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ORIGINAL ARTICLE

# Understanding the Multi-Functional Role of TCTP in the Regeneration Process of Earthworm, *Perionyx excavatus*

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**Abstract**

***BACKGROUND:*** Regeneration is a highly complex process that requires the coordination of numerous molecular events, and identifying the key ruler that governs is important to investigate. While it has been shown that TCTP is a multi-functional protein that regulates cell proliferation, differentiation, apoptosis, anti-apoptosis, stem cell maintenance, and immune responses, but only a few studies associated to regeneration have been reported. To investigate the multi-functional role of TCTP in regeneration, the earthworm *Perionyx excavatus* was chosen.

***METHODS:*** Through pharmacological suppression of TCTP, amputation, histology, molecular docking, and western blotting, the multi-function role of TCTP involved in regeneration is revealed.

***RESULTS:*** Amputational studies show that *P. excavatus* is a clitellum-independent regenerating earthworm resulting in two functional worms upon amputation. Arresting cell cycle at the G1/S boundary using 2 mM Thymidine confirms that *P. excavatus* execute both epimorphosis and morphallaxis regeneration mode. The pharmacological suppression of TCTP using buclizine results in regeneration suppression. Following the combinatorial injection of 2 mM Thymidine and buclizine, the earthworm regeneration is completely blocked, which suggests a critical functional role of TCTP in morphallaxis. The pharmacological inhibition of TCTP also suppresses the key proteins involved in regeneration: Wnt3a (stem cell marker), PCNA (cell proliferation) and YAP1 (Hippo signalling) but augments the expression of cellular stress protein p53.

***CONCLUSION:*** The collective results indicate that TCTP synchronously is involved in the process of stem cell activation, cell proliferation, morphallaxis, and organ development in the regeneration event.

**Keywords** TCTP · Buclizine · Wnt3a · p53 · Hippo signaling

## 1 Introduction

The homeostatic process known as 'regeneration' directs the recovery of damaged or lost tissues [1]. Generally, epimorphosis and morphallaxis are the main principles of regeneration [2–4]. There are differences and similarities between the two processes. After wound induction, the primary tissue injury response invites stem cells from the niche [5, 6] and will accumulate at the wound site that depends on signals for healing and regeneration [7]. The cellular mechanism of apoptosis [8] and immune responses are essential for the morphallaxis mode of regeneration,

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and tissue-specific stem cells are not required in this process [9] But epimorphosis requires tissue-specific stem cells, apoptosis, and immune responses [10, 11]. For example, *Hydra vulgaris* follows only morphallaxis [12], limb regeneration of *Xenopus laevis* froglet follows the epimorphosis mode of regeneration [13], and planarians follow both the regeneration principles [8]. Earthworms like *Eudrilus eugeniae* and *Perionyx excavatus* can regenerate their tails, segments, or even entire bodies from fragments that break off from the original worm. The pieces need a portion of the digestive and reproductive systems for regeneration. Earthworms can also regenerate lost tissue through the growth of new cells. This type of regeneration is more limited than fragmentation regeneration and can occur when the earthworm has minor injuries or is damaged but not fragmented [6].

During restoration, the earthworm's body undergoes several cellular and molecular changes. The regeneration process begins with forming a blastema, a group of undifferentiated cells that can differentiate into the specific cell types required to regenerate the lost body part. The blastema grows and differentiates into the tissues needed for the new body part, such as muscles, nerves, and skin. Apoptosis is critical in regenerating body parts in *E. eugeniae* and other earthworms and maintaining tissue homeostasis in these organisms [14]. During the regeneration process, apoptotic cells help break down and remove damaged tissue, stimulating the proliferation of new cells in the blastema. In addition to its role in tissue remodeling during regeneration, apoptosis also plays a role in maintaining tissue homeostasis in earthworms. Studies have shown apoptotic cells in the reproductive organs of earthworms during spermatogenesis, where they help remove excess cells and maintain the proper balance of germ cells and support cells [15].

When cells die because of stress or damage, it can cause the remaining cells to divide more, a process termed "apoptosis-induced compensatory proliferation (AICP)." Stem cells must proliferate and differentiate for the compensation, but this whole process depends on various signaling molecules, such as Wnt and Hh, produced from apoptotic caspases in response to cell damage [16–19]. Apoptotic executioner caspases proceed with Apoptosis Induced Apoptosis (AIA) and AICP to compensate for lost parts [20, 21]. Those driving caspases may belong to Feeder cells [22, 23]. Collective reports indicate that most of the animal models such as *Hydra*, planarians, newts, *Drosophila melanogaster*, and mouse follow AICP for the reformation of lost parts [16, 24–28]. In earthworms, apoptotic cells were observed throughout their cell renewal period of regeneration [14]. On another side, in juvenile *E. eugeniae* worms, the movement of stem cells from the clitellum to the wound site was observed [5]. However, the connective link between apoptosis and stem cells is still unclear.

According to the recent report of transcriptomic analysis of *E. eugeniae*, while more than 3986 genes are upregulated during their regeneration [29], silencing a single protein called Translationally Controlled Tumor Protein (TCTP) influences the entire progress of regeneration of *E. eugeniae* regeneration [30]. TCTP has both apoptotic and anti-apoptotic functions [31, 32] and can decide the fate of cells [33]. TCTP also plays a remarkable role in regeneration [30] and regulates major cellular mechanisms like proliferation, cell homeostasis, and survival [34], maintaining the stability of p53 [32]. For example, reciprocal repression between TCTP and p53 was observed in knockout mice models [35].

Researchers have used chemicals and siRNAs for silencing TCTP, such as Buclizine and Nutlin-3a [30]. Seo and Efferth [43] confirmed that buclizine could block the TCTP protein in MCF-7 cell lines at the cellular level. The tropical earthworm, *Perionyx excavatus*, has a unique regeneration ability [36]. The survival capacity of *P. excavatus* is higher than other earthworm species [37]. *P. excavatus* is a regeneration animal model for the central nervous system and tissue regeneration [37, 38]. Within two weeks of the amputation, *P. excavatus* achieves complete regeneration, including rebuilding vital organs [37]. Recently, Bae et al. [37] observed the epimorphosis mode of head regeneration in the earthworm *P.* excavatus [37], but the role of morphallaxis in earthworms is vague. Martinez et al. [39] suggest that the earthworm might follow both epimorphosis and morphallaxis regeneration patterns due to high predatory injury, but clear scientific evidence is unclear [40].

We observed that *P. excavatus* follows both epimorphosis and morphallaxis forms of regeneration in the present study. In addition, we report that TCTP governs morphallaxis along with the epimorphosis pattern of regeneration. Also, we provide direct evidence that TCTP is a multi-functional protein that regulates many functions associated with the regeneration mechanism, especially in association with AICP.

## 2 Materials and methods

### 2.1 Earthworm rearing and maintenance

We reared the Earthworm, *Perionyx excavatus*, from the stock maintained in the Centre for Molecular and Nanomedical Sciences, International Research Centre, Sathyabama Institute of Science and Technology, Chennai, Tamilnadu, India. Earthworms were maintained according to the standard protocol [39–42]. Cow dung and leaf litter

were fed to worms in equal amounts and kept in the plastic tub at the appropriate moisture condition.

### 2.2 Cell cycle arresting assay

To block epimorphosis, 2 mM thymidine was injected into the 24th segment of the worms (No: 10). Nuclease-free water injection was used for the control set of worms (No: 10). An injection was continued for up to 10 days. After the second day of injection, the worms were amputated on the 10th segment and observed for their regeneration kinetics with a 15 cm scale and camera.

### 2.3 Buclizine injection and pharmacological suppression of TCTP

In this experiment, three dose levels of Buclizine (Mankind Pharma Ltd, Chennai, India), namely 140 μg, 160 μg, and 200 μg, were injected into the post-clitellum regions (24th segment) of *P. excavatus* worms once per day throughout the experiments. The control worm was injected with 1X PBS. Following 2nd day of the initial injection, the worms were amputated at the 10th segment and observed for regeneration ability. The regeneration process was monitored and documented using a Canon digital camera (IXUS 285 HS).

### 2.4 Regeneration studies

We used the eight groups of mature *P. excavatus* worms in this study to understand the importance of TCTP in regeneration. Each group consisted of ten worms, and the treatment is as follows: 1. *in vivo* regeneration analysis, 2 and 3. Control and Buclizine treatment for TCTP silencing, 4 and 5. Control and Thymidine treatment for cell arrest analysis, and 6. Buclizine and thymidine treatment for combinatorial toxicology. 7 and 8. Control and Nutlin-3a treatment for TCTP silencing. For *in vivo* regeneration analysis, selected worms (group 1) were amputated in the 10th segment (anterior region-head) using a cruzine carbon steel surgical scalpel blade (size 15) and maintained in the worm bed. Every 24 h, the amputated worms were documented with the help of a canon digital camera (IXUS 285 HS).

### 2.5 Influence of Nutlin-3a in regeneration

To study the importance of TCTP in regeneration, mature *P. excavatus* worms were selected. Two batches were selected, each containing 10 worms, 10 for control and another 10 for Nutlin-3a treatment. The first batch of ten worms acted as a control and was injected with DMSO. The second batch of ten worms was injected with Nutlin-3a in the 24th segment at a dose of 5 μg/g. The control and treated worms were amputated at the 10th segment (anterior) and maintained in the soil. The worms were anesthetized by ice before amputation. The blastemal formation was carefully observed in both batches of worms with the help of a Canon digital camera (IXUS 285 HS).

### 2.6 Histology

Thymidine-treated 7th day regenerated worms, 5th day Nutlin-3a treated samples and their respective control samples were subjected to histological sectioning. Both the regeneration blastema of the control and treated worms were cut using sterile scalpels along with the adjacent two segments and fixed in 10% formaldehyde (Cat.F0080; Rankem, Mumbai, India) for 24 h. To remove formaldehyde, tissues were gently washed with distilled water and dehydrated with isopropanol and acetone after the tissue was cleared using xylene. Each step was performed for one hour at 50 °C. Following the xylene removal step, tissue impregnation was incubated in paraffin wax, and the 5 μm sections were made using a microtome [30]. Eosin and hematoxylin stains were used to distinguish the internal arrangements and were observed in Euromex bScope Epi-Fluorescence HBO Microscope (Catalogue Number: BS.3153-PLFi).

### 2.7 Western blotting

All experiment samples were quantified using Lowry's method, and an equal volume (80 μg) of protein samples was loaded onto SDS page gel electrophoresis and applied to run at 60 volts for 2 h 30 min using Bio-Rad gel systems. The resolved proteins in the SDS-PAGE gel were transferred to the PVDF membrane. The membrane was blocked with 5% BSA in TBST buffer and then incubated with either of the following primary antibodies of Anti-TCTP (Abcam [Cambridge, UK], ab37506; dilution, 2.5:5000), Anti-p53 (Abcam, ab26; dilution, 2.5:5000) and Anti-β-actin (Abcam, ab8226; dilution, 1:5000), Anti-Wnt3a (Abcam, ab19925; dilution, 0.5:5000), Anti-PCNA (Abcam, ab18197), Anti-H3 (Abcam, ab1791; dilution, 1:5000) and Anti-YAP1 (Abcam, ab62751; dilution, 0.5:5000) for overnight at 4 °C. After washing, the secondary antibody of Anti-rabbit IgG-HRP (1:10000) or Anti-mouse IgG-HRP (1:10000) was added to protein transferred membrane. The substrate ECL was used as a developer solution. ChemiDoc XRS documented the developed membrane, Bio-Rad (Hercules, CA, USA); the band intensity was analyzed using ImageJ analysis software (NIH, Bethesda, MD, USA).

### 2.8 Homology modelling of TCTP protein

TCTP of *Lumbricus rubellus* (Humus earthworm) was selected as the target sequence for the study. The one-dimensional FASTA sequence of the target protein, TCTP, were retrieved from the UniProt protein sequence database with accession number 018477. The structural blast was performed with the TCTP sequence of *L. rubellus* against the PDB database to identify the template structure for homology modelling. The homology model of *L. rubellus* TCTP protein was generated by SWISS-MODEL homology modelling server with the crystal structure of human histamine releasing factor-translationally controlled tumor protein (HRF-TCTP) (PDB ID: 5O9M) as the template structure. The quality and reliability of the constructed model were evaluated by PROCHECK, ERRAT, and VERIFY3D servers.

### 2.9 Ligand preparation for docking

The structure of the ligand, buclizine, was constructed using the 2D/3D ChemBio Draw Ultra software application, version 12 (Cambridge Soft, Cambridge, MA, USA), and then copied into the ChemBio 3D Ultra software application, version 12, to create the 3D structure. Then the structure was energy minimized by the MMFF94 method using the geometry optimization function in Chem Draw 3D software, and the lower energy conformations were selected for molecular docking studies.

