

Stage-resolved analysis of posterior regeneration in *Ophryotrocha labronica* reveals early pluripotency activation and conserved regenerative programs

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Research Article

Keywords: Annelida, Blastema, Cell proliferation, Nervous system, Ophryotrocha, Stem cells, Transcriptomics.

Posted Date: September 22nd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10589385/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Regeneration is a widespread post-embryonic process across metazoans and particularly diverse among annelids, offering a valuable system to explore how complex tissues are restored and why regenerative capacity varies across species. While morphological aspects of regeneration, including nervous system reconstruction, have been extensively described, the underlying cellular and molecular mechanisms remain poorly resolved in many non-model annelids, particularly at stage-specific resolution.

Results

Here, we characterise posterior regeneration in the dorvilleid annelid *Ophryotrocha labronica* by integrating morphological, cellular, neuroanatomical, and transcriptomic approaches. We define a temporal framework from wound healing to complete tail regeneration within five days, focusing on mid-early proliferative and differentiation stages. Proliferation and stem cell-related genes contribute to blastema formation and early differentiation, while neurogenesis-related genes activate shortly after injury and persist throughout regeneration, suggesting a sustained role in coordinating blastema development. This process involves both local and migratory proliferative cells, preceded by early activation of pluripotency and neural markers. Comparative analyses further reveal both conserved and lineage-specific mechanisms in *O. labronica*, highlighting shared molecular pathways as well as evolutionary divergence across annelids.

Conclusions

Our results provide stage-resolved insights into the molecular regulation of posterior regeneration in *O. labronica*. Conserved signalling pathways (Notch, Hedgehog, Wnt) alongside candidates such as NOX2/CYBB, previously described in vertebrate regeneration, highlight shared and lineage-specific mechanisms underlying regenerative diversity. These findings underscore the value of non-model annelids for understanding the evolution and diversity of regenerative strategies across metazoans.

BACKGROUND

Regeneration, the ability to restore lost body parts or tissues, is a widespread biological process that emerged early in metazoan evolution, although it has been secondarily lost in several bilaterian clades [1, 2]. This ability varies from limited tissue repair to whole-body regeneration, reflecting high diversity in regenerative potential across animals [2, 3]. Among metazoans, annelids stand out as excellent model systems for studying regeneration [4]. With nearly 21,000 species described [5], annelids combine complex body organization with whole-body regeneration in numerous species, providing clear

ecological advantages against predation or injury [2]. Most annelids can regenerate posterior segments, while certain lineages can also restore anterior structures [3, 6]. Both capacities appear to be ancestral to the phylum and have been independently lost multiple times, making annelids valuable for investigating the evolution and molecular mechanisms underlying regeneration [6].

During normal growth, posterior elongation in adult annelids depends on the posterior growth zone (PGZ), a proliferative region harbouring stem/progenitor cells with a conserved molecular signature linked to the germline multipotency program (GMP) [7–9]. Following posterior amputation, the PGZ is lost, so proliferative cells emerge at the wound site before a new PGZ is re-established, marking a transition to an injury-induced regenerative program. Regeneration then proceeds through a conserved sequence: rapid wound closure, formation of a proliferative blastema, and subsequent differentiation and repatterning to restore missing tissues. This epimorphic process is driven by intense cell proliferation, sometimes followed by morphallactic remodelling of pre-existing adjacent segments [10–13]. Early proliferative activity is often detected close to the ventral nerve cord (VNC), suggesting the nervous system promotes blastemal proliferation and provides positional information to re-establish the anteroposterior axis [10, 14–16].

At the molecular level, numerous studies have identified regeneration-specific gene expression programs underlying these events, comprising transcription factors and RNA-binding proteins associated with wound epithelium formation, pygidial reconstruction, and blastema segmentation and elongation [12, 11]. Several of these genes are also linked to GMP programs, a robust molecular signature of stem cells, relating to a longstanding question in annelid regeneration: the cellular origin of blastemal proliferative cells. Two classical, non-mutually exclusive hypotheses remain under debate: blastemal cells may derive from migratory pluripotent or multipotent stem-like populations [17–19], or originate locally through dedifferentiation of resident cells followed by redifferentiation at the wound site [20, 21]. Evidence for both mechanisms exists across species with variable regenerative abilities. Supporting the first hypothesis, a pool of stem cells contributing to regeneration have been described in several annelids. In *Enchytraeus japonensis*, mesodermal stem cells termed neoblasts participate particularly in initial [22, 23]. In *Pristina leidyi* and *Capitella teleta*, both GMP clusters and migratory cell populations relocate to the wound, though their functional contribution remains unclear [24–26]. In contrast, in *Platynereis dumerilii* or *Syllis gracilis*, no cell migration has been observed, and regenerated tissues appear to rely predominantly on dedifferentiation of pre-existing cells, consistent with the *de novo* expression of GMP genes at the wound site [27–30]. Together, this evidence indicates that multiple cellular strategies likely underlie blastemal proliferation, with their relative contributions varying across lineages, potentially correlating with differences in regenerative capacity and reproductive mode.

Dorvilleid annelids provide a powerful model for studying regeneration due to their remarkable variation in regenerative ability. While some dorvilleids can regenerate both anterior and posterior ends from only a few segments [14, 31], others, including *Ophryotrocha*, are restricted to posterior regeneration. Regenerative abilities have been described in *O. puerilis* and *O. notoglandulata* [32–35], and more recently the first detailed molecular characterisation of early regeneration was reported in *O. xiamensis* [36].

Ophryotrocha labronica is a rapidly developing and easily culturable species offering an experimentally tractable system for studying posterior regeneration. Its derived position within Dorvilleidae, together with the diversity of regenerative strategies across the family [37, 38], offers a valuable comparative framework for understanding the evolution and molecular mechanisms of regeneration in annelids.

In this study, we combine morphological, cellular, and transcriptomic approaches to characterize the complete posterior regeneration process in *O. labronica*. By analysing external and internal morphology (SEM, CLSM, DAPI, EdU) and reconstructing neural architecture (immunostaining), we examine the role of the regenerating nervous system and the dynamics of cell proliferation at the wound site.

Transcriptomic profiling further reveals conserved and lineage-specific features defining the molecular identity of each regeneration stage and reconstructs the temporal progression of blastema formation and differentiation. We evaluate these results within a broader annelid comparative framework and provide new insights into conserved and highly coordinated mechanisms of regeneration across the phylum.

RESULTS

Basal proliferative activity and neural architecture in non-regenerating adults

Cell proliferation dynamics and nervous system anatomy were first analysed in non-amputated *O. labronica* individuals (Fig. 1A) to establish a baseline for comparison (Fig. 1B-D, Fig. 2). Proliferating cells were distributed throughout the body, with clear dorsoventral differences. EdU+ cells were evenly distributed across the epidermis of all segments (Fig. 1B), whereas in the parapodia and anal cirri they formed discrete medio-ventral clusters (Fig. 1C). Despite inter-individual variability, labelled cells consistently concentrated around the jaws (Fig. 1D) and pygidium (Fig. 1E), where a dense EdU+ population marks the PGZ (Fig. 1C, E).

The central nervous system comprises a brain and a ventral nerve cord (VNC; Fig. 2A-B). The neuropil of the brain, in the dorso-anterior part of the prostomium, is formed by a central core of commissural neurites that connect posteriorly via the circumesophageal connectives (cc) to the VNC. Multiple sensory cells of the anterior prostomium form minor neural bundles that enter the brain anteriorly while the sensory neurons of the nuchal organs and the two eyes form the larger olfactory and optic nerves that enter the brain laterally (Fig. 2C). Two prominent circumesophageal connectives connect the VNC to the brain (Fig. 2B-C). These connectives bifurcate into a dorsal (dcc) and a ventral (vcc) branch adjacent to each other (Fig. 2D). The stomatogastric nervous system connects anteriorly to the brain neuropil by four stomatogastric nerves (stn) that converge into a stomatogastric commissure (sc) and extend numerous neurites posteriorly (Fig. 2D). The ventralmost stomatogastric nerves branch out towards the mouth, jaws and pharynx, while the most prominent dorsal nerves extend towards the pharyngeal bulb and the oesophagus (Fig. 2D).

The VNC consists of a relatively thin ventromedian nerve (vmn) and two thicker peripheral main nerve strands (mn) well separated from each other (Figs. 2B, E-F). One pair of ganglia (g) per segment are connected by numerous commissures. From each ganglion at least four nerves extend (Fig. 2F). The anteriormost, elongates towards the anterior base of the parapodium (pn1), and continues dorsally connecting with a lateral and laterodorsal commissures. The second, and thickest, one (pn2) extends towards the parapodium branching into dorsal and ventral neurite bundles that loop around it. Adjacent to the previous, a third thin nerve emerges and extends towards the parapodium. The fourth and posteriormost nerve (pn4) is also thin and extends towards the posterior edge of the parapodial base. In each segment, numerous sensory cells connect to the parapodial nerves through thin neurites.

The pygidium does not show a distinct ganglion but a thin terminal commissure (tc) anterior to the anus, the VNC separates and each main nerve strand branches out into the anal cirri (cin), the ciliary bands and the anal area. The ventromedian nerve also branches out connecting to the terminal commissure (Fig. 2F). Multiple ciliated sensory cells distributed in the anal cirri and ciliary bands connect to the VNC via thin neurite bundles (Fig. 2F).

The peripheral nervous system comprises the epidermal plexus, segmental nerves branching off from the connectives and longitudinal nerves extending from the brain (Fig. 2A-C). There are a pair of dorsolateral (dln), lateral (ln), and ventrolateral (vln) longitudinal nerves extending along the trunk interconnected by segmental nerves and converging posteriorly at the pygidium (Fig. 2A). Some non-neuronal structures include transverse ciliated bands (cb) of sensory/motor cilia in the middle of each segment (Fig. 2A) and a pair of nephridia (n) at the anterior half of each segment (Fig. 2B).

