrichment of cilium associated genes (e.g. *Ccdc11*, *Rsph3*, *Iqcd*, and *Iqub*) (Figure 6A) indicates that in all three models a key set of genes up-regulated early are components of motile cilia. While this feature has not been reported in either planaria or hydra, the role of cilia in wound response and regeneration has been noted in mammals (Joiner et al., 2015) and zebrafish (Hellman et al., 2010), and a recent transcriptomic study of regeneration in a related cnidarian (*Nematostella vectensis*) suggested a similar involvement of ciliogenesis in that model (Schaffer et al., 2016). It is plausible that the up-regulation of cilia-associated genes reflects an early need for sea star larvae to re-establish the ciliated bands, which are critical for effective swimming and feeding. However, the common up-regulation in all of these models suggests that the generation of cilia may be involved more generally in wound healing, perhaps in re-epithelialization of the wound or in communicating cell signals.

There are a number of key regulatory genes that are commonly up-regulated early in all of these models, including orthologs of a number of tumor suppressor genes. The similarities between tumor suppressors and regeneration has been discussed previously (Pearson and Sánchez Alvarado, 2008; Pomerantz and Blau, 2013), and these results present another context in which related processes may be intertwined. In the sea star we observed the cessation of normal patterns of proliferation prior to the emergence of the distinct wound-proximal proliferation in the regenerative blastema. The observed early up-regulation of a suite of tumor suppressor genes (i.e. *Abl*, *Menin*, *Frk, Pten*, *Rbbp6L, Plk2,* and *Wee1*) (Figure 6A), several of which are also up-regulated early in the other models, and down-regulation of ribosome biogenesis genes may be associated with this response. There is also an early signature of general cell cycle arrest in the hydra transcriptome (Petersen et al., 2015). While planarian neoblasts continue to proliferate at sites distal