### 2.10 Molecular docking studies

A molecular docking process was carried out using Autodock vina to reveal the binding analysis of *L. rubellus* (closely related species of *P. excavatus*) TCTP protein vs. Buclizine (PubChem ID: 6729). The protein's binding site was adjusted using a grid box and x, y, and z-axis values. The grid box (60ÅX 60Å X60Å) is centered at 4.066, 20.626, − 11.479, and the spacing is 1. All other parameters were kept as default. An exhaustiveness of 10 was assigned for docking throughout the docking process, and 10 mode numbers were assigned to achieve reliable results. Post-docking analyses were carried out using Discovery Studio.

## 3 RESULTS

### 3.1 Anterior regeneration and tissue growth in the earthworm, *P. excavatus*

The earthworm *P. excavatus* is a bright brown segmented worm, and its clitellum segments are located between 13 to 17th segments, as shown in Fig. 1A. The worms were amputated at the 50th segment, i.e., away from the clitellum segments, and observed for their regeneration ability. The amputated anterior segments with head and clitellum can regenerate the tail on the 8th day of post-amputation. Similarly, the other portion of amputated posterior segments without clitellum segments form the regenerative head on the 8th day of post-amputation, as shown in the Fig. 1B. To understand the wound healing and regenerative dynamics of anterior regeneration, the earthworms are amputated at the 10th segments and observed every 24 h, as shown in Fig. 1C–G. Following 24 h of post-amputation, perfect sealing of amputation sites occurs with the formation of deep furrow (Fig. 1C), and wound healing was distinctly observed following 48 h of post-amputation (Fig. 1D). The worm starts to restore their lost tissue by forming a pre-blastemal structure following 72 h of post-amputation (Fig. 1E) and with increased size in 96 h (Fig. 1F). In proceeding days, the blastema starts to elongate further and differentiate to form the new lost segments following 120 h of post-amputation as shown in Fig. 1G.

### 3.2 *P. excavatus* follows both epimorphosis and morphallaxis patterns for their regeneration

To further investigate the regeneration mechanism of clitellum-independent *P. excavatus* worm, the worms were injected with 2mM Thymidine to arrest the cell proliferation or synchronize the cells in the G1/S phase. The worms injected with 2mM Thymidine as described in materials and method are amputated on the 2nd day and observed for regeneration ability on the 2nd, 4th, 6th, and 8th day of post-amputation (Fig. 2A–H). The result shows that in the control worm, the blastema starts to project out on the 2nd day of post-amputation, but in the 2mM thymidine-injected worm, it is visible only with wound healing. On successive days of the 4th, 6th, and 8th days of post-amputation, the size of the regenerative bud is 1/3rd reduced in 2mM Thymidine injected worm when compared with the control worms. The delayed regeneration following cell proliferation inhibition with 2mM thymidine injection represents the worms following morphallaxis and epimorphosis for their regeneration. Comparative histological analysis of the 8th day of the regenerative bud of control and 2 mM thymidine-treated worms shows that in control worms, the bud segments are well elongated along with septum formation however this was not observed in Thymidine treated groups (Fig. 2I, J). Also, in the control worm, the functional mouth is formed along with a well-developed prostomium, but in 2 mM thymidine-treated worms, it initiates the formation of the intestine without forming a functional mouth and prostomium (Fig. 2I, J).

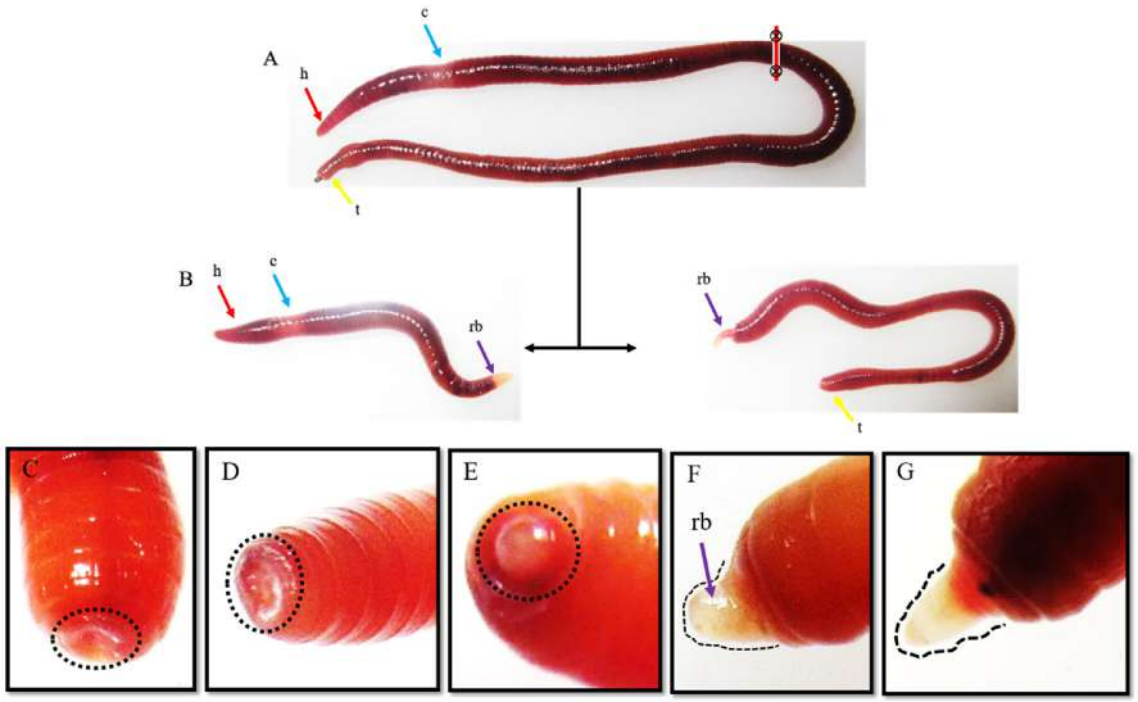


**Fig. 1** Regeneration dynamics in clitellum independent earthworm, *P. excavatus*: **A** The earthworm, *P. excavatus*, appears red-brown and possesses an anterior head followed by clitellum, intestine, and anus. **B** amputation of earthworm at post-clitellum segments (50th segments), resulting in the development of two individual worms following eight days of post-amputation. **C** Following 24 h of post-amputation at the 10th segment, the wound sites were sealed by the contraction of surrounding tissues. **D** proliferative cells fill the deep furrow following 48 h of post-amputation **E** Pre-blastema structure was visibly observed on 72 h of post-amputation **F** Blastemal size elongated following 96 h post-amputation **G** Elongated blastema starts to differentiate and form visible segments following 120 h of post-amputation

### 3.3 Pharmacological suppression of TCTP using Buclizine, modelling of TCTP protein, and molecular docking of TCTP and Buclizine

Buclizine is an antihistamine and anticholinergic compound used to prevent the symptoms caused by histamine activity. Buclizine has effectively inhibited the TCTP protein in human cells [43]. Buclizine was injected into the worm in different concentrations of 140 µg, 160 and 200 µg and observed for the regeneration ability to identify the role of TCTP in *P. excavatus* regeneration, as shown in Fig. 3A, L. The results show that different buclizine concentrations hinder regeneration on the 1st, 4th, and 6th day of post-amputated worms to a level of 1/4th compared to the control worms. Also, we observed no significant difference among the different concentrations of buclizine used in these experiments. Therefore, we preferred a lower concentration of 140 µg for further Western blotting investigations. Protein samples are prepared from 3rd and 5th-day control regenerating and buclizine-injected regenerated worms and subjected to Western blotting against TCTP (19 kDa) and $\beta$-actin proteins (Fig. 3M). The results indicate that in control regenerating worms, TCTP upregulated in succeeding days of the 3rd and 5th days of post-amputation. On the other hand, in the buclizine-treated worms, TCTP is completely inhibited on 3rd day of buclizine-injected regenerated worms but negligibly observed on 5th day. The $\beta$-actin is used as a positive control, and its molecular weight is 42 kDa. The graphic representation of normalized values is shown in Fig. 3N.

We performed homology modelling by generating a *L. rubellus* TCTP 3D protein structure. Human histamine-releasing factor-translationally controlled tumor protein (HRF-TCTP) (PDB ID: 5O9M) was selected as the template structure for protein model building. The template 5O9M exhibited 47.90% sequence identity and a GMQE score of 0.71 with *L. rubellus* TCTP protein. The above parameters confirm that the template 5O9M could be the best template for homology modelling. The PROCHECK server evaluated the stereochemical quality of the protein model. The results of the PROCHECK server (Fig. 3O) confirmed that 93.2% of residues were in the most favored regions, and none were placed in disallowed areas. The above result confirms that the generated 3D structure of *L. rubellus* is a reliable and good-quality model. In addition to the stereo-chemical assessment, the local errors of unbounded atomic interactions were assessed by the ERRAT server (Fig. 3P). The ERRAT analysis showed an

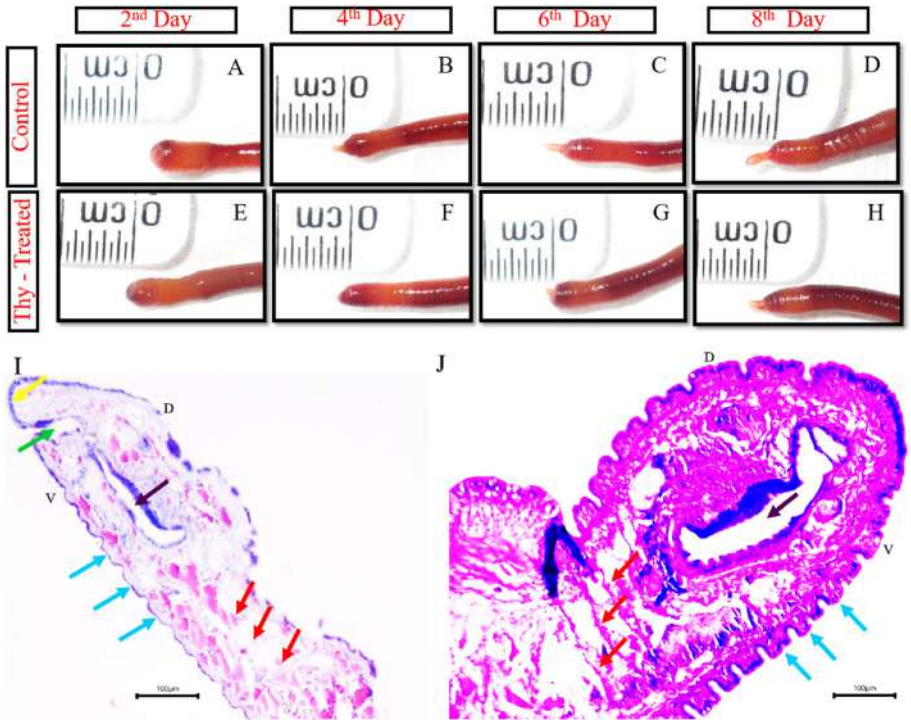


**Fig. 2** Regeneration following 2 mM Thymidine (cell cycle inhibitor) injection: **A** Control worm heals wound on 2nd day of post-amputation. **B** Blastema size of 0.2 mm was observed on the 4th day after post-amputation. **C** regenerative bud elongated to 0.3 mm on the 6th day. **D** blastemal size almost doubled (0.5 mm) on the 8th day upon restoration. (E) wound healing was adequately executed in Thymidine treated worm on the 2nd day of post-amputation. **F** blastemal growth is restricted and shows only 0.1 mm size following the 4th day of post-amputation. **G** unmeasurable very little blastemal growth progress was observed until the 6th day of post-amputation. **H** blastemal size is slightly elongated to 0.2 mm size following the 8th day of post-amputation. **I** histology of 8th day control blastemal tissue shows well-differentiated prostomium, mouth, septum, and elongated segments. **J** histology of 2 mM Thymidine injected 8th day regenerative blastema with less developed structures lacking prostomium, mouth, and with truncated regenerative body segments. Arrows (red represents septum; blue represents newly regenerated segments; yellow indicates prostomium; green represents functional mouth; purple represents intestinal tract). "D" represents the dorsal side of the earthworm, and "V" specifies the ventral surface of the earthworm

overall quality factor of 98.5% for the homology-modelled 3D structure of *L. rubellus*. Further, the compatibility of the 3D atomic model of *L. rubellus*, TCTP protein with its 1D sequence was assessed by VERIFY 3D server. It is evident from the result that 99.40% of the residues with an average 3D-1D score were achieved. Therefore, all the above results confirmed the modelled 3D structure of *L. rubellus* TCTP protein as the quality model for further theoretical studies.

According to molecular docking results, buclizine formed hydrogen bonding and hydrophobic interactions with *L. rubellus* TCTP protein with a binding energy of − 6.1 kcal/mol. Two hydrogen bonds were interacting with the TCTP residues ASN49 and ALA 45 with a bond length of 3.6 Å and 3.5 Å (Fig. 3Q). Hydrophobic interactions were displayed with four residues: ALA 50, ILE 41, ALA 62, and ALA 45, as shown in Table 1. A notable feature in the docking analysis was that the residue ALA 45 exhibited hydrogen bonding and non-bonded interactions towards the ligand buclizine. The above results suggest that the residue ALA 45 and the adjacent flanking residues are the active site region of *L. rubellus* TCTP protein.