Morphological stages during posterior regeneration

O. labronica exhibit only posterior regeneration, fully restoring the pygidium and the PGZ, and adding one segment within five days post-amputation (dpa) (**Fig. S1**). Regeneration proceeds through reproducible stages of roughly one day each, with no differences between males and females and the first new segment appearing between 4–5 dpa (mean = 4.54, N = 63; **Fig. S1Q**). Wound margins open immediately after amputation (**Fig. S1B, G**), are fully closed by 12 hours post-amputation (hpa) (Fig. 3A, **Fig. S1C, H**). A small outgrowth corresponding to the early blastema appears at 1 dpa (stage 1; Fig. 3B, **Fig. S1D, I**) and continues expanding; by 2 dpa (stage 2) a central depression marks anus reopening (Fig. 3C, **Fig. S1E, J**). At 3 dpa (stage 3), the pygidium is nearly restored, with two terminal cirri buds and reappearance of the ciliary band (Fig. 3D, **Fig. S1K, N**). By 4 dpa (stage 4), anal cirri elongate further and segmental grooves appear within the differentiating blastema (**Fig. S1L, O**). At 5 dpa, the pygidium is fully developed, anal cirri reach control-comparable lengths, and a new segment with parapodia is present (**Fig. S1M, P**). Segment addition then continues for several days until adult tail proportions are reached, as in normal PGZ-driven growth. These results indicate that regeneration *sensu stricto* concludes by 3 dpa; accordingly, only the first four stages (12 hpa, 1, 2, 3 dpa) were considered for subsequent analyses, as later stages mainly reflect tissue differentiation and normal segmental growth.

Cell proliferation dynamics during blastema formation

To assess the contribution of local proliferating cells, we performed EdU incorporation at each regenerating stage (12 hpa, 1, 2, 3 dpa; Fig. 4A). Labelling for 1 hour followed by immediate fixation revealed no EdU+ cells at 12 hpa, reflecting an injury-induced proliferative arrest (Fig. 3E). Moderate proliferation appeared at 1 dpa, with EdU+ cells bilaterally near the gut forming early regenerative structures (Fig. 3F). By 2–3 dpa, strong EdU signal was detected in the medio-ventral blastema, indicating active localized proliferation (Fig. 3G, H); at 3 dpa, a dense EdU+ band at the upper blastema boundary suggests PGZ re-establishment (Fig. 3H).

To assess whether cells proliferating before amputation migrate to the wound site, we labelled proliferative cells one hour pre-amputation (Fig. 4B). No EdU+ signal was found around the wound at 12 hpa (Fig. 3I) or in the blastema at 2–3 dpa (Fig. 3K, L), where labelled cells were instead distributed elsewhere in the body, mostly in the parapodia. However, at 1 dpa some EdU-labelled cells appeared in the blastema and adjacent segment alongside broader body distribution (Fig. 3J), suggesting a possible contribution of migratory proliferating cells during early blastema formation.

Re-establishment of the ventral nerve cord during regeneration

We used acetylated tubulin labelling to track VNC re-establishment (Fig. 3M-P). At 12 hpa, disorganized nerve fibres branch from the main strands toward the wound (Fig. 3M). By 1 dpa, axonal extensions reach the new tissue and connect to the wound epithelium (Fig. 3N). At 2 dpa, lateral nerves toward the parapodia emerge alongside a ventromedian nerve extension into the growing blastema, with the anus fully reopened as shown by internal tubulin labelling in the ciliated hindgut (Fig. 3O). By 3 dpa, the fourth lateral nerve pair (pn4) is reconstructed to the posteriormost parapodial base, and the regenerated VNC appears fully reorganized, with each strand branching into the anal cirri (cin) with multiple axons extending distally to innervate these structures (Fig. 3P).

De novo transcriptome assembly and annotation

We generated 14 RNA-seq libraries, including three biological replicates for each of the four regeneration stages (12 hpa, 1, 2, 3 dpa) and two replicates for control worms, yielding 287.5 million paired-ended raw reads (~ 53 GB). Of those, 276.6 million reads (96.21%) were retained after trimming low-quality bases and reads shorter than 36 bp (**Supplementary Table 1**). Trinity assembly generated a high-quality and complete *de novo* reference with 535,801 transcripts (mean GC 40.06%), with N50 of 1,673 bp and Ex90N50 of 1,919 bp. BUSCO completeness against Metazoa was 99.3% (**Supplementary Table 2**).

Of assembled transcripts, 89,748 (16.75%) had a BLAST hit against the UniProtKB (SwissProt). TransDecoder predicted 130,825 proteins, of which 69,549 (53.16%) were annotated via BLASTP. Combining transcriptome and proteome data yielded 90,548 unique annotated Trinity transcripts (16.9%). While roughly half the predicted proteome was annotated, only ~ 17% of the transcriptome

received functional annotation, reflecting the limited genomic resources available for this non-model species.

Patterns of gene expression across regeneration stages

A total of 1,497 transcripts were significantly differentially expressed across all pairwise comparisons ($n = 10$; $p < 0.001$, $\log_{2}FC > 2$) (**Supplementary Information 2**). Notably, most of them were detected in two specific pairwise comparisons: Control vs. 1 dpa and 1 dpa vs. 3 dpa (**Fig. S2**), reflecting major transcriptional shifts during early blastema formation and differentiation.

To identify the most relevant biological changes at each stage, we focused on sequential comparisons, comparing each regeneration timepoint with the immediately anterior one (Control vs. 12 hpa, 12 hpa vs. 1 dpa, 1 dpa vs. 2 dpa, and 2 dpa vs. 3 dpa). This approach identified 402 differentially expressed genes (DEGs; **Fig. 5, Supplementary Information 2**). During wound healing (Control vs. 12 hpa), 125 DEGs were found (83 down-, 42 upregulated), including mitotic regulators KIF20A and CDC20, acetylcholine receptor subunit ACHA3, and neoblast marker PCDH9 (**Fig. 5, 6A-B**). At 1 dpa, 84 DEGs appeared (45 down-, 39 upregulated), including nerve regeneration genes BIRC2 and UBB and the migration gene AAMP (**Fig. 5, 6A, C**). The highest DEG count occurred at 2 dpa (136 DEGs: 60 down-, 76 upregulated), dominated by proliferation-related genes including cyclins CCNE1 and TSC1 and cell cycle regulators GPSM1 and MIA2 (**Fig. 5, 6A, D**). At 3 dpa, 57 DEGs were found (29 down-, 28 upregulated), including Wnt pathway components UBA2 and SCYL2, regulator SOCS5, and myogenic marker CANP3 (**Fig. 5, 6A, E**), consistent with early differentiation. Full DEG lists per comparison are in **Supplementary Information 2**.

Clustering analysis identified 30 gene clusters with specific expression profiles (**Supplementary Information 3 and 4**). From these, we selected five clusters showing clear stage-specific upregulation: clusters 4, 16, 23, 18, 26, corresponding to control, 12 hpa, 1, 2, and 3 dpa respectively (**Fig. 6F-I; Supplementary Information 5**).

We identified 26,909 genes with peak expression in control individuals, of which 933 were annotated and uniquely associated with non-regenerating individuals, with no upregulated isoforms of the same gene ID at any other regeneration stage (**Supplementary Information 6**). At 12 hpa, 16,674 genes peaked (921 annotated and unique), including conserved stem cell markers *vasa*, *nanog*, *brat*, and *frizzled class receptor 10*. The highest number peaked at 1 dpa (57,308 genes; 3,404 annotated and unique), including cyclins, *elav* isoforms, and matrix metalloproteases possibly involved in blastema formation. At 2 dpa, 44,437 genes peaked (2,712 annotated and unique), including neural markers previously reported during annelid regeneration, like *netrin*, *IMPACT* and *noelin*. At 3 dpa, 40,596 genes peaked (1,929 annotated and unique), including *notch1* and several *Wnt* isoforms. Developmental signalling pathways such as *BMP* and stem cell marker *pumilio* showed sustained expression across most stages (**Supplementary Information 6**).

Unique gene sets for each cluster were used for GO enrichment analysis to uncover the biological functions associated with each regeneration stage (**Supplementary Information 5 and 7, Fig. 7**). At 12

hpa, enrichment centred on DNA repair and genomic stability (MF). At 1 dpa, terms related to nervous system development, purine/nucleotide binding (MF), and cell cycle (BP) were enriched, many persisting at 2 dpa and highlighting DNA biogenesis in blastema proliferation. Catalytic activity (MF) and intracellular transport/organisation (BP) terms were enriched throughout the process (**Supplementary Information 7**, Fig. 7).

DISCUSSION

Neural organisation in adult stages

The nervous system of *O. labronica* shows the typical Pleistoannelida organisation with at least two brain commissures connected to a ventral ganglionated cord via the circumesophageal connectives and their ventral and dorsal roots [39], consistent with previous descriptions of dorvilleids such as *Parapodrilus psammophilus*, *Dorvillea bermudensis*, and *O. diadema* [14, 40, 41]. The VNC consists of a thin median nerve and two thicker peripheral nerve strands that remain relatively close to each other compared with other annelid groups such as Dinophilidae or Capitellidae [14, 25, 42].

Conserved tissue remodelling, immune response, and epigenetic regulation initiate early regeneration

Wound healing marks the onset of regeneration and involves rapid closure of the wound by the contraction of the circular musculature [11, 12]. In *O. labronica*, the wound is covered by a wound epithelium by 12 hpa (Fig. 2A; **Fig. S1C, H**), faster than *D. bermudensis* (21 hpa; [31], but slower than in *Aeolosoma viridae* (6 hpa; [43]). While visible blastemal outgrowth is not observed until 1 dpa, the 12 hpa stage already shows extensive tissue remodelling and activation of conserved regenerative pathways. Among upregulated genes at this stage (**Supplementary Information 2**) is *Ejrup5*, also upregulated during *Enchytraeus japonensis* regeneration and linked to cell adhesion [44], suggesting a conserved role in wound epithelial closure. FGF signalling pathway members also show elevated expression supporting a conserved role in coordinating early blastema initiation [45]. In *O. labronica* *FGF18* and *FGFR1* peaked at 12 hpa, with *FGFR1* remaining elevated until 1 dpa (**Supplementary Information 6**), while *in situ* hybridization in *A. virens* showed *FGF18/FGFR1* expression from 4 hpa in the nervous system, wound epithelium, and mesodermal cells near the injury site [45]. Additional genes previously linked to head regeneration in *Pristina leidy* but poorly characterized in annelids, including *MARK3* kinase and *GAS6* [46], are also upregulated at this stage, further supporting active tissue remodelling (**Supplementary Information 6**).