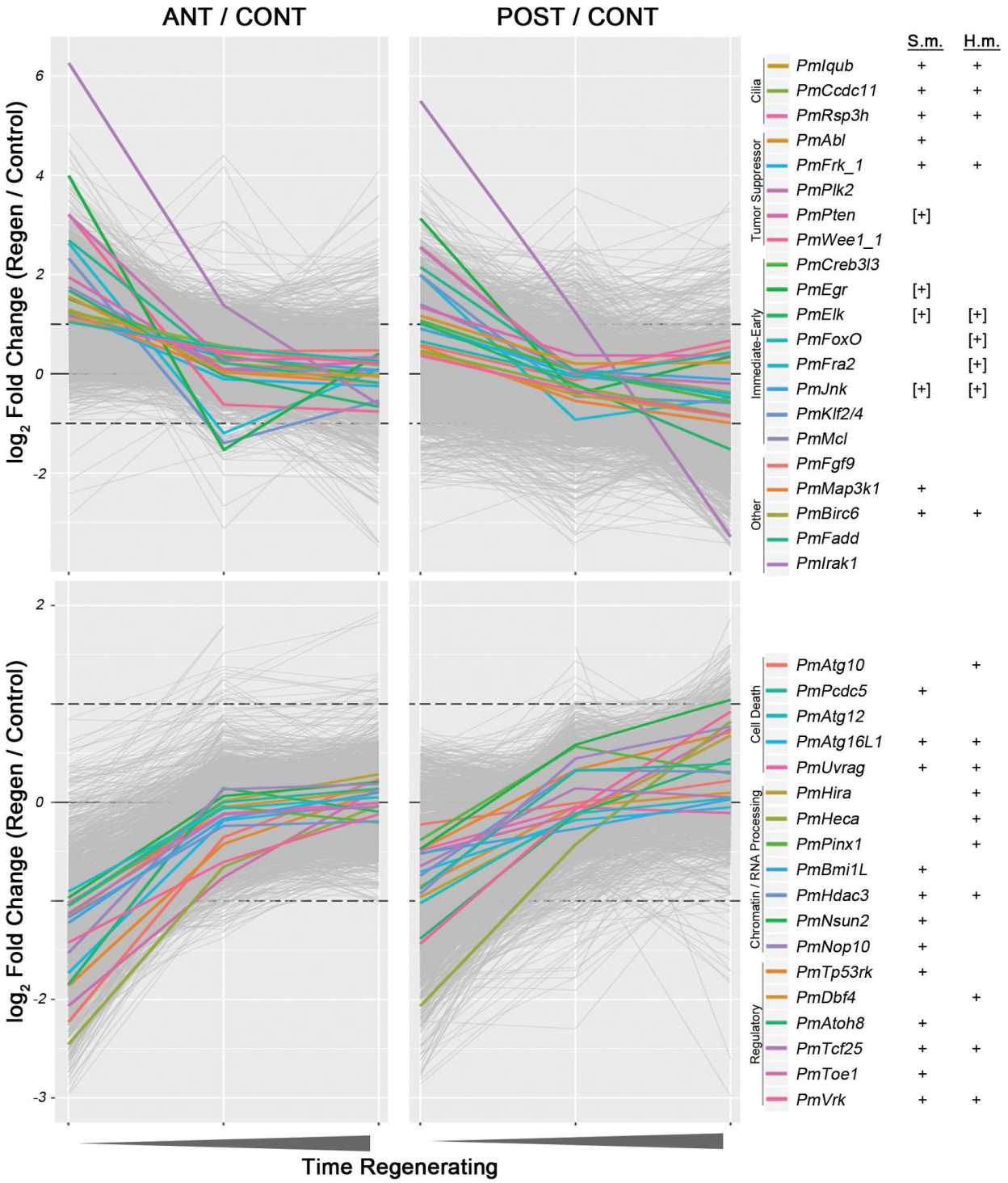


**Figure 6.** Evolutionarily conserved early regeneration response. These plots show sea star gene log fold change values for genes differentially expressed early in both anterior and posterior regenerating segments compared with non-bisected sibling control larvae. Genes up-regulated in both segments (top row) correspond to cluster I and genes down-regulated in both segments (bottom row) correspond to cluster II. All genes assigned to each cluster are plotted in grey. Several genes, either referenced in the text or representative of functions considered, are indicated with colored lines. Next to the key for each gene is an indication (+) of whether an ortholog for that gene was found in an analogous cluster in either the planaria (S.m.) or hydra (H.m.) datasets. Indicators in brackets (e.g. "[+]") are those was no overlapping ortholog identified by our analyses, but genes with the same name were implicated by published datasets.

to the injury even during blastemal proliferation, inactivation of planarian *PTEN* gene homologs resulted in defective regeneration due to neoblast hyperproliferation (Oviedo et al., 2008). These results indicate that a common early feature of WBR in these systems is the modulation of regulators of cell proliferation.

Genes associated with cell death pathways are also commonly differentially expressed early in these models. We note conserved down-regulation of several genes in the autophagy pathway (e.g. *PmAtg16L1, PmAtg12*, *PmAtg10, PmAtg14*, and *PmUvrag*) (Figure 6B) and an increase in the expression of genes involved in regulation of apoptotic and autophagic death (e.g. *PmFadd*, *PmBirc6*, and *PmUlk1*). An early repression of autophagic death is important for effective regeneration in hydra (Chera et al., 2006) while early apoptotic events are necessary for the observed increase in i-cell proliferation (Chera et al., 2009). Apoptotic cell death has also been ascribed to remodeling activities during planarian regeneration and is linked to neoblast proliferation via *Jnk* signaling (Almuedo-Castillo et al., 2014; Pellettieri et al., 2010). Therefore this modulation in cell death pathways during early regeneration may represent an important prelude to cellular proliferation observed during later stages.