### 3.4 TCTP and its role in morphallaxis following combinatorial injection of buclizine and thymidine

*P. excavatus* regenerates through epimorphosis (cellular proliferation) and morphallaxis (trans-differentiation), as confirmed by 2 mM thymidine injection. There is currently no report available in the academic literature that comprehensively covers the morphallaxis mechanism, specifically concerning trans-differentiation, within the context of TCTP. We administered a combination of 2mM Thymidine (an epimorphosis blocker) and Buclizine (a TCTP suppressor) to investigate the matter and show the results in Fig. 4A–I. Well-differentiated blastema was observed in the control group of worms on the 8th day, as depicted in

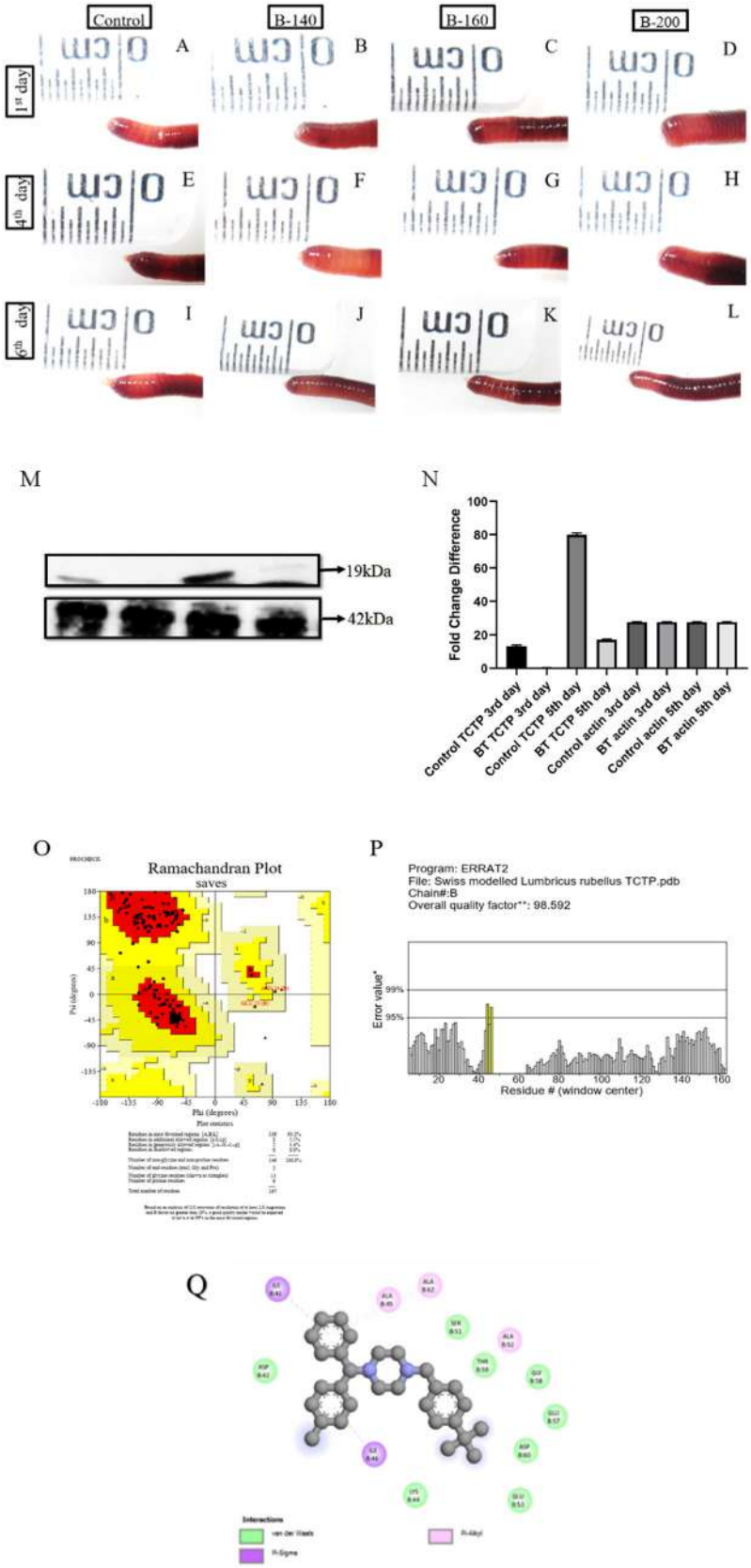


**Fig. 3** Pharmacological suppression of TCTP using buclizine and its effects on regeneration: **A–D** represents control worm (1X PBS), 140 µg buclizine, 160 µg buclizine, and 200 µg buclizine injected worms respectively following day-1 of post-amputation. **E–H** represents the 4th day post amputated worms correspondingly from the control worm (1X PBS), 140 µg buclizine, 160 µg buclizine, and 200 µg buclizine injected worms. In all the buclizine-injected worms, the regenerative bud size is hindered. Similarly, **I-L** represents the 6th day regenerative bud from the control worm, 140 µg buclizine, 160 µg buclizine, and 200 µg buclizine-injected worms, respectively. In all the buclizine-injected worms, regeneration is 1/4th reduced. M. Western blotting image represents TCTP (19 kDa) and β-actin (42 kDa) expression in 3rd day control regenerating worm, 3rd day buclizine injected regenerating worm, 5th day control regenerating worm and in 5th day buclizine injected regenerating worms respectively. N. graphical representation of Western blotting results shows the fold difference of TCTP and the expression of β-actin in control regenerating worms and the 5th day buclizine injected regenerating worms. O. Stereo chemical validation of predicted *L. rubellus* TCTP protein using Ramachandran plot. P. Evaluation of *L. rubellus* TCTP protein for unbounded atomic interactions by ERRAT server. Q. *In-silico* prediction of molecular-level interaction between Buclizine and TCTP protein

**Table 1** *In-silico* docking analysis of TCTP versus Buclizine

| S. no | Protein | Ligand | Affinity | RMSD (I. B.) | RMSB (U. B.) | No. of bonds | Peptide– llele interaction (non-bound) | Distances | Category |
|---|---|---|---|---|---|---|---|---|---|
| 1 | TCTP (*Lumbricus terrestris*) | Buclizine | − 6.1 | 4.633 | 8.898 | 2 hydrogen bonds | :UNK0:C - B:ASN49:O | 3.64063 | Hydrogen Bond |
| | | | | | | + | :UNK0:C - B:ALA45:O | 3.58072 | Hydrogen Bond |
| | | | | | | 6 hydrophobic bonds | B:ALA50:CB - :UNK0 | 3.9661 | Hydrophobic |
| | | | | | | | :UNK0:Cl - B:ILE41 | 5.40736 | Hydrophobic |
| | | | | | | | :UNK0:Cl - B:ALA62 | 4.17849 | Hydrophobic |
| | | | | | | | :UNK0:C - B:ALA50 | 4.28267 | Hydrophobic |
| | | | | | | | :UNK0 - B:ALA45 | 4.19758 | Hydrophobic |
| | | | | | | | :UNK0 - B:ALA45 | 4.92024 | Hydrophobic |

Fig. 4A–C. The combinational injection of Buclizine and Thymidine completely inhibited the regeneration process for eight days post-amputation (Fig. 4D–F). However, there were no observable adverse effects on the physiological survival of the worm. The findings above suggest that TCTP governs cellular proliferation (epimorphosis) and substantially influences the morphallaxis (trans-differentiation) mechanism of regeneration, as evidenced by the total hindrance of regeneration. When we injected the worms with a double dose (two doses per day) of either 2 mM Thymidine or Buclizine, their physiological functions shut down, leading to their death within 6 days. The Mantel-Cox test was utilized to calculate and present the survival rate of worms treated with a double dose of Buclizine and Thymidine, as illustrated in Fig. 4G. The study revealed a reciprocal repression of the TCTP/p53 pattern in samples treated with buclizine/thymidine, as evidenced by the significant upregulation of p53 expression in TCTP-blocked samples (Fig. 4H–I).

### 3.5 Inhibition of TCTP through Nutlin-3a and their counter effect on essential regenerative proteins

Nutlin-3a indirectly promotes TCTP degradation by activating p53. To further understand the function of TCTP in regulating the key regenerative proteins, the TCTP is pharmacologically inhibited using Nutlin-3a in three different dosages of 5, 7, and 9 µg injection. When compared with the control worms, the Nutlin-3a injected worms show an overall reduced bud size (5th day) in all the concentrations (Fig. 5A [I–IV]). It is also evident that increasing the level of Nutlin-3a gradually hinders the regeneration process in the earthworm *P. excavatus.* For further histological and western blotting experiments, Nutlin-3a (9 µg) injected worm samples were taken for analysis. Comparative histology of the regenerative bud tip between control (Fig. 5B) and 9 µg Nutlin-3a injected worms (Fig. 5C) shows that the outermost epithelial layer is well organized and thicker in control worm but which is lacking in Nutlin-3a treated worms. Also, the next inner layer, the outer epithelial layer, has a thickened outer covering in the control worm, which is more thinned in treated worms. The bud interior shows more compact structures in control worms but is highly diffused without any organized form observed in Nutlin-3a treated worms. Collectively, Nutlin-3a confirms that it had a direct influence on cell package and implies the role of TCTP in holding all-essential cellular functions towards regeneration. To confirm further Western blotting was performed in control and Nutlin-3a treated worms against PCNA (cell proliferation), Wnt3a (stem cell marker), and YAP1 (organ formation ruler and Hippo signaling). The results revealed that TCTP influences all the critical regeneration proteins upon inhibiting with Nutlin-3a (Fig. 5D). PCNA, Wnt3a, and YAP1 are remarkably reduced in all TCTP-inhibited worms, and it is evident that TCTP is a multi-functional protein that governs many functions associated with the regeneration mechanism. The graphical representation of the influence of Nutlin-3a in inhibiting TCTP and its associated proteins in regeneration have shown in Fig. 5E.

## 4 Discussion

*P. excavatus* is a topsoil earthworm that is habitually subjected to injury by predators, and correlating it with its enormous posterior regeneration ability represents its evolution nature to survey following an injury [36]. Regeneration studies show that the earthworm, *P. excavatus*, has an enormous regeneration ability. The worms