GO enrichment reveals terms for anatomical structure development (GO:0048856) and cytoskeleton/cell projection organization (GO:0007010, GO:0008092, GO:0030030, GO:0120036; Fig. 7), many persisting at 1 dpa, indicating structural reorganization beginning immediately after wound closure and continuing through blastema initiation. Multiple matrix metalloproteases peak at 1 dpa (**Supplementary Information 6**), likely promoting blastema growth via extracellular matrix remodelling, as in *E. japonensis* [47]

Wound healing is also accompanied by an immune response, essential for phagocytosis of pathogenic cells and debris [11, 12]. DMBT1, linked to epithelial regeneration and inflammation [48, 49], is significantly upregulated at 1 dpa (**Supplementary Information 3**), aligning with its upregulation at the same stage in *O. xiamensis* [36] and suggesting a conserved role mediating inflammatory response and tissue repair.

Epigenetic and post-translational mechanisms are also prominent early on. At 12 hpa, JMJD6, an epigenetic regulator of stem cell function via DNA methylation [50, 51] is upregulated (Fig. 6A), while *DNMT1*, which maintains methylation during DNA replication and is essential for regeneration across taxa [52–54], is upregulated later at 2 dpa. Similarly, in *Platynereis dumerilii*, DNA methylation plays a critical role in early regeneration [27], supporting a sustained contribution of methylation-dependent regulatory programs throughout blastema development. Enrichment at 1 dpa of proteasome-mediated ubiquitin-dependent catabolism (GO:0043161) and protein ubiquitination (GO:0016567) further underscores post-translational regulation in early regenerative programs (Fig. 7).

Together, these findings indicate that posterior regeneration in *O. labronica* initiates through a coordinated program integrating cytoskeletal/extracellular remodelling, immune activation, and epigenetic regulation, necessary for blastema formation and tissue reconstruction.

Early activation of proliferation programs drives blastema growth and PGZ restoration

Epimorphic regeneration requires proliferation for blastema formation, though active proliferation is often depleted during wound closure, with a shutdown lasting up to a day before blastemal proliferation begins [11, 12, 31]. A similar pattern occurs in *O. labronica* at 12 hpa, with DNA replication largely absent (Fig. 2I), yet cell cycle-related genes are already significantly upregulated (**Supplementary Information 3**; Fig. 6A, B), including mitotic regulators such as *KIF20A*, *CDC20*, and the RNA polymerase III transcription factor *BDP1*, involved in liver and bone regeneration, and associated with tumour proliferation in vertebrates [55–58]. This early induction prior to detectable EdU incorporation suggests proliferative programs are activated molecularly before widespread S-phase entry—possibly a preparatory phase [10], or alternatively a rapid mitotic activation of pre-existing G2-arrested cells, as proposed in planarians [59–61]. Under this second scenario, early M-phase regulators like kinesins and cyclins, would reflect activation of pre-existing populations, while later EdU incorporation would reflect de novo S-phase entry. Future mitotic labelling should help resolve which mechanism operates at the wound site.

EdU labelling is detectable by 1 dpa around the gut, expanding thereafter (Fig. 3B, F; **Fig. S1D, I**), and increases markedly by 2 dpa around the reopened anus and across tissue layers (Fig. 3G), consistent with blastemal proliferation contributing to regeneration of all germ layers in *P. dumerilii* or *Capitella teleta* [25, 27, 31].

This transition is accompanied by a coordinated transcriptional shift: sustained proliferation/cell-cycle gene activation between 12 hpa and 1 dpa is followed by a pronounced transition between 1 and 2 dpa

coinciding with active blastema expansion (Fig. 6A; **Supplementary Information 2**). At 1 dpa, upregulated splicing factors SREK1 and U2AF4 suggest a role for post-transcriptional regulation, while activation of *indian hedgehog* signalling points to a conserved cell-division mechanism, consistent with *O. xiamensis* [36]. This signature persists at 2 dpa with upregulation of CCNE1, TSC1, GPSM1, and MIA2, all key drivers of cell division and tumour progression in vertebrates [62–66]. Similarly, the enrichment of cell cycle progression and nucleotide metabolism (i.e., purine nucleotide binding (GO:0017076), ribonucleotide binding (GO:0032553), and cell cycle process (GO:0022402); Fig. 7; **Supplementary Information 7**), highlight the highly proliferative nature of the developing blastema, which is consistent with early enrichment of translation, ribosome biogenesis, and proliferation reported other annelids such as *Lumbriculus variegatus*, *O. xiamensis* and *P. dumerilii* [36, 67, 68].

By 3 dpa, proliferation remains active but coupled to early signs of differentiation, including the emergence of the anal cirri buds (**Fig. S1K, N**). Sustained EdU incorporation and *cyclin B* isoforms expression (Fig. 3H; **Supplementary Information 6**) is accompanied by a transcriptional shift toward cell fate specification and remodelling. The appearance of a band of proliferating cells above the pygidial region is consistent with PGZ re-establishment, as reported at comparable stages in other annelids [27].

Stem cell programs support blastema formation

Conserved stem cell programs are key to blastema formation, where pluripotency/progenitor markers are commonly activated (reviewed in [12], reflecting recruitment of pre-existing stem cells, local dedifferentiation, or both. Many annelid models show *de novo* upregulation of stemness genes [29, 30, 67], though migratory contributions from pre-existing stem-like cells are also reported [23–25] (discussed below).

In *O. labronica*, key stemness/pluripotency regulators including *vasa*, *nanog*, *brat*, *frizzled class receptor 10*, and *pumilio*, peak at 12 hpa, with *pumilio* remaining strongly expressed through 2 dpa (**Supplementary Information 6**), consistent with other annelid models [8, 28, 36, 47, 67, 69, 70]. Additional factors linked to stem cell maintenance, KAT6A and PCDH9 [71–73] are also upregulated at 12 hpa (Fig. 6A, B; **Supplementary Information 2**).

As regeneration progresses, this stem cell signature is maintained through expression of regulators of progenitor identity and genomic integrity (Fig. 6A, B; **Supplementary Information 2**): the expression of *UBE3C* and *EED* at 2dpa suggest ongoing transcriptional repression required for stem cell identity [74, 75], while *RAD51*, essential for homologous recombination and stem cell maintenance in planarians and vertebrates [76–78], together with *Ap2*, *nanos* and *argonaute*, and *tudor 2*, remain enriched from 1–3 dpa (**Supplementary Information 6**). This is consistent with patterns reported in *A. virens* and *P. dumerilii* [27, 67, 79] and supports a persistent progenitor-like state through early blastema development.

This signature is tightly coupled to DNA repair and genomic stability pathways, with enrichment during 12 hpa–1 dpa of telomere organisation (i.e., chromosome, telomeric region (GO:0000781), telomere

maintenance via lengthening (GO:0010833)) and DNA repair-related pathways (i.e., DNA repair (GO:0006281), DNA damage response (GO:0006974); Fig. 7), as previously observed in *O. xiamensis* [36].

Contribution of cell migration to blastema initiation

Beyond local proliferation, migratory cells also contribute to early blastema formation. Pre-amputation EdU-labelled cells appear around the gut and wound epithelium at 1 dpa, indicating migration from uninjured tissue (Fig. 3J) and suggesting a separate population, potentially stem or pluripotent cells, though a contribution from differentiated or quiescent/slow-cycling cells activated upon injury cannot be excluded [23–25].

Similar migratory behaviours occur in other annelids, though cell origin remains debated. In *C. teleta*, stem cell-like migratory cells peak at 3 dpa, contributing mainly to later differentiation [25]. Likewise, in *P. dumerilii*, slow-cycling cells labelled at least 2 days before amputation and mostly originating from the segment adjacent to the amputation plane, give rise to most blastemal proliferating cells, possibly after partial differentiation [20, 27, 80]. In *O. labronica*, migratory cells appear predominant during early blastema formation, with limited contribution at later stages (Fig. 3K, L). This is consistent with the upregulation of the migration gene *AAMP* at 1 dpa [81] (**Fig. 6 A, C**). Despite unclear cell origin, these observations highlight the diversity of cellular strategies underlying annelid regeneration.

Neural contribution is essential for blastema development

The nervous system is a central regulator of annelid regeneration, promoting blastemal proliferation, tissue organization, and positional cues, with the VNC also serving as scaffold for neural redevelopment [14, 16]. Tubulin labelling shows axons extending from the VNC into the wound site by 12 hpa; by 1 dpa these penetrate the wound epithelium, and as regeneration progresses, they branch and organize to eventually innervate the anal cirri (Fig. 3M-P).

The relevance of the nervous system in early regeneration is also evidenced at the molecular level. At 12 hpa upregulated neural genes include the acetylcholine receptor subunit *ACHA3* and multiple *elav* isoforms (Fig. 6A; Supplementary Information 2, S6), suggesting early activation of conserved neural developmental pathways. *ACHA3* is linked to synaptic re-establishment during neuromuscular regeneration [82–84], and is also reported during *Perionyx excavates* anterior regeneration [85]. Similarly, *elav*, a conserved marker of nervous system development, peaks between 12 hpa and 1 dpa, consistent with its expression in the posterior wound epithelium of *Platynereis dumerilii* [27].