Finally a suite of immediate early genes are commonly up-regulated in these models. We observed strong early up-regulation of *PmJnk*, *PmEgr*, *PmMcl*, *PmCreb3l3*, *PmElk*, *PmFra2*, *PmFoxO*, and *PmKlf2/4* in sea star larvae (Figure 6A). We also observed up-regulation, although non-significant, of *PmFos* and *PmJun*. Many of these genes have important functions that have been described in other regeneration contexts. *PmEgr* is one of the most strongly up-regulated genes in both anterior and posterior regenerating sea stars and is also one of the earliest and strongest wound-proximal genes induced during planarian regeneration (Wenemoser et al., 2012). The conserved early down-regulation of the *Egr* repressor *Toe1* in both sea stars and plarnaria suggests these genes are parts of concerted early response in these contexts. There is evidence that several of these early genes are regulated by upstream MAPK and related signaling pathways (Bahrami and Drabløs, 2016). A recent study of the sea urchin *Elk* gene demonstrated that *SpElk* is a target of MAPK signaling (ERK) and regulates both *SpRunt1* and *SpEgr* expression during embryogenesis (Rizzo et al., 2016). In planaria, MAPK signaling (*Jnk*) is necessary for activating the early *Runt1* and *Egr* expression following wounding (Almuedo-Castillo et al., 2014). *Jnk* signaling in hydra has been shown to regulate *FoxO* expression (Bridge et al., 2010), which is an important regulator of hydra i-cells (Boehm et al., 2012).

These overlapping sets of genes differentially expressed early reflect a common response to the bisection insult. It is plausible that these genes are a key shared characteristic between highly regenerative species is 8a specific response to injury that permits the regeneration program. However it is equally potential that the key to understanding the commonality among these highly regenerative species is in how they interpret this common early response and connect this to specific downstream regenerative processes.

## CONCLUSION

While the capacity for larval sea stars to undergo WBR has been appreciated for over two decades, there has not yet been a systematic characterization of the cellular and molecular processes involved. In the present study we demonstrate that larval sea stars exhibit many stereotypical characteristics found in other models of WBR. Through our transcriptome analyses, we detect an early wound-response phase involving significant alterations in the expression of stress response genes, signaling genes (including MAPK, $Ca^{2+}$) and a broad shut-down of energetically expensive anabolic processes (e.g. ribosome biogenesis). The first few days following bisection are marked by a global decrease in the number and distribution cycling cells compared to what is typically observed in growing, non-bisected larvae. This coincides with, or perhaps slightly precedes, the re-establishment of developmental axes, specifically the AP axis ablated by bisection. Re-patterning of the AP axis is observed both through in situ hybridization as well as transcriptome measurements. These observations are facilitated by our extensive prior knowledge of sea star developmental patterning programs and, indeed, genes described by the developmental gene regulatory network are enriched in these clusters. Notably, through both our transcriptome and in situ experiments we observe that axis respecification occurs prior to the onset of wound-proximal cell proliferation, which is the last phase assayed in the present study. This is the first description of concerted, wound-proximal cell proliferation in regenerating sea star larvae. Given that this wound-facing region in both regenerating segments is the primordium from which larval tissues will regenerate, we define this proliferative zone as the regeneration blastema. In this study we have only monitored the first half of the regeneration process up until the emergence of this blastema. Complete regeneration in these larvae takes a total of 10-14 days (Oulhen et al., 2016; Vickery and McClintock, 1998).

In this work we sought to leverage the power of comparing regeneration in a variety of contexts to identify common features. For example, we clustered gene expression levels to identify genes similarly differentially expressed in both anterior and posterior regenerating sea star larval segments. These patterns were then used as a basis for comparison to planaria and hydra regeneration datasets. In the present study we compared regeneration in species that last shared a common ancestor approximately 580 million years ago, at the base of the metazoa. This is the broadest direct comparison of regeneration yet described, encompassing three of the major groupings of animals (Deuterostome, Protostome, and basal Eumetazoan). We find evidence for conservation of both broad functional classes as well as specific orthologs involved with the regenerative process among these models. These commonalities are summarized in Figure 7. The most remarkable signature of shared genes and processes is among genes both up- and down-regulated early in these models. We are potentially most empowered to detect such an overlap among early genes as temporal synchrony between the models likely diverges later in the time course. Nonetheless early changes to $Ca^{2+}$ and