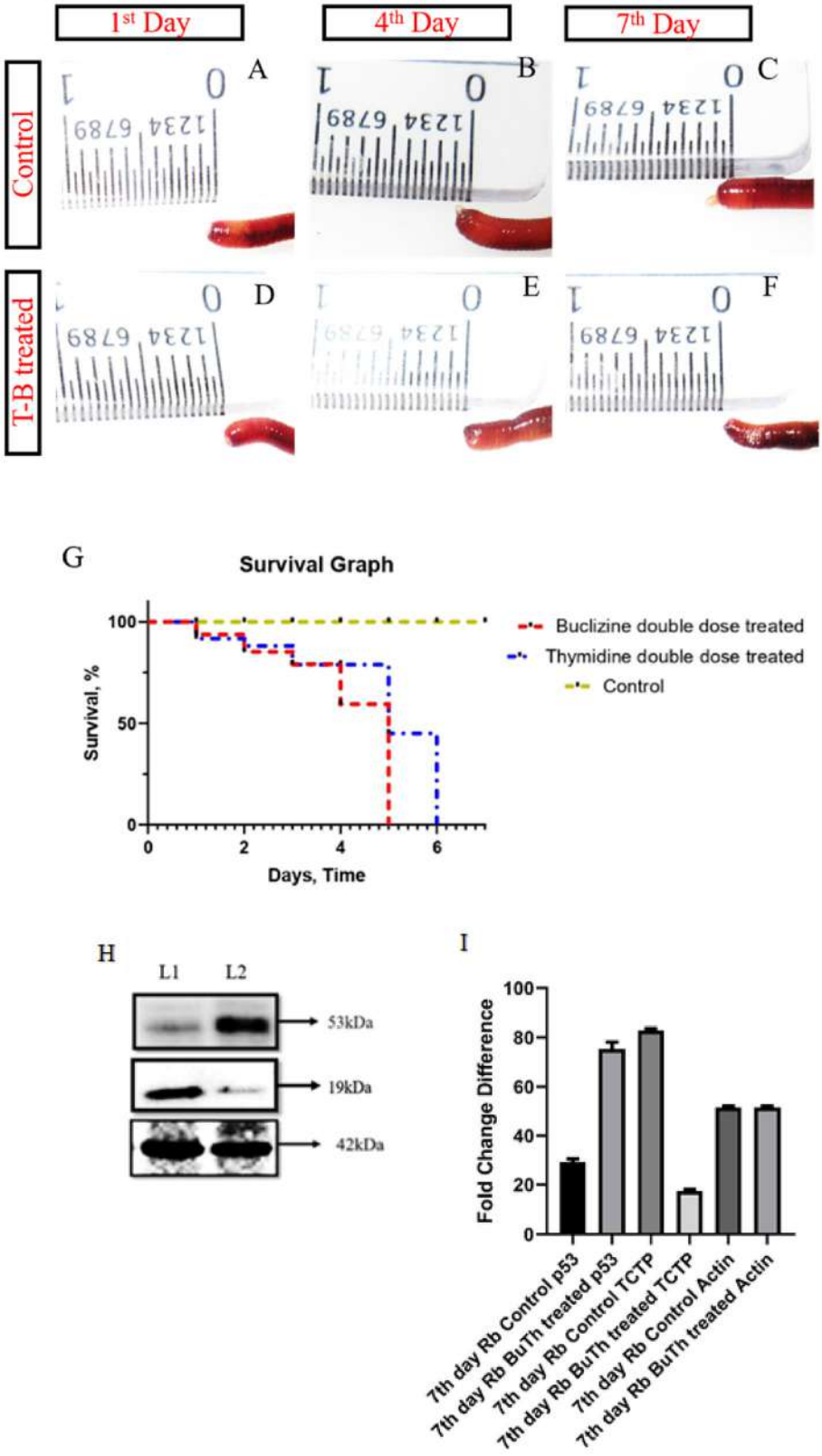


**Fig. 4** Combinatorial injection of Buclizine and Thymidine: **A** control worm following day-1 post-amputation at 10th segment. **B** the amputated worms form blastema on the 4th day. **C** on the 7th day, the amputated worm forms elongated blastema. **D** Buclizine and Thymidine injected worm following 1st day of post-amputation. **E** complete inhibition of regeneration on 4th day of post-amputation in Buclizine and Thymidine injected worm. **F** Combinatorial injected worms show no sign of regeneration even after seven days of post-amputation. **G** Survival graph for the Control *P. excavatus*, buclizine, and Thymidine double dose-treated worms. After treatment, the live and dead worms on different days are counted and plotted in the survival (Kaplan–Meier) curve. Statistical analysis was accomplished using the log-rank test (Mantel-Cox), and the obtained $p$ values $< 0.05$ were considered significant. **H** Expression of p53, TCTP, and β-actin in 8th day Control (L1) and 8th day Buclizine and Thymidine treated samples (L2). High expression of p53 and lower expression of TCTP was observed in Buclizine and Thymidine treated samples (p53–53 kDa; TCTP − 19 kDa; Actin − 42 kDa). **I** Band intensity of Western blotting image is quantified and represented with a bar diagram. Statistical significance is achieved when the $p$ value is $< 0.05$

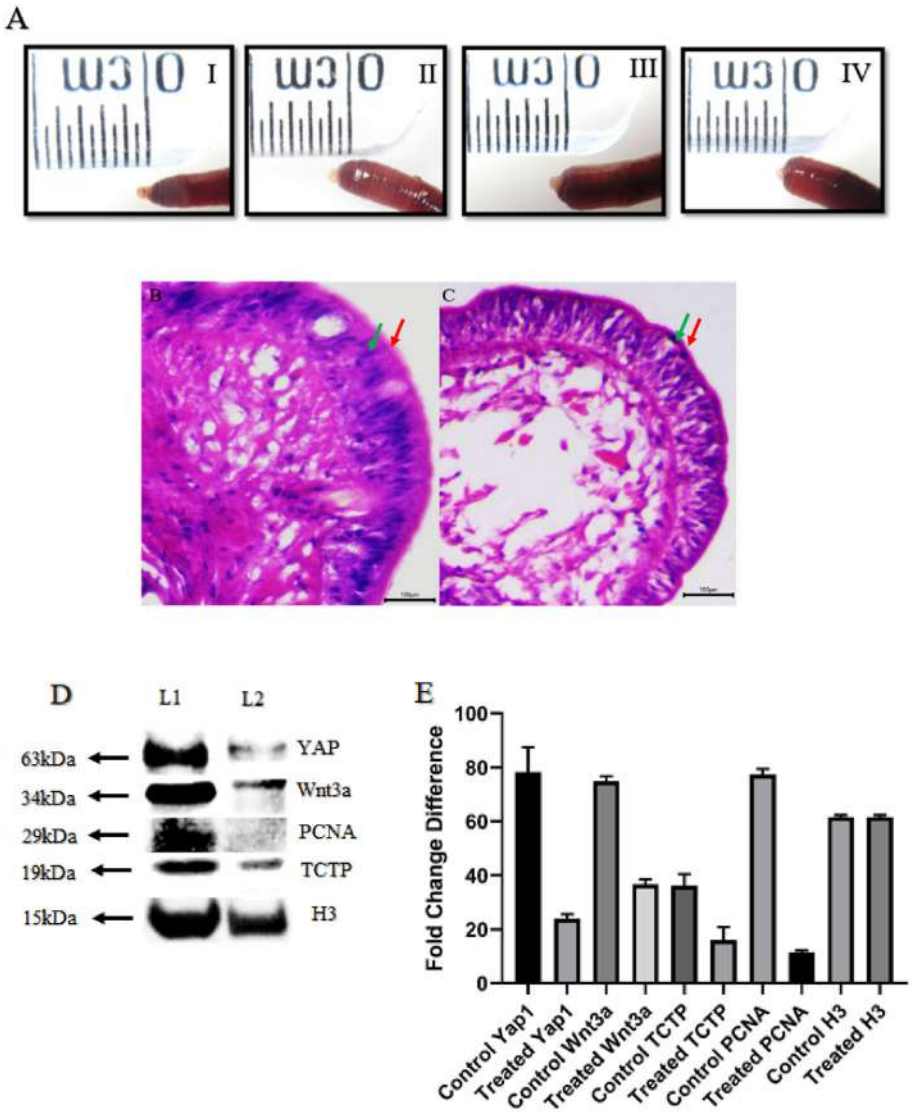


**Fig. 5** TCTP and its connecting link with regenerative protein: **A** (I). Control amputated worm with 5th day bud. II, III and IV represent progressive regeneration suppression in Nutlin-3a injected worms with 5, 7, and 9 µg, respectively. **B** Histology of control 5th -day regenerative blastema with well-organized tissue structures has the most thickened outer and inner epithelial layer. **C** In Nutlin-3a injected worms, the 5th day bud is not well organized, loosely packed with internal bud tissues and thinner layers of the outer and inner epithelium. **D** Western blotting image represents that when compared to the control samples (7th day regeneration—Lane 1), all frame of regenerative key proteins was notably reduced in Nutlin-3a treated samples (7th day regeneration—Lane 2). Nutlin-3a is known for TCTP silencing, and according to the result, TCTP silence influences organ formation (YAP1), stem cell activation (Wnt3a) and cell proliferation (PCNA). E. Quantification of YAP1, Wnt3a, TCTP, PCNA and H3 expression are done based on the band intensity and represented using bar diagram. The experiments were repeated in triplicate to analyze the statistical significance, representing their value as mean ± SD. $p$ value $< 0.05$ was considered statistically significant data. The red arrow represents the "Outermost epithelial layer"; the Green arrow represents the "Inner layer of epithelial tissue."

amputated at the post-clitellum segment (30th segment) can regrow as an individual worm. The data confirm that *P. excavatus* is a clitellum-independent worm that does not requires clitellum segments for their regeneration as it is necessary for clitellum-dependent worms [5]. The pre-blastema was observed within 48 h, and within another 24 h, the blastema developed into a well-developed structure and further developed rapidly in the following hours. The data represents that anterior head regeneration is more vigorous because it needs to restore all the vital organs like mouth, tubular heart, simple brain and other organ systems to survive and perform the normal functions. Following injection of 2mM Thymidine, the regeneration potential of the earthworm was suppressed to 1/3rd level, representing the earthworm. *P. excavatus* can perform regeneration to a certain extent by utilizing morphallaxis when blocking

epimorphosis. The epimorphosis mode of head regeneration was early reported in the earthworm *P. excavatus* [37], in which a high proliferative mass of cells forms the regenerative blastema. The blastema-like structure is observed in the arm regeneration of starfish, which adopts the intermediate mechanism of both morphallaxis and epimorphosis for their regeneration [44]. From histology, it confirms that the regenerative ability of the earthworm is suppressed in 2 mM Thymidine injected worms with a lack of development in their internal structures like functional mouth, septum, and segment elongation, which indirectly implies the factors or signals necessary for regeneration are not regulated correctly [45].

Following the pharmacological suppression of TCTP using an antihistamine drug, buclizine, the worm shows reduced regeneration ability which implies the critical role of TCTP in determining the regeneration ability of the worm. TCTP determines the cell fate on both ends, on a positive side through the influence of DNA damage and on a negative side as regulated by p53 [46]. The positive side of TCTP in determining the cell fate is revealed upon regeneration in that TCTP is upregulated on succeeding days of regeneration, and their pharmacological suppression hinders regeneration. TCTP also plays a crucial role in promoting the pathways related to cancer progression [47]. Therefore more studies are needed to understand their regulatory mechanism in determining the cell fate with controlled (Regeneration) and uncontrolled (Cancer) regulation upon many extracellular stimuli [48]. The modelled TCTP protein using earthworm sequences and their close interaction with buclizine and *in-vivo* results conclude that antihistaminics are the potent lead compounds in inhibiting the TCTP, which have a broad medicinal scope in treating cancers [48]. TCTP is a multi-functional protein that plays a crucial role in cell proliferation, cell growth, and apoptosis by interacting with many regulatory proteins [34]. TCTP's part is also well documented in regenerative models involving epimorphosis or cell proliferation [30, 34], but its role is not revealed in the aspect of morphallaxis. The combinatorial injection of 2 mM Thymidine and Buclizine inhibit both epimorphosis and TCTP protein, respectively, resulting in complete regeneration loss, but the worms survived without any physiological stress. The data confirms that 2 mM Thymidine injections block epimorphosis and conjoined inhibition of TCTP blocks morphallaxis, which can completely block the regeneration events in the earthworm, *P. excavatus.* The clitellum-independent worms have a vast regeneration ability because regeneration is not restricted or dependent only on the clitellum segments, and in such worms, TCTP governs both epimorphosis and morphallaxis. There are also high possibilities with more multi-functional ability of TCTP protein with animals with more regenerative ability and animals with less regenerative ability. In these aspects, research is needed to conclude it in the near future. Surprisingly in combinatorial injected amputated worms, the p53 level increased compared to the non-injected regenerating worms. p53 also reverses the cell cycle, allowing cells to repair their DNA and inducing apoptosis in severe DNA damage [49]. In the present study, the amputated worm is subjected to double stressful conditions, notably cell cycle arrest and TCTP suppression, and in that conditions, the worm expresses abundant p53, representing that p53 promotes cell survival to repair and rescue. Several *in-silico* and *in-vitro* research have examined buclizine as an inhibitor of TCTP, but neither study reported on *in-vivo* models [43, 50]. Here we reported the potential *in-vivo* interactions of Buclizine and TCTP with visible suppression of TCTP expression and regeneration in the earthworm model. TCTP is a multi-functional protein inhibiting them with Buclizine targets TCTP and interplays with the TCTP interacting proteins [51].

Unlike buclizine, the inhibitory effect of Nutlin-3a in targeting TCTP is well documented in many *in-vivo* models [30, 52, 53]. The delay of posterior segment regeneration and wound closure following amputation was reported in Nutlin-3a injected clitellum dependent, *Eudrilus eugeniae* earthworm. Similarly, in these present studies, upon anterior regeneration, Nutlin-3a suppresses regeneration; histologically, it is evident with the improper cellular package. The data indicates that TCTP is linked with many regenerations-associated proteins, such as those involved in cell proliferation, cellular morphallaxis, cell differentiation, apoptosis, immune response, stem cell activation, and organ development. Inhibiting TCTP with Nutlin-3a suppresses the regeneration mechanism together with influences from other proteins like PCNA (cell proliferation), Wnt3a (stem cell marker), and YAP1 (organ formation ruler and Hippo signaling). Following amputation, the microenvironment at the wound site provides signals for triggering regeneration and in which DNA damage-induced responses like apoptosis [54], stem cell migration [55] play a significant role in determining the regeneration potential. TCTP determines cell fate by regulating major cellular functions like apoptosis and proliferation [32, 56, 57]. Notably, the TCTP protein is known for its anti-apoptotic role [32, 58, 59] and also act as an apoptotic protein in some context of abrogate DNA repair [60]. Like TCTP, Wnt3a is also known for its pleiotropic cellular functions regulating cell proliferation, cell renewal, cellular differentiation, apoptosis, and motility [61]. Compared to other WNTs, Wnt3a is remarkably important in determining regeneration potential in *in-vitro*, *ex-vivo*, and *in-vivo* conditions [62]. Following pharmacological suppression of TCTP, the Wnt3a expression decreases and directly indicates the tight regulation between TCTP and Wnt3a upon

regeneration. The connective link between TCTP and β-catenin is reported in *in-vitro* and *in-vivo* cancer models [63], and in the present study the link between TCTP and Wnt3a upon regeneration is evident. The pharmacological inhibition of TCTP suppress the YAP1 signals upon regeneration and it represents the crosstalk between TCTP and YAP1. Regeneration occurs through a highly co-ordinated process and in that YAP/TAZ or Hippo pathway regulates the cell-cell interaction that determines the organ size and development [64]. YAP/TAZ complex also have a role in determining the cell fate by controlling the genes related with cell proliferation and apoptosis [65].