The strongest neural signature occurs at 1 dpa, coinciding with blastema formation and DNA replication onset (Fig. 3B, F), with upregulation of multiple genes associated with neural regeneration and neurogenesis (**Fig. 6A, C; Supplementary Information 2**), including *BIRC2*, *UBB*, and *NOX2/CYBB* [86–88]. The upregulation of *Wnt* components *COW* and *PODN*, further point to neuromuscular development and myogenesis [89, 90]. Consistent with comparable-stage observations in *P. dumerilii* [67], we found enrichment of GO terms related to nervous system development (GO:0007399), neurogenesis (GO:0022008), and neuron projection (GO:0043005; Fig. 7).

Neural remodelling continues at 2 dpa (**Fig. 6A; Supplementary Information 2**), with the upregulation of glucosidases like *MOGS* and *GANAB*, *PTBP1*, and *GPM6A*, involved in axonal growth and synaptic development [91–94]. *Netrin*, *IMPACT* protein and the *collapsin response mediator protein*, also peak at this stage, consistent with their roles in axon guidance and neural regeneration in annelids and planarians [67, 95, 96], and in neuronal migration in *E. japonensis* [97, 44]. By 3 dpa, *Ash1* expression peaks, as also reported at later stages in *P. dumerilii* [67], alongside the Wnt components *UBA2* and *SCYL2* are upregulated at 3 dpa, both required for neurogenesis and neuronal maintenance [58, 98–100]. Together, these patterns suggest continued neurogenesis during later blastemal stages, matching anatomical observations of denser lateral nerves and progressive neural reconnection to the anal cirri (Fig. 3P).

Regulatory signals for muscle development and tissue differentiation

Following wound healing and blastemal proliferation, regeneration transitions toward differentiation, evidenced by myogenesis, morphogenesis, and patterning genes at 2–3 dpa (Fig. 6A; **Supplementary Information 2 and 6**). Myogenic regulators, including *ADAR*, previously linked to muscle regeneration in *Perionyx excavates* [101], and *myoD*, a conserved muscle progenitor marker upregulated during *C. teleta* regeneration [102], are both upregulated at 2 dpa, marking muscle restoration onset. This is followed at 3 dpa by *CANP3*, a muscle-specific protease involved in myogenic maturation [103], *SNX13*, implicated in muscle lineage specification [104], and *myosin 6*, previously reported during annelid muscle regeneration [67]. This aligns with morphological observations of progressive muscle fibre differentiation in *Platynereis dumerilii* and *A. virens*, [16, 27].

Alongside myogenesis, broader differentiation proceeds: *ack-like*, linked to cytoskeletal organization in late *Pristina leidyi* asexual development [46], suggests active remodelling, while *TYMP* [105] and *SOCS5* [106, 107] indicate a coordinated transition from proliferation to differentiation.

Central to this transition is progressive body patterning. Canonical Wnt/ β -catenin components, including β -catenin and multiple Wnt isoforms, are upregulated between 1–3 dpa (**Supplementary Information 6**), consistent with their conserved role in posterior specification across annelids [27, 108, 109] and other regenerative invertebrates [110, 111]. *NKX1*, which regulates medio-lateral patterning in planarians [112], is activated earlier in *O. labronica* than in *Platynereis dumerilii* [67], suggesting distinct temporal deployment of conserved patterning mechanisms. Induction of *delta-like protein 1* and *distal-less* further supports posterior structure morphogenesis, including the anal cirri [27, 67], indicating coordinated PGZ restoration and terminal differentiation onset. This shift is reflected functionally in enrichment of tissue morphogenesis (GO:0048729), regulation of cell communication (GO:0010646), regulation of signalling (GO:0023051), and signal transduction (GO:0009966; Fig. 7).

Moreover, the high biosynthetic demands of blastema maturation are reflected in upregulated *FARSB* and *DHDDS* [113, 114] and enrichment of phosphoric ester hydrolase activity (GO:0042578; Fig. 7), consistent with late-stage transcriptional profiles in *P. dumerilii* [67]. Overrepresentation of GO categories

related to intracellular anatomical structure and organelle organization (GO:0031090, GO:0043231, GO:0098588; Fig. 7) indicates extensive intracellular remodelling through protein synthesis, vesicular trafficking, and organelle biogenesis, reflecting the substantial energetic investment required for tissue restoration across regenerative invertebrates (reviewed in [115]).

A conserved regulatory framework underlies posterior regeneration in annelids

Together, these transcriptomic dynamics show that posterior regeneration in *O. labronica* follows a coordinated sequence from wound response, blastemal proliferation, and tissue differentiation, broadly conserved across annelids and matching stage-specific patterns in the closely related *O. xiamensis* [36] and other species including *D. bermudensis*, *A. virens*, and *P. dumerilii* [16, 27, 31, 67].

Sustained activation of Wnt, Hedgehog, and Notch signalling pathways (Fig. 8; **Supplementary Information 6**) is broadly implicated in annelid regeneration and axial patterning [67, 116, 117], while persistent upregulation of glutamine/glutamate metabolism reflects the sustained biosynthetic demands of continuous tissue remodelling. Notably, *vitamin D3 receptor*, *cubilin*, and *alanyl aminopeptidase*, remains consistently expressed across all regenerative stages (**Supplementary Information 6**), suggesting a role in coordinating the entire regeneration process rather than specific transitions [28, 44, 46, 68].

These findings reveal a coordinated regulatory framework underlying annelid posterior regeneration, while identifying previously unreported candidate genes that may broaden understanding of how regeneration evolved within the phylum.

CONCLUSIONS

This study provides an integrative characterization of posterior regeneration in *O. labronica*, combining morphological, neuroarchitectural, cellular, and time-series transcriptomic analyses. Based on comparisons with other annelid models, we propose a framework integrating the key cellular and molecular events driving regeneration (Fig. 8): the blastema becomes visible by 1 dpa, initiates differentiation by 3 dpa, and gives rise to the first new segment after 5 dpa.

Our data show that molecular activation of proliferative programs precedes robust DNA synthesis in the developing blastema. While localized EdU incorporation increases markedly between 2–3 dpa, EdU-positive cells labelled before amputation are already detectable in the blastema by 1 dpa, likely representing pre-existing migratory cells that had entered S phase before injury, and suggesting early blastema formation may partly rely on cells already cycling before amputation. At the same time, strong upregulation of cell-cycle regulators, stem cell markers, and pluripotency genes as early as 12 hpa indicates rapid activation of regenerative programs following wound healing, driving later proliferative expansion and widespread DNA synthesis.

Neural markers are activated early and remain expressed throughout regeneration, highlighting the importance of neural signalling and nervous system remodelling in blastema formation. Finally, our study identifies both conserved annelid regeneration genes and genes previously associated mainly with vertebrate regenerative systems, reinforcing the value of comparative studies in non-model annelids for identifying conserved mechanisms, novel candidate regulators, and the temporal dynamics shaping regeneration.

MATERIALS AND METHODS

O. labronica culture and morphological characterisation

A culture of *Ophryotrocha labronica* La Greca & G. Bacci, 1962 from the type locality (Livorno, Italy) was maintained in 1L glass containers filled with 35 ppt artificial seawater prepared from 0.22 µm-filtered water. Animals were kept at a constant temperature of 21 °C and fed dried spirulina powder every two weeks following water exchange. Under these conditions, worms reproduced continuously, with multiple egg masses present simultaneously.

Posterior regeneration was characterised based on general morphological changes using a rapid visual staging approach. Adult individuals (i.e. those with more than 12 segments) were anaesthetised in 7% MgCl₂ in seawater and amputated at the posterior end, removing the 2–3 segments and the pygidium. Regenerating worms were maintained individually in 12-well plates at 21°C under starvation conditions until complete regeneration of the posterior region. Morphological progression of posterior regeneration was monitored daily using an Olympus SZ40 stereomicroscope, and representative images of each stage were acquired (**Fig. S1**). A total of 63 worms were closely monitored to assess these morphological changes and record the duration of each stage (see details in results section). Resulting data were visualised as violin plots using ggplot2 v.3.5.2 in R.

Further morphological characterisation of each stage was performed using scanning electron microscopy (SEM). Specimens from each stage were fixed overnight at 4°C in KINFix [118], dehydrated through a graded ethanol series up to 100% ethanol, critical-point dried (Emitech K850), gold-coated (25 nm, Q150T-S), and imaged using a Hitachi S-3000N SEM at the Servicio Interdepartamental de Investigación (SIdI) of the Universidad Autónoma de Madrid (UAM).

Immunostaining, cell proliferation, and confocal laser scanning microscopy (CLSM)

For the study of nervous system regeneration, worms were relaxed in 7% MgCl₂ in seawater for 5 minutes and fixed overnight at 4°C in KINFix. Samples were rinsed several times in phosphate-buffered saline 1x (PBS 1x, pH 7.4) and permeabilised for 1h at RT in a solution of 1% PBSt (PBS 1x + 1% Tween-20) and 1% DMSO. This was followed by an incubation in the blocking solution (1% PBSt + 2.5% BSA) overnight at 4°C. Mouse monoclonal anti-acetylated α-tubulin (Sigma-Aldrich T7451) was diluted 1:400

in blocking solution, and samples were incubated for 72 hours with shaking at RT. After multiple rinses in PBSt to remove the primary antibodies, samples were incubated for 72 hours with shaking at RT in goat anti-mouse 488 secondary antibody (Invitrogen A10680) at a final concentration of 1:400 in blocking solution. The antibody was then removed with several washes in 0.1% PBSt before counterstaining.

Additionally, we tracked cell proliferation by incubating complete specimens in 0.5 mM 5-ethynyl-2'-deoxyuridine (EdU; ClickTech EdU Cell Proliferation Kit 555 for Imaging, Roth 1Y72.1, and Click-iT EdU Imaging Kit, Invitrogen, C10338) in seawater for one hour in two different experiments (Fig. 4). In the first one, amputated worms on each selected regeneration stage were incubated in EdU during one hour before fixation (Fig. 4A). In the second experiment, EdU was administered for one hour before amputation and worms were allowed to regenerate until the different selected stages before fixation (Fig. 4B). Non-amputated worms, used as control, were incubated for one hour before fixation. All worms were fixed in KINFix at 4°C for 48 hours and rinsed several times in 1% PBSt before EdU detection. For EdU staining, samples were incubated for 30 minutes in a freshly prepared reaction cocktail following the manufacturer's instructions. When both immunostaining and EdU experiments were performed on the same samples, incubations with the primary antibody were done after EdU labelling and before EdU detection.