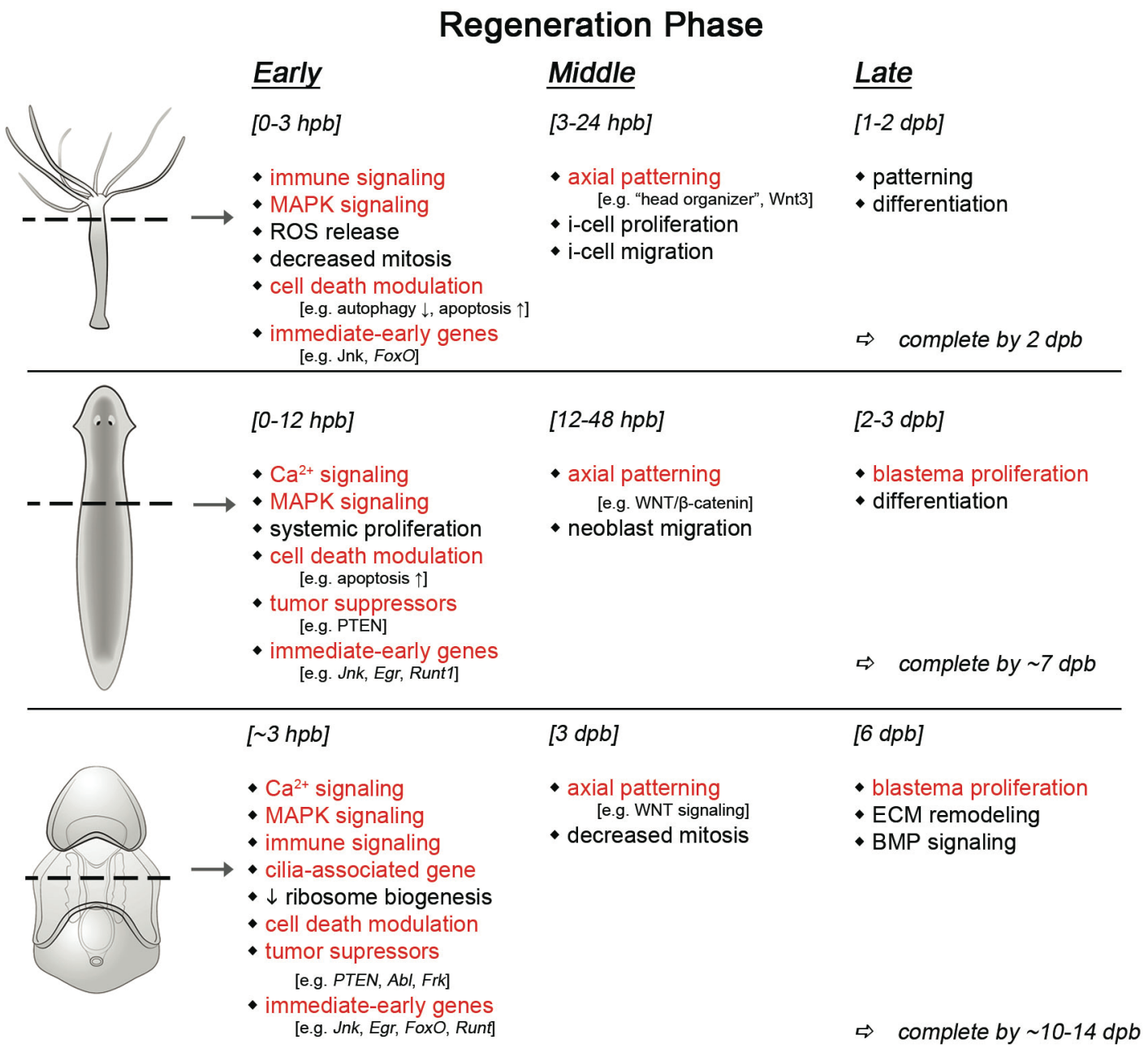


**Figure 7.** Summary of similarities between WBR models. The reported features of regeneration at early, middle, and late stages of regeneration, with respect to the datasets considered in this study, are indicated. Features detected in the sea star model in our study that are shared with the other two models are highlighted in red. Some aspects are considered in common based on shared gene expression (e.g. MAPK signaling) whereas others are based on cytological observations (e.g. blastema proliferation).