Apoptosis, stem cell activation, cellular proliferation, and organ development are essential for regeneration. Our studies conclude that TCTP governs both epimorphosis and morphallaxis during regeneration. Inhibiting TCTP impairs the regeneration mechanism by inhibiting all keyframes of regenerative proteins, including PCNA (proliferation), Wnt3a (Stem cell activation), and YAP1 (Hippo signaling). The cellular stress following pharmacological suppression of TCTP also initiates the p53 expression in the context of anti-apoptotic responses. Collectively, the present studies reveal the regulatory role of TCTP in connection with all critical regenerative proteins.

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**Author contributions** KR (M-Tech, SRF, Ph.D Scholar) were involved in the writing original draft, conceptualization, data curation and figures. JDSC ( Ph.D, Associate Professor (Research)) were involved in the writing original draft, conceptualization, data curation, investigation, supervision and project administration. KSC (M.Sc, M.Phil, OVDF Fellow, Ph.D Scholar) were involved in conceptualization and data curation. KM (Ph.D, Assistant Professor) were involved in bioinformatics analysis (protein preparation). PD (M.Sc), LN (M.Sc), SG (M.Sc) were involved in minor experiments.

**Data availability statement** The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Ethical statement** The experiments are carried out using lower invertebrate, earthworm therefore ethical statement is not needed. Necessary care is taken in experimental procedure that are intended to avoid unnecessary pain and sufering to the experimental animals.

## References

1. Carlson BM. Principles of regenerative biology. Amsterdam: Elsevier; 2011.
2. Agata K, Saito Y, Nakajima E. Unifying principles of regeneration I: epimorphosis versus morphallaxis. Dev Growth Differ. 2007;49:73–8.
3. Morgan TH. Sex limited inheritance in Drosophila. Science. 1910;32:120–2.
4. Morgan TH. Regeneration. Stuttgart: Macmillan; 1901.
5. Selvan Christyraj JD, Azhagesan A, Ganesan M, Subbiah Nadar Chelladurai K, Paulraj VD, Selvan Christyraj JRS. Understanding the role of the Clitellum in the regeneration events of the earthworm Eudrilus Eugeniae. Cells Tissues Organs. 2019;208:134–41.
6. Sivasubramaniam S. The earthworm Eudrilus Eugeniae: a model organism for regenerative biology. J Genet Genomic Sci. 2021;6:23.
7. Owlarn S, Klenner F, Schmidt D, Rabert F, Tomasso A, Reuter H, et al. Generic wound signals initiate regeneration in missing-tissue contexts. Nat Commun. 2017;8:2282.
8. Pellettieri J. Regenerative tissue remodeling in planarians: the mysteries of morphallaxis. Seminars in cell and developmental biology. Amsterdam: Elsevier; 2019. p. 13–21.
9. Sugiyama T, Fujisawa T. Genetic analysis of developmental mechanisms in Hydra. II. Isolation and characterization of an interstitial cell-deficient strain. J Cell Sci. 1978;29:35–52.
10. Abnave P, Ghigo E. Role of the immune system in regeneration and its dynamic interplay with adult stem cells. Semin Cell Develop Biol. 2019;87:160–8.
11. Bergmann A, Steller H. Apoptosis, stem cells, and tissue regeneration. Sci Signal. 2010;3:re8.
12. Bosch TCG. Why polyps regenerate and we don't: towards a cellular and molecular framework for hydra regeneration. Dev Biol. 2007;303:421–33.
13. Suzuki M, Yakushiji N, Nakada Y, Satoh A, Ide H, Tamura K. Limb regeneration in Xenopus laevis froglet. ScientificWorldJournal. 2006;6:26–37.
14. Bodó K, Kellermayer Z, László Z, Boros Á, Kokhanyuk B, Németh P, et al. Injury-induced innate immune response during segment regeneration of the earthworm, Eisenia andrei. Int J Mol Sci. 2021;22:2363.
15. Liu T, Wang L, Chen H, Huang Y, Yang P, Ahmed N, et al. Molecular and cellular mechanisms of apoptosis during dissociated spermatogenesis. Front Physiol. 2017;8:188.
16. Fan Y, Bergmann A. Apoptosis-induced compensatory proliferation. The cell is dead. Long live the cell! Trends Cell Biol. 2008;18:467–73.
17. Huh JR, Guo M, Hay BA. Compensatory proliferation induced by cell death in the Drosophila wing disc requires activity of the apical cell death caspase dronc in a nonapoptotic role. Curr Biol. 2004;14:1262–6.
18. Jung Y, Witek RP, Syn WK, Choi SS, Omenetti A, Premont R, et al. Signals from dying hepatocytes trigger growth of liver progenitors. Gut. 2010;59:655–65.
19. Ryoo HD, Gorenc T, Steller H. Apoptotic cells can induce compensatory cell proliferation through the JNK and the wingless signaling pathways. Dev Cell. 2004;7:491–501.
20. Pérez-Garijo A, Steller H. Spreading the word: non-autonomous effects of apoptosis during development, regeneration and disease. Development. 2015;142:3253–62.
21. Fogarty CE, Bergmann A. Killers creating new life: caspases drive apoptosis-induced proliferation in tissue repair and disease. Cell Death Differ. 2017;24:1390–400.

22. Huang Q, Li F, Liu X, Li W, Shi W, Liu FF, et al. Caspase 3–mediated stimulation of tumor cell repopulation during cancer radiotherapy. Nat Med. 2011;17:860–6.
23. Llames S, García-Pérez E, Meana Á, Larcher F, del Río M. Feeder layer cell actions and applications. Tissue Eng Part B Rev. 2015;21:345–53.
24. Chera S, Ghila L, Wenger Y, Galliot B. Injury-induced activation of the MAPK/CREB pathway triggers apoptosis-induced compensatory proliferation in hydra head regeneration. Dev Growth Differ. 2011;53:186–201.
25. Fujimoto K, Yamamoto T, Kitano T, Abé SI. Promotion of cathepsin L activity in newt spermatogonial apoptosis induced by prolactin. FEBS Lett. 2002;521:43–6.
26. Li F, Huang Q, Chen J, Peng Y, Roop DR, Bedford JS, et al. Apoptotic cells activate the phoenix rising pathway to promote wound healing and tissue regeneration. Sci Signal. 2010;3:ra13.
27. Oka T, Adati N, Shinkai T, Sakuma K, Nishimura T, Kurose K. Bisphenol A induces apoptosis in central neural cells during early development of Xenopus laevis. Biochem Biophys Res Commun. 2003;312:877–82.
28. Pellettieri J, Fitzgerald P, Watanabe S, Mancuso J, Green DR, Sánchez Alvarado A. Cell death and tissue remodeling in planarian regeneration. Dev Biol. 2010;338:76–85.
29. Paul S, Balakrishnan S, Arumugaperumal A, Lathakumari S, Syamala SS, Arumugaswami V, et al. The transcriptome of anterior regeneration in earthworm Eudrilus eugeniae. Mol Biol Rep. 2021;48:259–83.
30. Subramanian ER, Gopi Daisy N, Sudalaimani DK, Ramamoorthy K, Balakrishnan S, Selvan Christyraj JD, et al. Function of translationally controlled tumor protein (TCTP) in Eudrilus eugeniae regeneration. PLoS One. 2017;12:e0175319.
31. Koziol MJ, Gurdon JB. TCTP in development and cancer. Biochem Res Int. 2012;2012:105203.
32. Rho SB, Lee JH, Park MS, Byun HJ, Kang S, Seo SS, et al. Anti-apoptotic protein TCTP controls the stability of the tumor suppressor p53. FEBS Lett. 2011;585:29–35.
33. Telerman A, Amson R. The molecular programme of tumour reversion: the steps beyond malignant transformation. Nat Rev Cancer. 2009;9:206–16.
34. Chen SH, Lu CH, Tsai MJ. TCTP is essential for cell proliferation and survival during CNS development. Cells. 2020;9: 133.
35. Amson R, Pece S, Lespagnol A, Vyas R, Mazzarol G, Tosoni D, et al. Reciprocal repression between P53 and TCTP. Nat Med. 2012;18:91–9.
36. Banik D, Chaudhuri PS. Regeneration ability in seventeen top soil and sub soil earthworm species. J Environ Biol. 2017;38:393.
37. Bae YS, Kim J, Yi J, Park SC, Lee HY, Cho SJ. Characterization of *Perionyx excavatus* development and its head regeneration. Biology (Basel). 2020;9:273.
38. Cho SJ, Lee MS, Tak ES, Lee E, Koh KS, Ahn CH, et al. Gene expression profile in the anterior regeneration of the earthworm using expressed sequence tags. Biosci Biotechnol Biochem. 2009;73:29–34.
39. Martinez VG, Menger GJ 3rd, Zoran MJ. Regeneration and asexual reproduction share common molecular changes: upregulation of a neural glycoepitope during morphallaxis in Lumbriculus. Mech Dev. 2005;122:721–32.
40. Chellathurai Vasantha N, Rajagopalan K, Selvan Christyraj JD, Subbiahanadar Chelladurai K, Ganesan M, Azhagesan A, et al. Heat-inactivated coelomic fluid of the earthworm Perionyx excavatus is a possible alternative source for fetal bovine serum in animal cell culture. Biotechnol Prog. 2019;35:e2817.
41. Gopi Daisy N, Subramanian ER, Selvan Christyraj JD, Sudalai Mani DK, Selvan Christyraj JR, Ramamoorthy K, et al. Studies on regeneration of central nervous system and social ability of the earthworm Eudrilus eugeniae. Invert Neurosci. 2016;16:6.
42. Johnson Retnaraj Samuel SC, Elaiya Raja S, Beryl Vedha Y, Edith Arul Jane A, Amutha K, Dinesh SM, et al. Autofluorescence in BrdU-positive cells and augmentation of regeneration kinetics by riboflavin. Stem Cells Dev. 2012;21:2071–83.
43. Seo EJ, Efferth T. Interaction of antihistaminic drugs with human translationally controlled tumor protein (TCTP) as novel approach for differentiation therapy. Oncotarget. 2016;7:16818.
44. Yokoyama H, Ogino H, Stoick-Cooper CL, et al. Wnt/β-catenin signaling has an essential role in the initiation of limb regeneration. Dev Biol. 2007;306:170–8.
45. Michalopoulos GK. Liver regeneration. Liver Biol Pathobiol. 2020. https://doi.org/10.1002/9781119436812.ch45.
46. Acunzo J, Baylot V, So A, Rocchi P. TCTP as therapeutic target in cancers. Cancer Treat Rev. 2014;40:760–9.
47. Bommer UA, Kawakami T. Role of TCTP in cell biological and disease processes. Cells. 2021;10:2290.
48. Seo EJ, Fischer N, Efferth T. Role of TCTP for cellular differentiation and cancer therapy. Results Probl Cell Differ. 2017;64:263–81.
49. Feroz W, Sheikh AMA. Exploring the multiple roles of guardian of the genome: P53. Egypt J Med Hum Genet. 2020;21:1–23.
50. Kumar R, Maurya R, Saran S. Identification of novel inhibitors of the translationally controlled tumor protein (TCTP): insights from molecular dynamics. Mol Biosyst. 2017;13:510–24.
51. Bommer U-A, Telerman A. Dysregulation of TCTP in biological processes and diseases. Cells. 2020;9:1632.
52. Zuber J, Rappaport AR, Luo W, et al. An integrated approach to dissecting oncogene addiction implicates a myb-coordinated self-renewal program as essential for leukemia maintenance. Genes Dev. 2011;25:1628–40.
53. Kang JH, Lee SH, Lee JS, Oh SJ, Ha JS, Choi HJ, et al. Inhibition of transglutaminase 2 but not of MDM2 has a significant therapeutic effect on renal cell carcinoma. Cells. 2020;9:1475.
54. Ryoo HD, Bergmann A. The role of apoptosis-induced proliferation for regeneration and cancer. Cold Spring Harb Perspect Biol. 2012;4:a008797.
55. Sahu S, Sridhar D, Abnave P, Kosaka N, Dattani A, Thompson JM, et al. Ongoing repair of migration-coupled DNA damage allows planarian adult stem cells to reach wound sites. Elife. 2021;10: e63779.
56. Hsu YC, Chern JJ, Cai Y, Liu M, Choi KW. Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase. Nature. 2007;445:785–8.
57. Telerman A, Amson R. TCTP/tpt1-Remodeling signaling from stem cell to disease. Berlin: Springer; 2017.
58. Bommer UA, Thiele BJ. The translationally controlled tumour protein (TCTP). Int J Biochem Cell Biol. 2004;36:379–85.
59. Lee HJ, Song KH, Oh SJ, Kim S, Cho E, Kim J, et al. Targeting TCTP sensitizes tumor to T cell-mediated therapy by reversing immune-refractory phenotypes. Nat Commun. 2022;13:2127.
60. Omabe K. Translationally controlled tumor protein: a key target to abrogate DNA repair and therapeutic resistance in cancer. J Cancer Res Ther Oncol. 2022;10:1–17.
61. He S, Lu Y, Liu X, Huang X, Keller ET, Qian CN, et al. Wnt3a: functions and implications in cancer. Chin J Cancer. 2015;34:1–9.
62. Chang CY, Liang MZ, Wu CC, Huang PY, Chen HI, Yet SF, et al. WNT3A promotes neuronal regeneration upon traumatic brain injury. Int J Mol Sci. 2020;21:1463.
63. Gu X, Yao L, Ma G, Cui L, Li Y, Liang W, et al. TCTP promotes glioma cell proliferation in vitro and in vivo via enhanced β-catenin/TCF-4 transcription. Neuro Oncol. 2014;16:217–27.
64. Halder G, Johnson RL. Hippo signaling: growth control and beyond. Development. 2011;138:9–22.