Samples were counterstained with DAPI (1 µg/mL) or DRAQ5 (1:1000) for 20 minutes, and after several washes with 1x PBS, individuals were mounted on glass slides in 75% glycerol in PBS 1x with a coverslip elevated above each specimen with modelling clay. Slides were stored horizontally at – 20°C in the dark until CLSM imaging. Specimens were imaged using a Leica TCS SP5 spectral confocal microscope with either a 20x or 40x oil immersion objective, using a sequential scanning method between stacks at the SIdI-UAM. Z-stacks (1024 × 1024 × 8 bit) with 5 µm interval were acquired, along with bright-field images for positional information in a separate channel. Images were compiled with Fiji v.2.0 (Wayne Rasband, National Institutes of Health) and edited with Adobe Photoshop v26.11.0. Final figure plates were assembled in Adobe Illustrator v29.6.1.

RNA extraction and sequencing

To further characterise posterior regeneration in *O. labronica*, we applied a comparative transcriptomic approach to explore stage-specific gene expression profiles compared to a control group of non-amputated individuals. We analysed four timepoints within the first three days of regeneration (12 hpa, 1, 2, and 3 dpa) to focus on proliferative activity and early differentiation events. For each stage, samples consisted of pools of five individuals (two males and three females) fixed in *RNA/later* solution (Invitrogen) at 4°C overnight and stored at – 20°C for up to weeks before RNA extraction. We generated three biological replicates for each regeneration stage and two replicates for the non-amputated control group, resulting in a total of 14 samples.

Fixed individuals were placed in 1.4 mm Ceramic PowerBead Tubes (Qiagen) with 1 mL of TRIzol Reagent (Invitrogen) and homogenised using the soft mode (2 cycles of 15 s with 30 s pause at 5,800 rpm) in a Precellys Evolution Touch Homogenizer (Bertin Technologies). Total RNA was extracted using

the PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. RNA quantity and integrity were assessed using the High Sensitivity RNA ScreenTape Analysis with the 4150 TapeStation System (Agilent). Total RNA samples were sent to Novogene (Cambridge) for library preparation and sequencing using the Illumina HiSeq 2500 v4 platform, generating 150 bp paired-end reads. Raw sequences were deposited in the NCBI Short Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/Traces/sra/>) under accession numbers: XXX–BioProject XXX. (**Table S1** of **Supplementary Information 1**).

Sequence processing, transcriptome assembly and functional annotation

The quality of the raw reads was assessed and visualised using FASTQC v.0.12.1 (Andrews, 2010). Adapter sequences and low-quality bases (phred scores < 30) were trimmed off with Trimmomatic v.0.39 [119] using the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Subsequently, de novo transcriptome assembly was performed with Trinity[120] v.2.13.2 using the clean reads from all replicates, including control and regenerating samples. Assembly statistics were generated using the TrinityStats tool from Trinity, and completeness of the assembled transcriptome was assessed in the web server gVolante [121] (<https://gvolante.riken.jp/>), with default settings using the ortholog search pipeline BUSCO v.5.1.2 and the metazoan database (metazoa_odb10, last accessed September 7, 2023) on the Completeness Assessment Server version 2.0.0. De novo transcriptome assembly and BUSCO completeness statistics are shown in **Table S2** of **Supplementary Information 1**.

The resulting *O. labronica* reference transcriptome was annotated using DIAMOND[122] v.2.1.8 against the UniProtKB/Swiss-Prot database (The UniProt Consortium, 2023) with an e-value cutoff of 1e-5 in BLAST homology searches. Candidate coding regions of the assembled transcriptome were identified using TransDecoder v5.5.0 (<https://github.com/TransDecoder/TransDecoder/wiki>). Open reading frames were first detected, followed by homology searches against the UniProt database and protein domain identification using the Pfam database with an e-value cutoff of 1e – 5. Predicted protein-coding sequences were refined based on the supporting evidence for coding transcripts and applying the --single_best_only parameter. Individual searches were performed with BLASTX in diamond using the proteome as the database.

Differential gene expression and clustering analyses

Transcript expression level for each replicate and stage was obtained by mapping the reads from each library to the reference transcriptome using BOWTIE2 [123] v.2.4.4 and estimating transcript abundance with RSEM [124] v.1.3.1 under the Trinity toolkit [125]. Differential expression analysis was performed on Trinity isoforms for all pairwise comparisons among stages (control, 12 hpa, 1 dpa, 2 dpa, 3 dpa) using the edgeR v.3.32.1 package implemented in Bioconductor 3.12, applying a false discovery rate (FDR) threshold of 0.01 and a log2 fold-change cutoff of 2 (**Supplementary Information 2**).

Differentially expressed genes between stages were graphically represented using a hierarchically clustered heatmap with the *pheatmap* package v.1.0.13 in R. Volcano plots were generated using the *EnhancedVolcano* package v.1.24.0 in R.

To better understand global transcriptomic dynamics during posterior regeneration, we performed soft clustering analysis using Mfuzz [126] v.2.54.0 in R using the average RSEM-normalised isoform read counts across replicates as expression values. We filtered out genes with missing values (NA) or standard deviation ≤ 0.001 . Data were standardised using the 'standardise' function in Mfuzz. The fuzzifier parameter 'm' was first estimated using the 'mestimate' function ($m = 1.9681$), but this resulted in poor clustering performance, with very low gene membership values. 'm' was therefore manually adjusted, and $m = 1.4$ was selected as it produced more distinct and biologically interpretable clusters with higher membership values. Minimum centroid distance was calculated using the 'Dmin' function to obtain the optimum number of clusters, which resulted in 30. Four regeneration-related clusters showing an expression peak of genes in each regeneration stage were selected for further analysis (**Supplementary Information 3**).

Core genes for each cluster were extracted as those with membership values above the α -core threshold, which was set at 0.5 using the 'acore' function. This filtering step retains only genes strongly associated with a given cluster, while increasing the α -core threshold reduces within-cluster variation by excluding genes with lower membership values (**Supplementary Information 4**). There was no overlap of Trinity isoforms among core genes of different clusters; however, gene IDs representing different isoforms were shared across clusters. To avoid redundancy in GO enrichment results and to better capture differences among regeneration stages, only genes uniquely assigned to a single cluster were retained (**Supplementary Information 5**).

Additionally, we compiled a curated database of genes previously reported in annelid regeneration studies and examined their expression profiles across the core gene sets of each Mfuzz cluster to identify the specific regeneration stages at which these genes exhibited higher expression levels (**Supplementary Information 6**).

Gene Ontology enrichment

To functionally characterise the gene sets associated with each regeneration timepoint, we performed Gene Ontology (GO) analyses using annotated and unique genes from the selected Mfuzz clusters and edgeR results, when possible. Analyses were conducted with g:Profiler [127] (<https://biit.cs.ut.ee/gprofiler>) using the g:GOST tool against the *Homo sapiens* background, applying a significance threshold of g:SCS-adjusted $p \leq 0.05$ (default settings). Multiple testing correction was performed using the g:SCS algorithm (**Supplementary Information 7**). Fold enrichment was calculated by comparing the frequency of genes associated with each term in the dataset to that in the annotated background set. Results were visualised using custom bubble plots generated with the R package *ggplot2* [128] v.3.5.2.

Abbreviations

CLSM
confocal laser scanning microscopy
DEGs
differentially expressed genes
Dpa
days post-amputation
EdU
5-Ethynyl-2'-deoxyuridine
GMP
germline multipotency program
GO
Gene Ontology
Hpa
hours post-amputation
PGZ
posterior growth zone
SEM
Scanning Electron Microscopy
VNC
ventral nerve cord

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Raw sequence files are available at NCBI SRA under BioProject XXXX.

Competing interests

The authors declare that they have no competing interests

Funding

This work was funded by the Madrid Government (Comunidad de Madrid-Spain) in the context of the V PRICIT (Regional Program of Research and Technological Innovation) (SI1/PJI/2019-00532), by the Spanish Government through the project MNCIN AEI/10.13039/501100011033 and by the European Union “Next Generation EU”/PRTR (CNS2023-145193) to PÁ-C. IdO was funded by an FPI-UAM 2022 fellowship from the Universidad Autónoma de Madrid. MH was supported by the Spanish Ministry of Science MICIU/AEI/10.13039/501100011033 and FSE+ grant RYC2022/037128-I. AV was supported by the Spanish Ministry of Science MCIN/AEI/10.13039/501100011033, European Union Next Generation EU/PRTR (grant IJC2020-045256-I) and by a fellowship from the ‘la Caixa’ Banking Foundation (ID 100010434), with the code LCF/BQ/PR24/12050011.

Authors' contributions

IdO: conceptualization, data curation, formal analysis, methodology, writing—original draft, writing—review and editing; MH: data curation, writing—review and editing; KW: resources, writing—review and editing; AV: conceptualization, methodology, supervision, visualization, writing—review and editing; PA-C: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Acknowledgements

We are grateful to Santi Gómez Escabia for his assistance with morphological experiments and to Víctor Ruiz López for his help with immunostaining. Silvia Núñez kindly helped with the original worm illustration used in the figures. We also thank Lola Morales García and Isidoro Poveda Barriga for their help at the SIDI-UAM facility.