MAPK signaling pathways, upregulation of ciliogenesis genes, upregulation of tumor suppressor genes, downregulation of autophagy genes, and activation of a suite of immediate early genes are common aspects of regeneration in these models. There is also a set of conserved genes that we hypothesize are commonly involved in axial respecification, most notably genes in the WNT signaling pathway. Although the temporal order of axial respecification prior to blastemal proliferation is common among these models, the genes underlying the proliferative response are poorly conserved.

These commonalities between highly diverged WBR models highlights a deep conservation in regeneration mechanisms among the metazoa. This work also underscores the potential power of such comparative inquiries in identifying the core components of the regenerative response and, potentially, how these components are altered in non-regenerative species.

## MATERIALS & METHODS

### *Animals and Regeneration Paradigm*

Adult *Patiria miniata* were obtained from the southern coast of California, USA (Pete Halmay or Marinus Scientific) and were used to initiate embryo cultures as previously described (Cheatle Jarvela and Hinman, 2014). *P. miniata* embryos were cultured in artificial seawater at 16 °C and fed *Rhodomonas les* ad libitum every 2 days along with fresh artificial seawater beginning at 4 days post fertilization (dpf). All studies of regenerating larvae were conducted with larval cultures beginning at 7 dpf at which point the larvae were manually bisected stereotypically through the foregut, midway along the transverse anterior-posterior axis (Figure 1b), with a #11 sterile scalpel. Resulting anterior and posterior segments, as well as control (uncut) larvae, were then transferred to separate 35 mm polystyrene dishes at a density of no more than 50 larval segments per ml of artificial seawater and cultured for the time indicated.

### *Whole-Mount Staining Procedures*

*P. miniata* larvae or regenerating larval segments, grown for the times indicated, were fixed in a solution of 4% paraformaldehyde in MOPS-fix buffer (0.1M MOPS pH 7.5, 2mM $MgSO_4$, 1mM EGTA, and 800 mM NaCl) for 90 minutes at 25 °C and transferred to a solution of 70% ethanol for long term storage at -20 °C. In situ hybridization experiments were performed as previously described (Hinman et al., 2003; Yankura et al., 2010) using digoxigenin-labeled antisense RNA probes. Labelling and detection of proliferating cells in *P. miniata* larvae was performed using the Click-it Plus EdU 488 Imaging Kit (Life Technologies), with the following modifications. Larvae were incubated in a 10 μM solution of EdU for 6 hours immediately prior to fixation in a solution of 4% paraformaldehyde in phosphate buffered saline (PBS). Larvae were fixed for a minimum of 90 minutes at 25 °C and subsequently transferred to a solution of 70% ethanol for storage at -20 °C. For detection of EdU incorporation, labeled embryos were transferred to a solution of PBS prior to following the

manufacturer's protocol. For both in situ and EdU staining, at least two independent biological replicate experiments were performed examining the pattern of at least 10 specimens per replicate.

### *RNA-seq, Read Mapping, and Transcriptome Assembly*

For transcriptome measurements, larvae were grown and bisected as described in results. RNA was collected from pools of approximately 300 sibling individuals of regenerating anterior segments, regenerating posterior segments, as well as uncut control larvae. Two biological replicate samples were prepared for each timepoint. RNA was extracted using the GenElute Mammalian Total RNA Kit (Sigma-Aldrich). Illumina TruSeq library preparation and HiSeq 2500 50 bp SR sequencing were performed (USC Epigenome Center).

RNA-seq reads were trimmed of residual adapter sequences and low quality bases (Trimmomatic v0.32, (Bolger et al., 2014)). High-quality reads were mapped to the *P. miniata* v1.0 genome assembly (Tophat v2.0.12, (Kim et al., 2013)) and, in total, 422.9 M uniquely mapping reads were recovered from the 18 samples at an average depth of 23.5 M reads per sample. Uniquely mapping reads were assembled into transcripts using Cufflinks (Trapnell et al., 2012) and the MAKER2 based gene predictions hosted at Echinobase were used to guide transcript assembly. Reads uniquely mapping to a gene (locus) from this Cufflinks transcriptome assembly were counted (HTSeq-count v0.6.1p1, (Anders et al., 2015)). Read counts were normalized and genes detected with more than 3 reads per million, corresponding to 50-120 uniquely mapping reads depending on the sample, in at least two samples were retained for further analyses, corresponding to 31,798 expressed genes. Raw and processed sequencing reads have been deposited into the NCBI Gene Expression Omnibus (in progress) and analysis scripts are available upon request.