65. Varelas X. The Hippo pathway effectors TAZ and YAP in development, homeostasis and disease. Development. 2014;141:1614–26.

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# The molecular mechanisms underlying the regeneration process in the earthworm, *Perionyx excavatus* exhibit indications of apoptosis-induced compensatory proliferation (AICP)

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## Abstract

Regeneration is a multifaceted biological phenomenon that necessitates the intricate orchestration of apoptosis, stem cells, and immune responses, culminating in the regulation of apoptosis-induced compensatory proliferation (AICP). The AICP context of research is observed in many animal models like in *Hydra*, *Xenopus*, newt, *Drosophila*, and mouse but so far not reported in earthworm. The earthworm *Perionyx excavatus* is used in the present study to understand the relationship between AICP-related protein expression and regeneration success in different conditions (normal regeneration and abnormal multiple bud formation). Initially, the worms are amputated into five equal portions and it is revealed that regeneration in *P. excavatus* is clitellum independent and it gives more preference for anterior regeneration (regrowth of head portion) than for posterior regeneration (regrowth of tail portion). The posterior segments of the worm possess enormous regeneration ability but this is lacking in anterior segments. Alkaline phosphate, a stem cell marker, shows strong signals throughout all the posterior segments but it decreases in the initial 1st to 15th anterior segments which lack the regeneration ability. While regenerating normally, it was suggested that the worm follow AICP principles. This is because there was increased expression of apoptosis signals throughout the regeneration process along with constant expression of stem cell proliferation response together with cellular proliferation. In amputated posterior segments maintained in vitro, the apoptosis signals were extensively detected on the 1st day. However, on the 4th and 6th days, caspase-3 and H2AX expression are significantly suppressed, which may eventually alter the Wnt3a and histone H3 patterns that impair the AICP and result in multiple bud formation. Our results suggest that AICP-related protein expression pattern is crucial for initiating proper regeneration.

**Keywords** Regeneration · *P. excavatus* · Apoptosis-induced compensatory proliferation · Wnt3a · H2AX

## Introduction

Regeneration is an intriguing biological phenomenon characterized by the restoration of lost body parts to their original form and function. This remarkable ability is observed across a diverse range of animal taxa. However, the regenerative capacity varies across different animal phyla, primarily influenced by the hereditary tissue regenerative responses. In certain organisms, such as planarians and *Hydra vulgaris*, remarkable regenerative capabilities have been observed. These simple animals possess the remarkable ability to regenerate their entire organism from even the smallest amputated fragment (Cebrià *et al.* 2018; Vogg *et al.* 2019). Certain phylogenetically primitive invertebrates and vertebrates exhibit an enhanced capacity for regeneration,

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wherein they are capable of regenerating specific lost body parts such as the head, tail, or appendages. This phenomenon has been observed in organisms such as the earthworm (Rajagopalan *et al.* 2022) and salamander (Gupta 2016). The regenerative capacity in phylogenetically advanced animals, such as mammals, is subject to significant limitations and strict regulations (Zhao *et al.* 2016). The observed variations in regenerative capacity among diverse animal species throughout the course of evolution can be attributed to several key factors. These factors encompass the inherent regenerative potential inherited by different organisms, the capacity for renewal and differentiation of stem cells, the ability to initiate regenerative signals and activate relevant genes, the potential for trans-differentiation and dedifferentiation processes, the influence of signals originating from immune cells, and the involvement of epigenetic regulators (Zhao *et al.* 2016).

Regeneration encompasses a multitude of pathways and a vast array of genes, including the Wnt signalling pathway (Lee *et al.* 2010; Gu *et al.* 2014), TGFB SMAD signalling pathway (Elston and Inman 2012; Mishra *et al.* 2018), JAK/STAT signalling pathway (Goyal *et al.* 2020), β-catenin/TCF-4 transcription (Yang *et al.* 2010; Gu *et al.* 2014), Hippo signalling pathway (Choi and Hsu 2007; Furth *et al.* 2018), Hh signalling pathway (Ho and Alman 2010), Notch (Dotto 2009), FGF (Rodriguez-Enfedaque *et al.* 2009), BMP signalling pathway (Koide *et al.* 2009; Voorneveld et al. 2015). etc. In the context of earthworm regeneration, specifically in *Eudrilus eugeniae*, the differential expression of over 10,000 genes has been observed (Paul *et al.* 2021). The interplay between regeneration-associated pathways and the expression of regenerative genes and proteins has been intricately linked to fundamental biological processes such as apoptosis, stem cell dynamics, and immune responses (Bergmann and Steller 2010). In the event that any one of these components is absent, the process of regeneration cannot occur successfully, as stem cells serve as the fundamental origin for tissue restitution (Bergmann and Steller 2010). However, the primary mechanism behind the regulation of stem cells is known as "apoptosis," a process by which cells undergo programmed cell death. In the specific context mentioned, this phenomenon is referred to as "apoptosis-induced compensatory proliferation" (Fogarty and Bergmann 2017).

Although the various species of earthworms exhibit nearly identical morphological structures, such as pre-clitellar, clitellar, intestinal, and anus segments, the placement of the clitellum varies across different species. The remarkable regenerative phenomena observed in various earthworm species are truly awe-inspiring, as they exhibit distinct regenerative capabilities even within closely related species. In clitellum-dependent earthworms, it has been observed that only the amputated segments containing an intact clitellum have the ability to regenerate. Conversely, those segments that lack clitellum segments have been found to be incapable of regeneration. This phenomenon has been documented in the species *Eudrilus eugeniae*, as reported by Rajagopalan *et al.* (2022). On the contrary, in clitellum-independent worms, the process of regeneration takes place even in segments that have been amputated and do not possess the clitellum. This results in the formation of multiple individual worms from a single amputated worm, as observed in the case of *Perionyx excavatus* (Cho *et al.* 2009). In our recent investigations, it was observed that the viability and regenerative capacity of *Eudrilus eugeniae* body segments were augmented when subjected to in vitro conditions (Rajagopalan *et al.* 2022). In order to elucidate the intricate mechanisms underlying regeneration, it is imperative to thoroughly investigate the regenerative potential in conjunction with the phenomenon known as AICP (apoptosis-induced compensatory proliferation).

The current state of earthworm models exhibits a deficiency in comprehensive studies pertaining to the analysis of the animal's internal cellular processes. Research has been conducted on the phenomenon of regeneration in earthworms, specifically exploring the role of either apoptosis or stem cells, but not both simultaneously. In a study conducted by Bodó *et al.* (2021), it was observed that during the process of regeneration, the occurrence of apoptosis was identified in earthworms (Bodó *et al.* 2021). Based on the research conducted by Christyraj *et al.*, it has been observed that the stem cells typically reside within the clitellum of the earthworm species. *E. eugeniae*, a species known for its migratory behavior, exhibits the remarkable ability to navigate towards wound sites and initiates the intricate process of regeneration, as observed in the study conducted by Selvan Christyraj *et al.* (2020). The comprehension of stem cell activation and apoptosis in the context of earthworm regeneration is crucial for the elucidation of the molecular mechanisms underlying this regenerative process. In this study, we present our findings regarding the expression patterns of important regenerative proteins, namely Wnt3a and H2AX in both in vivo and in vitro conditions. Wnt3a is a protein known for its ability to activate stem cells, while H2AX serves as an indicator of apoptosis and DNA damage response. The phosphorylated histone H3 (PHH3) can be used as a direct marker for cell proliferation in most of the studies (Ladstein *et al.* 2010; Habberstad *et al.* 2011; Villani *et al.* 2016; Elmaci *et al.* 2018), meanwhile nuclear histone H3 (H3, H3.1, H3.2) is used as a marker for measuring the total histones in many studies (Liu *et al.* 2022; Waidmann *et al.* 2022) but interestingly in our study we have observed the fold change difference in histone h3 protein during both in vivo and in vitro regeneration. Also, we observed the key regenerative proteins were expressed in a synchronized manner under in vivo conditions. However, in the in vitro setting,

we observed abnormal patterning, which we attribute to the asynchronous expression of Wnt3a, H2AX, and H3.

## Methodology

**The process of raising *P. excavatus*** *Perionyx excavatus*, a species of earthworm, was acquired and maintained in culture stocks within the confines of the Molecular Biology and Stem Cell Research Laboratory. This laboratory is situated within the Centre for Molecular and Nanomedicine Research Unit, which is part of the esteemed International Research Centre at Sathyabama Institute of Science and Technology in Chennai, India. The earthworms were maintained in accordance with previous studies (Gopi Daisy *et al.* 2016; Chellathurai Vasantha *et al.* 2019; Subbiahanadar Chelladurai *et al.* 2020) as previously documented. Worms were provided with a diet consisting of a mixture of cow dung and leaf litter, with a ratio of 70:30 respectively. The containers are upheld with an optimal level of moisture, while being exposed to the surrounding temperature of approximately 20–25 °C, within a well-ventilated environment. The worms exhibit a diurnal rhythm with a photoperiod of 12 h of light and 12 of darkness. This feeding regimen was repeated on a weekly basis, with the compost being replenished with fresh feed each time.

**In vivo regeneration** The process of in vivo regeneration refers to the natural ability of an organism to restore and repair damaged or lost tissues and organs within its own. For the purpose of conducting amputation studies, a total of six worms were initially placed within a plastic container, where they were given a period of 2 d to acclimatize and adjust to their new environment. After a period of 48 h, the annelids were subjected to a thorough rinsing using tap water, followed by submersion in cold water for a duration of 30 s, thereby inducing temporary immobilization. Following that, the annelids were subjected to amputation at various locations utilizing aseptic surgical instrumentation, specifically a sterile surgical blade manufactured by Surgeon, KEHR Surgical Private Limited, located in Kanpur, Uttar Pradesh, India. The amputation procedure was conducted to obtain distinct fragments comprising segments ranging from the 1st to the 17th, the 18th to the 36th, the 37th to the 50th, the 51st to the 75th, and the 76th to the 98th segments. The amputated segments were subjected to observation in order to assess the process of anterior (regrowth of head portion) and posterior (regrowth of tail portion) regeneration on day 5, day 9, and day 12 following amputation. In the context of protein sample preparation, an additional six worms were subjected to amputation at the intersegmental regions spanning from the 23rd to the 37th segments. Subsequently, these worms were provided with suitable conditions to initiate the process of regeneration. The protein samples were obtained from the regenerating termini of the amputated worms at day 1, day 4, and day 6 post-amputation. The regenerative process of various worm species was meticulously documented using a Canon digital camera (model IXUS 285 HS) in Chennai, India.

**BCIP/NBT staining** The BCIP/NBT solution serves as a substrate for the enzyme alkaline phosphatase, which exhibits a higher concentration in stem cells (Selvan Christyraj *et al.* 2020). The BCIP/NBT stain solution, which was acquired from Sigma Aldrich (catalogue number B1911-100 ml), India, was procured for use in the experiment. In the beginning, the worms underwent a thorough rinsing process with distilled water, followed by fixation using ice-cold methanol for a duration of 15 min. Following fixation, the nematodes underwent a thorough rinsing process using 1 × TBST (1 × Tris buffered saline with Tween-20) buffer. This buffer was applied three times, with each wash lasting for a duration of 5 min. The annelids were submerged in a solution of BCIP/NBT for a duration of 15 min, while being kept in a light-restricted environment at ambient temperature. Following this, the tissue samples underwent a subsequent washing step utilizing 1 × TBST buffer. Subsequently, the enzyme substrate reaction was brought to a halt by subjecting the samples to an incubation period in the stopping solution, consisting of a mixture of acetic acid (4), glycerol (1), and ethanol (2), for a duration of 15 min. The earthworm samples that had undergone processing were examined using a dissection light microscope (Optica Microscope Italy – Model No: SN621997), and meticulously recorded.