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Figures

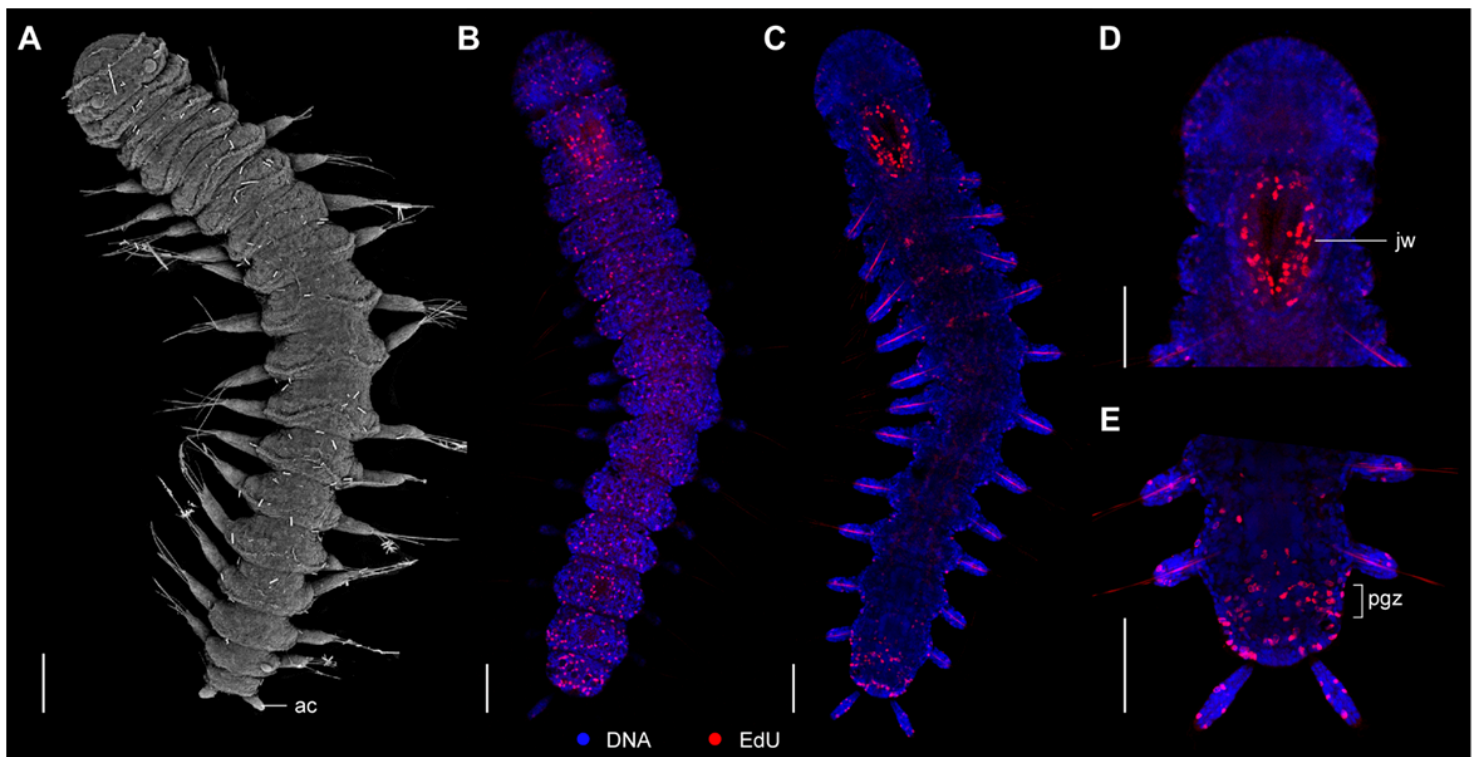


Figure 1

General morphology and proliferative activity of *Ophryotrocha labronica*. (A) SEM micrograph of an adult specimen. (B-D) Confocal z-stack projections of specimens co-labeled with EdU (red) and DAPI (blue) showing (B) dorsal epidermis, (C) medio-ventral view, (D) detail of the jaws in the head region, and (E) posterior region including the posterior growth zone (PGZ). Scale bars: 100 μ m. Abbreviations: ac, anal cirri; jw, jaws; pgz, posterior growth zone.

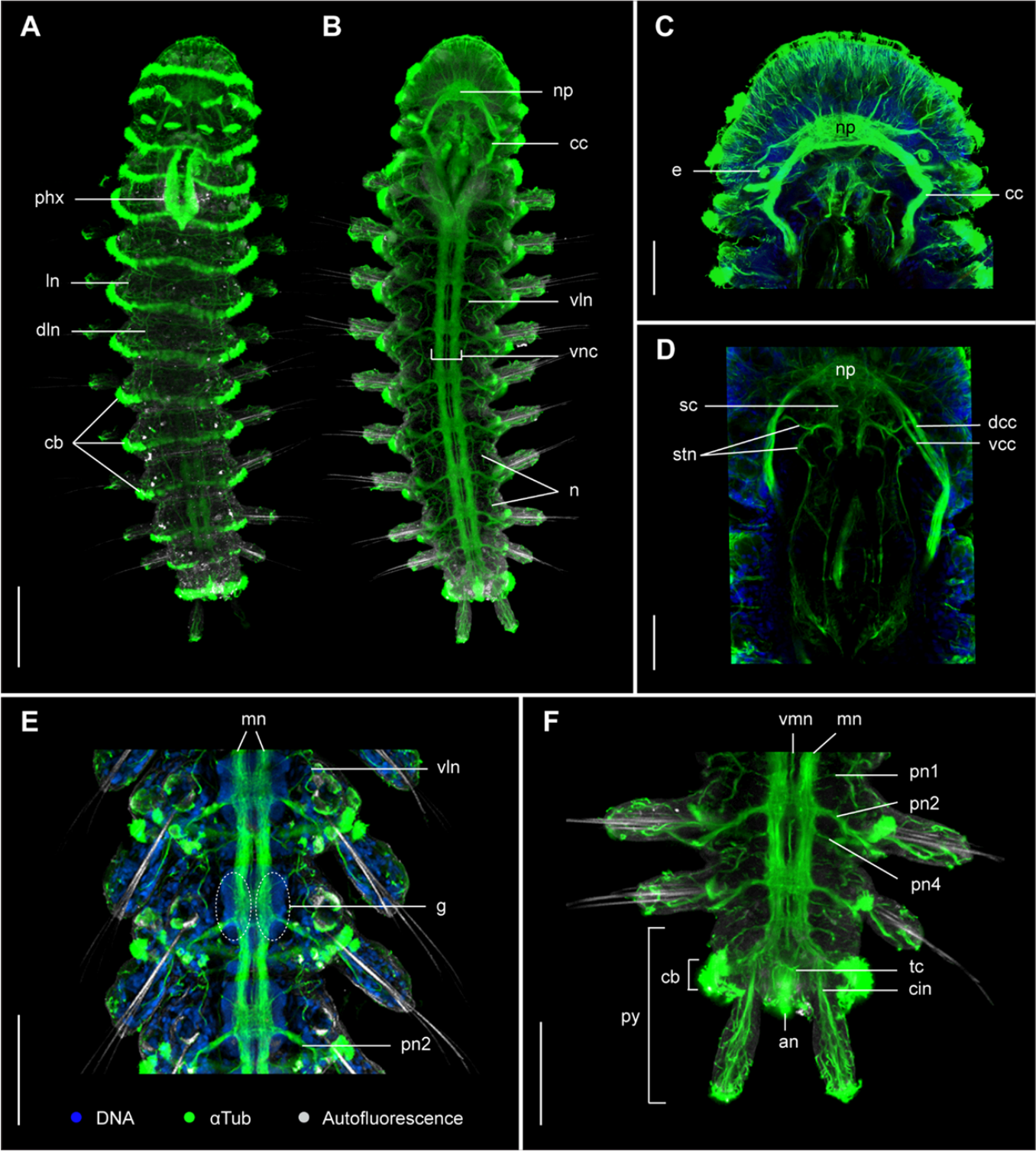


Figure 2

Acetylated α -tubulin-like immunoreactivity in the nervous system of *Ophryotrocha labronica*. Confocal z-stack projections of specimens co-labeled with α -tubulin and DAPI. Autofluorescence of the cuticle was kept for guidance. Color legend in (E) applies to all panels. Anterior is up in all panels. (A) dorsal overview, (B), ventral overview, (C) detail of the head region, (D) detail of the somatogastric nervous system, (E) detail of the ventral nerve cord and innervation of parapodia of the trunk, and (F) detail of the innervation of the posteriormost parapodia and caudal cirri. Scale bars: A, E: 100 μ m; C, D, F: 50 μ m. Abbreviations: an, anus; br, brain; cb, ciliated bands; cc, circumesophageal connectives; cin, caudal cirri nerves; dcc, dorsal circumesophageal connectives; dln, dorsolateral longitudinal nerve; e, eye; g, ganglion; ln, lateral longitudinal nerve; mn, main nerve strand; n, nephridia; phx, pharynx; pn(1-4), parapodium nerves; py, pygidium; sc, somatogastric commissure; stn, somatogastric nerves; vcc, ventral circumesophageal connectives; vmn, ventromedian nerve stand; vnc, ventral nerve cord; vln, ventrolateral longitudinal nerve.