### *Gene Ontology term annotation and Ortholog identification*

The newly assembled sea star genes were annotated in three ways: by identifying the reciprocal best BLAST hit (rBBH) between the sea star transcript and either sea urchin or mouse genes and using Blast2GO. 9,027 (28.4%) loci have an rBBH match to a sea urchin protein, 7,212 (22.7%) loci have an rBBH match to a mouse gene, and 9,617 (30.2%) assembled loci were annotated using Blast2GO. GO terms for each sea urchin and mouse gene were assigned to their respective rBBH match in the sea star set and these were used for enrichment analyses. Overall the results based on all three annotation methods are highly similar (Figure 3B and Figure S2). Reciprocal best BLAST hits (rBBH) were also used to identify putative orthologs between the sea star genes and the planaria and hydra transcripts. We found 5,220 *S. mediterranea* transcripts and 6,091 *H. magnipapillata* transcripts with an rBBH match to a sea star transcript.

### *Differential Expression Testing and Hierarchical Clustering*

Expression levels in biological replicate samples are highly correlated (pearson correlation coefficient = 0.985). Regenerating segments were compared to age-matched sibling uncut control larvae and differential

expression was assessed using a generalized linear model quasi-likelihood F test (edgeR, (Lund et al., 2012; Robinson et al., 2010)), controlling for sample batch. Differentially expressed genes (DEG) were defined as those changes detected below a p-value of 0.05 and with a fold change greater than 2-fold in either direction. Using these criteria there are 9,211 total DEG in at least one regenerating segment compared to the control larvae and at least one of the timepoints sampled, which represents 28.97% of all of the expressed genes detected.

The fold-change values for all 9,211 DEG relative to control larvae were clustered by first computing the euclidian distance matrix and then these values were then clustered using the “ward.D2” method provided as part of the R hclust function. The optimum number of clusters was determined by cutting the resultant dendrogram at various heights and empirically determining at which height the number of clusters began to plateau (h=42). The result was 8 distinct clusters. However, we noted that several clusters shared similar overall patterns (Figure S1). As the similar clusters shared very similar GO enrichments and expression patterns over the time course, we further grouped these into the final 5 clusters reported in the text. The grouping of clusters did not alter the enrichment of GO terms or our other downstream analyses (Figure S1).

For the planaria and hydra regeneration datasets, data was obtained from supplemental tables associated with each publication. The planarian data were reported as normalized read counts for the 15,422 transcripts detected. These counts were $\log_2$-transformed and then scaled to z-scores, or the number of standard deviations from the mean value for each transcript, and only those transcripts considered differentially expressed as reported by the authors were considered. This resulted in 7,975 transcripts that were then clustered in the same way as described above for the sea star transcripts. The hydra data were reported as binned z-scores for the 28,138 transcripts detected corresponding to lower, mid, and upper third of expression range for each transcript. We only clustered transcript values for which a positive reciprocal match was detected, leaving 5,779 transcripts for our analyses. The euclidian distance matrix was calculated, as with the other datasets, but to accommodate the binned nature of these data the hierarchical clustering was performed using the “average” method provided with the hclust R function. A fine-grained resolution of common gene expression dynamics across these species is not warranted without more closely aligning experimental designs, including sampling time points and normalization strategies. Therefore, for each of these datasets we sought very broad cluster classifications such that assigned genes are either either up-regulated early and down later or vice versa in their respective time course.The result is three clusters each for the *S. mediterranea* and *H. magnipapillata* datasets (Figures S3 and S4).

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## COMPETING INTERESTS

The authors declare no competing financial or non-financial interests associated with this work.

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