**In vitro regeneration** In order to facilitate in vitro regeneration, a cohort of 12 mature *P. excavatus* worms were selected and subjected to surgical amputation between the 33rd and 37th segments, considering a total of five segments in their body structure. The amputated segments were subjected to a triple wash with ice-cold distilled water and subsequently placed into filtered ice-cold 1X PBS (phosphate-buffered saline). The excised segments were subsequently relocated in a solution containing antibiotics, followed by immersion in a medium consisting of serum-free L15 medium, and ultimately transferred to a medium consisting of L15 medium with 10% FBS, as previously outlined in our investigation (Rajagopalan *et al.* 2022). After undergoing in vitro maintenance, all amputated segments were subjected to regular monitoring at 24-h intervals and were observed and recorded using an EVOS light microscope (ThermoFisher, Waltham, MA). The in vitro regeneration experiments were replicated in triplicate to obtain concurrent data.

**Western blotting** Protein samples were obtained from regenerative tissues of *P. excavatus* body segments, collected

from both in vivo (within the living organism) and in vitro (outside the living organism) sources. In a concise manner, the biological samples were subjected to homogenization using a 2X-sample buffer containing β-mercaptoethanol. Subsequently, the resulting homogenate was subjected to a thermal treatment at a temperature of 100 °C for a duration of 5 min. The protein samples were measured using the standard control, bovine serum albumin (BSA), in accordance with the Lowry's technique of protein quantification. Following that, 80 µg of each protein sample was subjected to resolution through a 12% SDS-PAGE utilizing the Mini-PROTEAN electrophoresis system manufactured by Bio-Rad Laboratories, located in Hercules, California. Proteins that had undergone resolution were subsequently transferred from the SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane using the Mini Trans-Blot Module, a product developed by Bio-Rad Laboratories. The protein transferred PVDF membrane underwent incubation with primary antibodies, specifically Wnt3a (rabbit polyclonal, Abcam-ab19925, Cambridge, UK), caspase-3 (mouse monoclonal, Abcam-ab208161), PCNA (rabbit polyclonal, Abcam- ab18197), H3 (Rabbit polyclonal, Abcam-ab1791), H2AX (Rabbit polyclonal, Abcam-ab20669), and β-actin (mouse monoclonal, Abcam-ab8227), at a dilution of 1:5000. This incubation took place at a temperature of 4 °C for the duration of the overnight period. After the incubation period, the PVDF membrane underwent a series of washes using ice-cold 1 × PBST buffer. Each wash was performed three times, with a duration of 5 min per wash. Subsequently, the cellular barrier was subjected to an incubation process wherein it was exposed to secondary antibodies conjugated with horseradish peroxidase (HRP). These secondary antibodies included anti-rabbit IgG, HRP (derived from goats, specifically the Sigma-A0545 strain), as well as anti-mouse IgG, HRP (derived from goats, specifically the Abcam-ab6789 strain). The dilution used for this incubation was 1:10,000, and the process was carried out at ambient temperature for a duration of 60 min. Following this, the PVDF membrane underwent a thorough washing process using ice-cold 1 × PBST buffer on three separate occasions. To visualize the protein bands of interest, the ECL substrate (BIO-RAD—Catalogue Number: 1705061, Clarity Western ECL Substrate, Hercules, CA) was employed. For attaining the statistical significance, the experiments were conducted three times, and their results are presented as mean ± SD. Statistical data was considered as significant when the $p$-value is $< 0.05$.

**Statistical analysis of western blot data using GraphPad Prism** The western blot data was subjected to statistical analysis using GraphPad Prism, a widely used software in biological research for data analysis and visualization. The protein expression was quantified utilizing the ChemiDoc XRS + imaging system (Bio-Rad—Catalogue Number: 1708265). The results were subjected to analysis using ImageJ, a software specifically designed for biological image analysis, developed by Bio-Rad in the USA. The normalization of regenerative key protein expressions in both the control and test groups was performed using β-actin expression. The statistical analysis was performed, utilizing one-way ANOVA, and the resulting values were graphically represented using GraphPad Prism 8.0.1.

## Results

The regenerative capacity and the kinetics of anterior and posterior regeneration in *P. excavatus* are of particular interest to biologists. We have used wild-type *Perionyx excavatus*, a species of earthworm that dwells on the surface of the soil and exhibits remarkable regenerative capabilities, making it a valuable organism for scientific investigations related to the process of regeneration (Fig. 1). The clitellum, a prominent anatomical feature of the worm, is located within the 13th to 17th segments. Prior to the clitellum, the segments preceding it encompass the head region, housing essential organs such as the brain, tubular heart, seminal vesicle, testes, and ovary. The segments following the clitellum consist of the intestinal segment, followed by the tail segment, and ultimately culminate in the anus segment, as depicted in Fig. 1. To investigate the regenerative capacity between segments, a series of amputations were performed on worms, dividing them into five distinct segments: segments 1 to 17, segments 18 to 36, segments 37 to 50, segments 51 to 75, and segments 76 to 98 (as depicted in Fig. 2*A*). After 5 d post-amputation, the regenerative abilities of all the amputated segments were examined. The results revealed that the anterior segments (head to clitellum region), specifically segments 1 to 17, were only capable of wound healing but failed to initiate regeneration. Consequently, these segments succumbed to death on the 6th day. Conversely, all other amputated segments displayed regenerative activity at both ends (as illustrated in Fig. 2*B*). The amputated segments that managed to survive and regenerate were subsequently monitored until the 12th day following amputation. The collected data unequivocally indicated that the rate of regeneration in the anterior region (regrowth of head region) was greater in comparison to the posterior region (regrowth of tail region) (Fig. 2*C*), as observed on the 5th day following amputation. The data categorically substantiated that the earthworm species *Perionyx excavatus* exhibits a pronounced inclination towards head regeneration, as evidenced by the conspicuous development of the prostomium and mouth segments (as depicted in Fig. 2*D*), in comparison to the regenerative processes


**Figure 1.** Morphology of the earthworm, *Perionyx excavatus*: The earthworm, *P. excavatus* has more than 100 repetitive body segments. The anterior 1 to 12 segments comprise the head which possess all vital organs like mouth, brain, tubular heart, seminal vesicles, testis, and ovary. Segments 13 to 17 form the clitellum which is important for reproduction and cocoon formation. The 18th to the terminal anus segments have intestine and tail. *m*, mouth (*sky blue arrow*); *h*, head region (*blue*); *c*, clitellum region (*green*); t, tail (*munsell yellow*).

observed in the intestines and tail. On the 12th day following the amputation event, the worms exhibited an incomplete regeneration of the 17th segment. Instead, they were only able to regenerate the first 14 segments (as depicted in Fig. 2*D*). This observation highlights the significance of head regeneration, as it encompasses numerous essential organs.

**The posterior segments of the earthworm *P. excavatus* exhibit discernible patterns of ALP signals** The findings from the amputation studies indicate that the anterior 1st to 17th segments (head to clitellum region), once amputated, exhibited a lack of survival and regenerative capacity. In contrast, the equivalent length of posterior segments (after clitellum region) that underwent amputation demonstrated remarkable regenerative ability. In order to elucidate the molecular basis underlying this phenomenon, an assay utilizing alkaline phosphatase (ALP) was conducted to monitor the signalling patterns exhibited by stem cells (Fig. 3*A–C*). Remarkably, we detected pronounced levels of alkaline phosphatase (ALP) signals in the posterior segments of the worms (Fig. 3*B, C*). Conversely, the 1st to 15th segments (Fig. 3*A*) exhibited a notable absence of intense ALP signals, with only faint signals observed primarily on the ventral surface, particularly in the nerve cords. Upon conducting a comparative analysis of the alkaline phosphatase (ALP) signals in different regions, namely the anterior (Fig. 3*A*), posterior mid-segments (Fig. 3*B*), and posterior tail segments (Fig. 3*C*), it was observed that the expression of ALP was notably higher in the posterior mid-segments, followed by the posterior tail segments, and lastly in the anterior segments.

**The capacity for survival and regeneration differs between posterior segments that have been amputated in vivo and those that have been amputated in vitro** In order to gain insight into the regenerative capacity of the posterior segments, the worms underwent amputation at the intersegmental region spanning from the 22nd to the 38th segments (as depicted in Fig. 4*A*). Subsequently, the specimens were subjected to analysis in order to assess their regenerative potential. The regenerating 17 segments exhibited signs of wound healing within 24 h after amputation, as observed on both sides of the amputation site (Fig. 4*B*). The severed segments underwent regeneration, resulting in the formation of pre-blastema on the 4th day after amputation. However, it is worth noting that the size of the pre-blastema formed varied, with a higher growth rate observed in the anterior regeneration (regrowth of head portion) compared to the posterior regeneration (regrowth of tail portion) (Fig. 4*C*). On the 6th day following the amputation event, the blastema, a specialized group of cells capable of regeneration, initiated the process of cellular differentiation. This led to the formation of distinct segments that became visible to the naked eye. Additionally, during this stage, the development of blood vessels within the regenerating tissue was observed (Fig. 4*D*). Fascinatingly, the worms that underwent amputation of fewer than 16 segments exhibited an inability to survive and regenerate when maintained in an in vivo setting. This can be attributed to the loss of segmental integrity, which compelled them to expel their intestinal tract. In contrast, the segments that were amputated (specifically, segments 33rd to 37th) consisted of only five segments. These segments were kept in an in vitro environment with L15 medium and were able to survive. Interestingly, these segments exhibited the formation of abnormal buds that protruded from the site of non-amputation, as depicted in Fig. 4*E–H*. After the amputation of these five segments, the worms were promptly transferred to L15 medium (as shown in Fig. 4*E*) and were subsequently maintained under

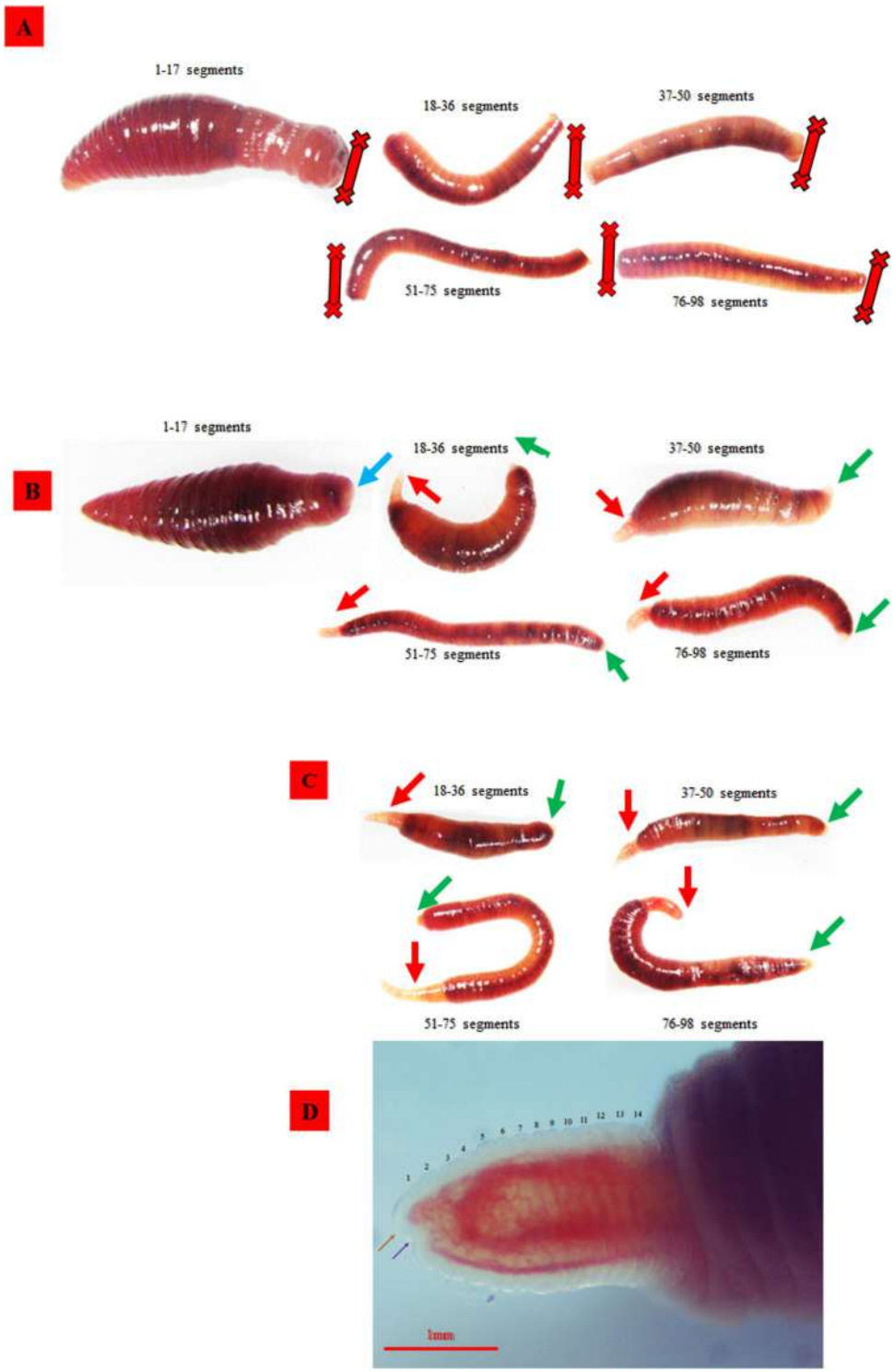


**Figure 2.** Regeneration potential of *P. excavatus* worm: (*A*) Both anterior and posterior segments of earthworms were amputated at equally as 1st to 16th segments, 17th to 36th segments, 37th to 50th segments, 51st to 75th segments, and 76th to 98th segments and represented as on the 0th hour. (*B*) Wound healing was observed in the anterior region (1–16 segments) of *P. excavatus* worm but in all amputated posterior segments the formation of blastema was observed in both ends of the 17th to 36th segments, 37th to 50th segments, 51st to 75th segments, and 76th to 98th segments on the 5th day of post-amputation. (*C*) Anterior region (1st to 16th segments) was dead on the 6th day but in all amputated posterior segments the blastema was elongated and forms the differentiated segments which are visible along with blood vessel formation as on the 9th day of post-amputation. In all the amputated posterior segments, the anterior regeneration kinetics is higher than the posterior regeneration. (*D*) Following 12 days of post-amputation of posterior segments, it forms well-developed prostomium and mouth while regenerating the anterior segments. Careful observation of regeneration represents that it forms only the first 14 segments instead of restoring the complete lost structure of 16 segments. *Sky blue arrow* (wound healing). *Red arrow*, anterior directional regeneration of posterior body segments. *Green arrow*, posterior directional regeneration of posterior body segments. *Violet arrow*, mouth of anterior directional regeneration of blastema of posterior body segments. *Brown arrow* depicts the prostomium region.