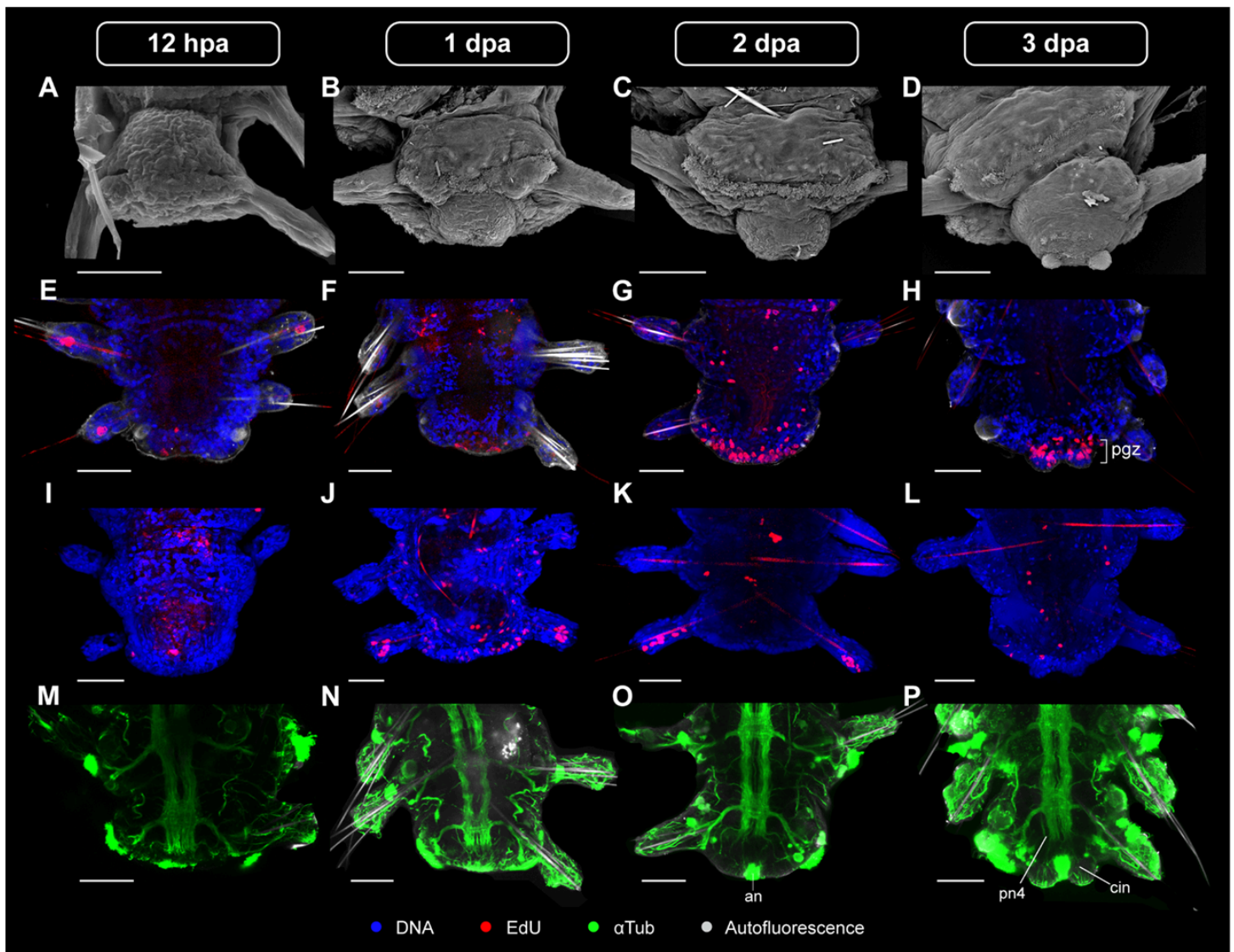


Figure 3

Morphological and cellular dynamics of posterior regeneration in *Ophryotrocha labronica*. All panels show posterior ends of amputated individuals in ventral view. Color legend in the bottom applies to all panels. Anterior is up in all panels. Time following amputation is indicated at the top. **(A-D)** SEM micrographs. **(E-P)** Confocal z-stack projections of EdU incorporation in proliferating cells **(E-L, red)** with nuclei counterstained (blue), and the nervous system labelled with acetylated α -tubulin **(M-O, green)**. EdU incorporation assays reveal proliferative activity under two experimental conditions: **(I-L)** EdU incorporated 1 h before fixation reveals local proliferation during regeneration, and **(M-P)** EdU incorporated 1 h before amputation to trace migration of proliferating cells in uncut worms. Scale bars: 50 μ m. Abbreviations: an, anus; cin, caudal cirri nerves; pgz, posterior growth zone; pn4, fourth parapodium nerves.

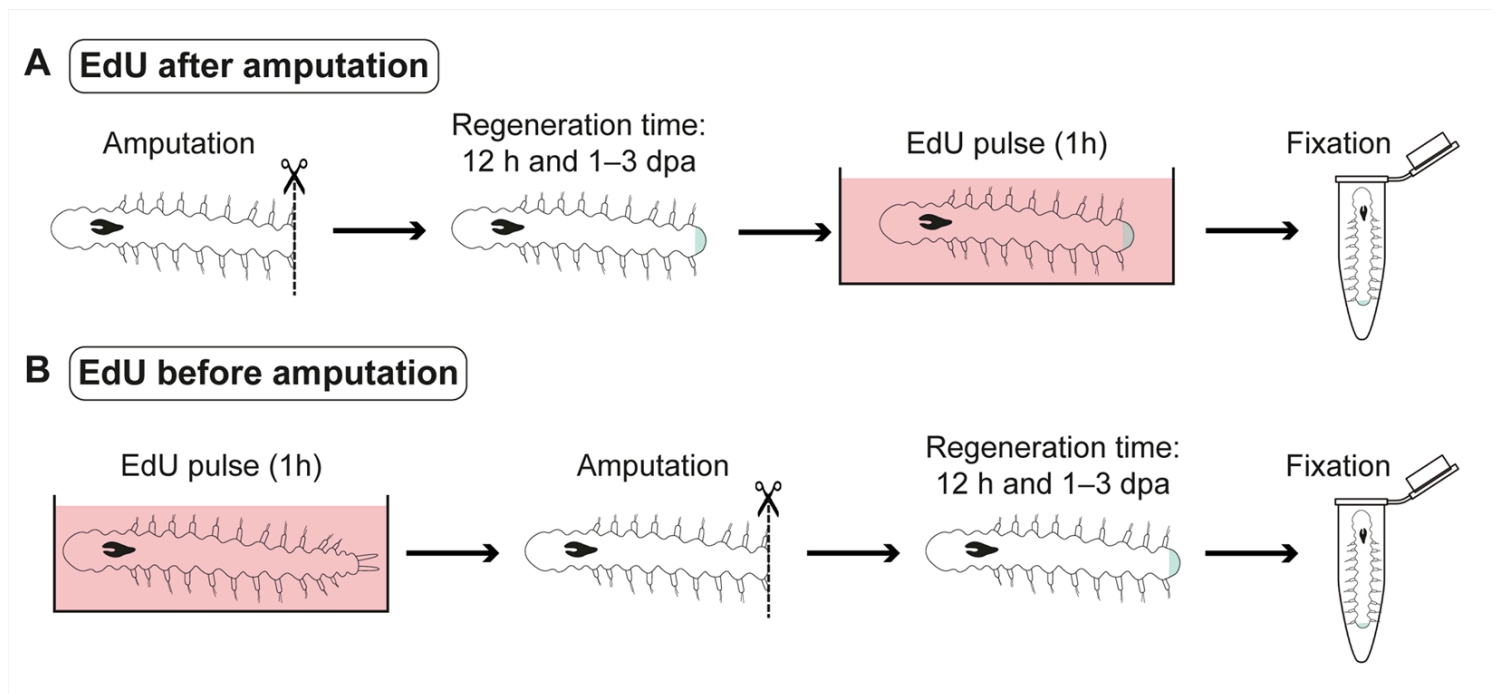


Figure 4

Experimental design for EdU incorporation assays. Schematic representation of EdU incorporation protocols. **(A)** EdU incorporation 1 h before amputation followed by regeneration for the indicated time prior to fixation. **(B)** EdU incorporation for 1 h at each regeneration timepoint after amputation and prior to fixation.

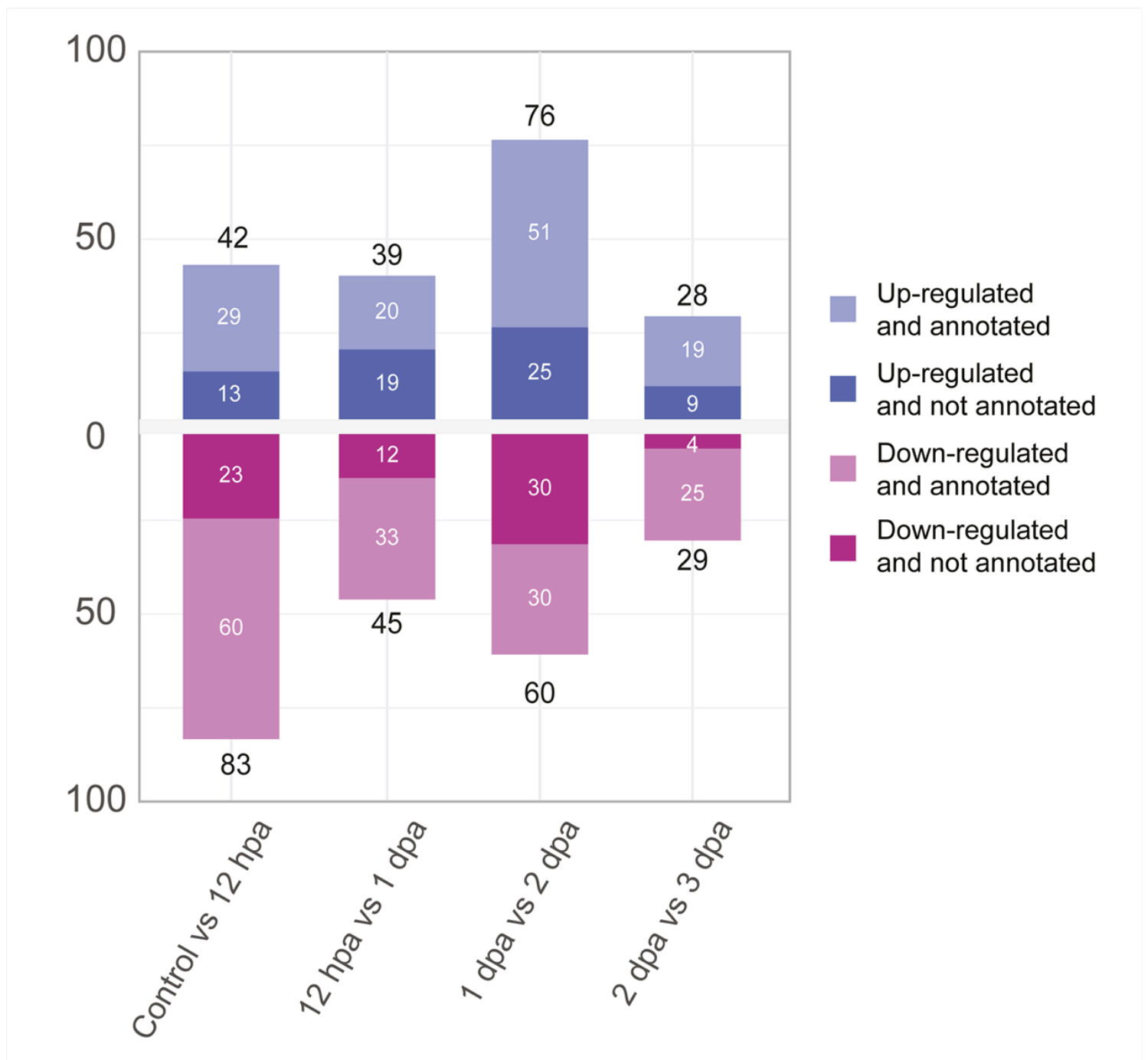


Figure 5

Differential gene expression across regeneration stages in *O. labronica*. Bar plots show the total number of upregulated and downregulated genes identified between sequential stages, separated into annotated and non-annotated genes.

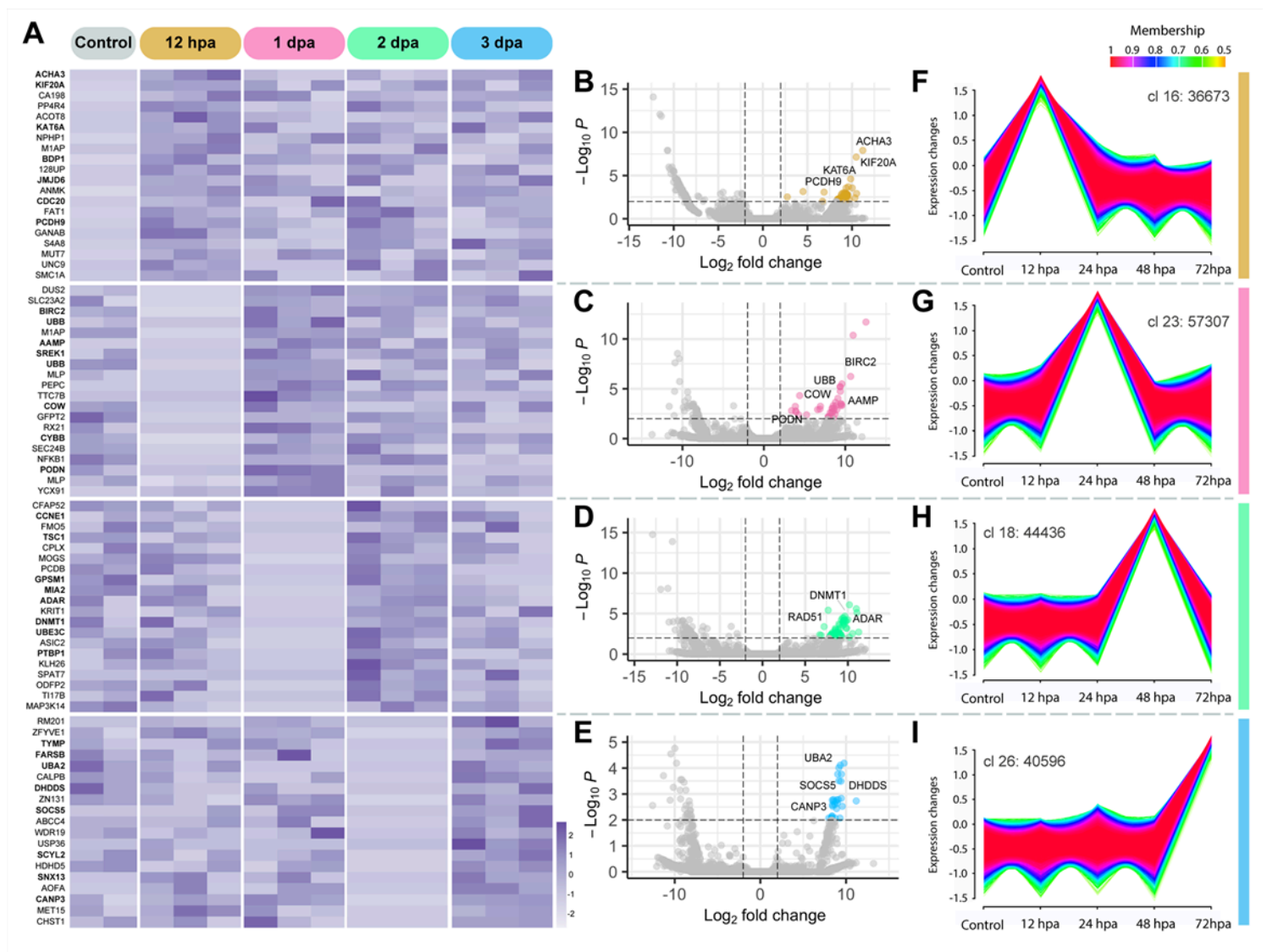


Figure 6

Gene expression dynamics during posterior regeneration. (A) Heatmap showing the top 10 upregulated and annotated genes resulting from the DEG analysis (fold change $\geq |2|$, p-value < 0.01) from sequential pairwise comparisons. Genes highlighted in bold are discussed in the main text. (B-E) Volcano plots displaying $-\log_{10} p$ -values as a function of fold change for each regeneration stage comparison; colored points indicate significantly upregulated genes. (F-I) Mfuzz clusters showing gene expression profiles across the regeneration process, each with a peak of expression at a specific timepoint. Red-colored lines correspond to genes with high membership value. The cluster number and the number of transcripts contained in each cluster is indicated in each profile.



Figure 7

Functional enrichment dynamics during posterior regeneration. Bubble plot showing enriched GO terms of the unique genes in the Mfuzz clusters at each regeneration stage, grouped by functional categories. Bubble size reflects the number of associated genes, and bubble colour indicates the significance level. Terms highlighted in bold are discussed in the main text.

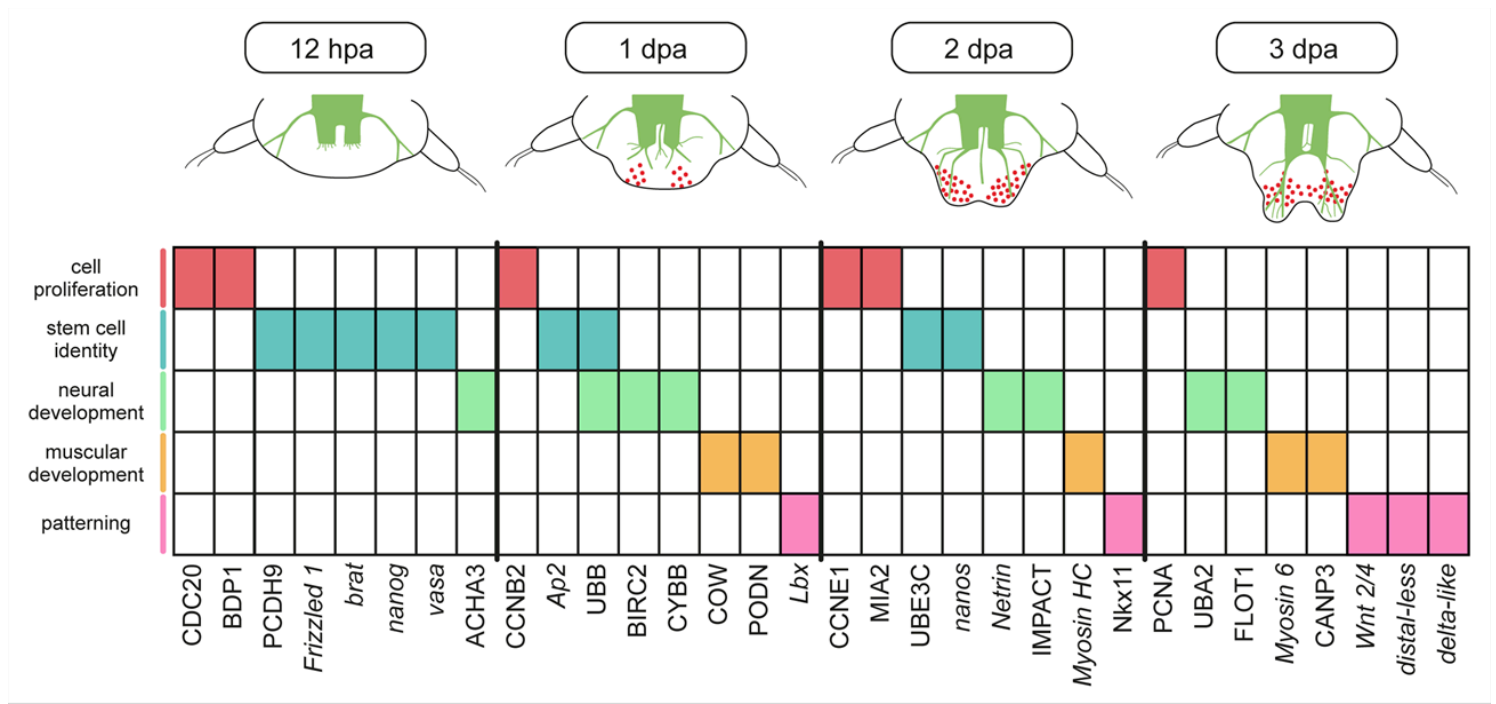


Figure 8

Overview of posterior regeneration in *O. labronica*. Schematic representation of key morphological events and associated gene functions during posterior regeneration in *O. labronica*.

Supplementary Files

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- [SupplementaryInformation1.pdf](#)
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