in vitro conditions. After a period of 24 h subsequent to maintenance, the worm exhibited the emergence of atypical patterning (as depicted in Fig. 4*F*), which became even more apparent after 36 h (as shown in Fig. 4*G*). Additionally, there was an observable discharge of coelomic fluid from the site of amputation. After a period of 48 h, the wound sites exhibited complete healing on both sides, accompanied by the presence of atypical bud patterning or projections across the various body segments (Fig. 4*H*). In order to gain further insight into the resilience and regenerative capabilities of worms under in vitro conditions, a separate group of worms had their segments amputated from the 33rd to the 37th segments. These amputated worms were then maintained in a medium consisting of serum-free L15 medium. The results

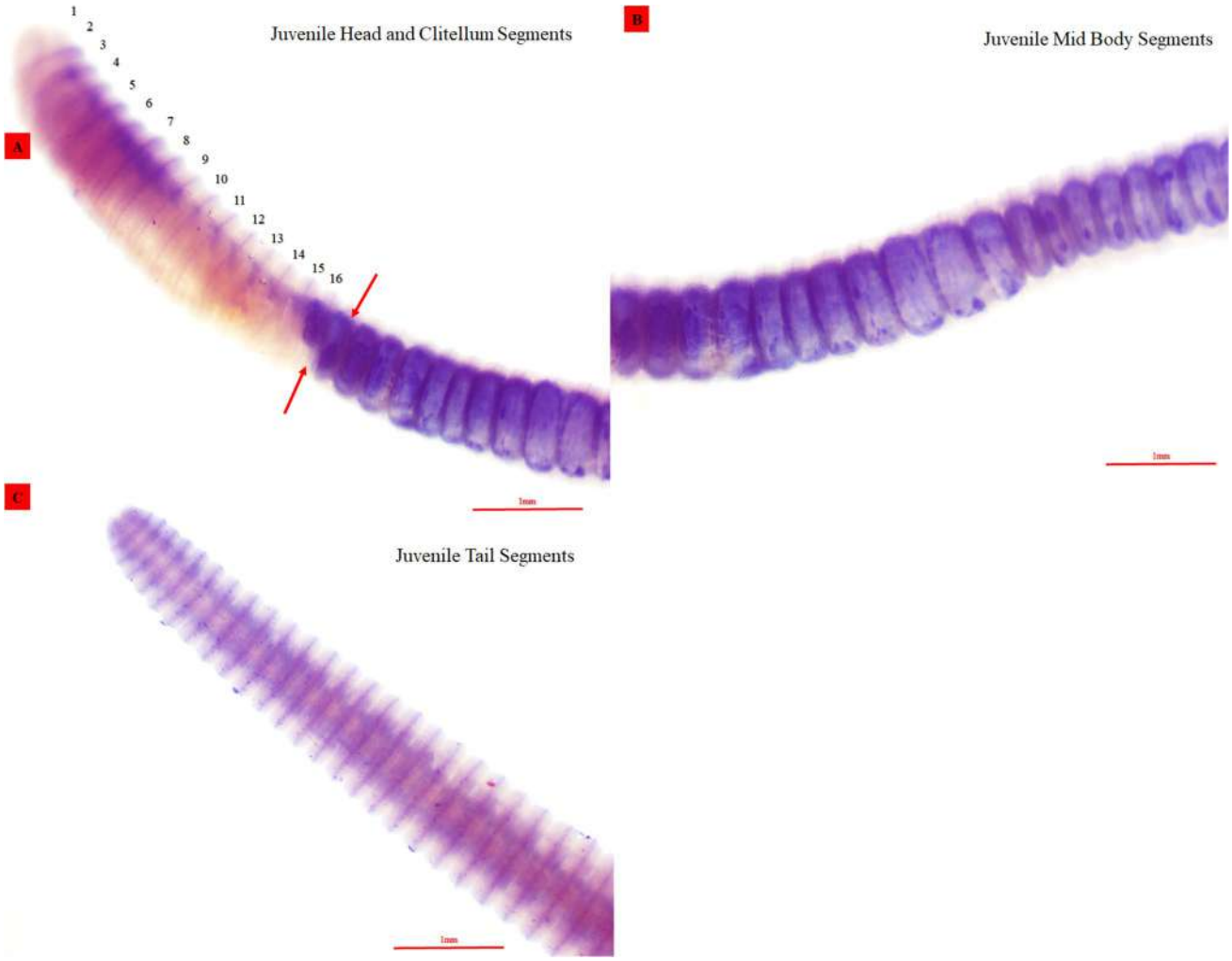


**Figure 3.** Distribution of ALP signals throughout the body of *P. excavatus:* (*A*) The first 15 segments lack the intense ALP signals but the presence of ALP signals was observed in the ventral nerve cord of each individual segment. On the other hand, high intensity of ALP signals was observed in all posterior segments beyond the 15th segments. (*B*, *C*) The mid-region of worm shows intense ALP signals throughout each segment. The ALP signal intensity is equally distributed in each individual segment's circumference, i.e., in dorsal, ventral, and lateral sides of the worm. *Red arrow*, high intense ALP signals observed on the 16th segment. *Sky blue arrow*, prostomium. *Purple arrow*, mouth. *Yellow arrow*, ventral nerve cord.

of this experiment can be observed in Fig. 4*I–L*. Surprisingly, it was observed that the amputated five segments of the worms were able to survive in the serum-free L15 medium. However, unlike in the 10% FBS-containing L15 medium, these worms failed to exhibit the abnormal pattern of bud formation (as depicted in Fig. 4*I–L*). Furthermore, the amputated annelids exhibited a notable inability to initiate the regenerative process in the vicinity of the amputation site for a duration of 48 h.

**The initiation of a proper regeneration mechanism requires the activation of apoptosis-induced compensatory proliferation** The study focuses on elucidating the molecular mechanism of regeneration in animal models, with AICP serving as the contextual framework (Rajagopalan *et al.* 2022). The worm segments that underwent amputation, specifically segments 22 to 36, displayed successful regeneration at both the amputation sites within a living organism (in vivo) and the amputated segments that were kept in controlled laboratory conditions (in vitro) with only five segments. These regenerated segments exhibited abnormal bud patterning and were subsequently used for Western blotting analysis. The primary proteins implicated in AICP, namely caspase-3 (apoptosis and stem cell regulation), PCNA (proliferation), H3 (histone h3—transcription regulation, DNA repair, DNA replication and novel marker for proliferation), Wnt3a (a marker for stem cells), and H2AX (a marker for apoptosis and chromatin remodelling), were concurrently examined on the 1st, 4th, and 6th days following amputation to assess the AICP pattern (Fig. 5*A–D*). In the context

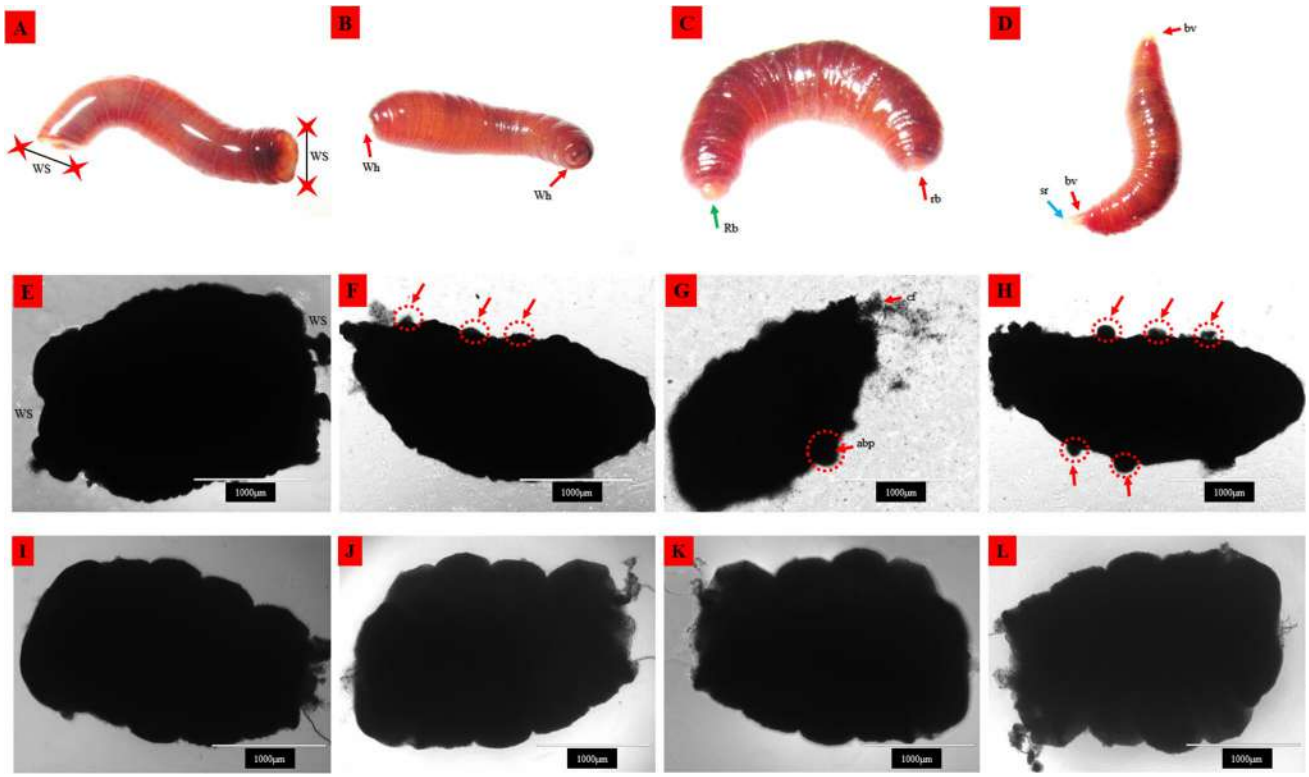


**Figure 4.** Survival and regeneration potential of amputated segments that are maintained in in vivo and in vitro conditions. (*A*) Worms are amputated in between the 22nd and 36th segments (15 segments) and observed on the 0th hour with wound site. (*B*) Wound healing observed after the 1st day of post-amputation (24 h). (*C*) On the 4th day, blastema forms in both the amputated site. (*D*) On the 6th day of post-amputation bud size elongation. (*E*) Amputated 33rd to 37th body segments under in vitro condition (L15 medium with 10% FBS) at the 0th hour of observation. (*F*) Initiation of abnormal projections in the body segments was observed following 24 h. The wound started to heal which is evident with the formation of curvature at the wound site. (*G*, *H*) Wound healing and multi-bud formation was observed after 36 and 48 h respectively. (*I–L*) amputated 33rd to 37th body segments that are maintained in in vitro condition in serum-free L15 medium and observed on the 0th hour, 24 h, 36 h, and 48 h respectively. Even though the worm survives, it is not able to heal the wound and unable to initiate abnormal bud formation. ws, wound site; wh, wound healing; rb, pre-stage regeneration of blastema; Rb, regeneration of blastema; bv, blood vessel formation; sr, segment restoration; abp, abnormal patterning.

of the natural regeneration process observed in live worms, it has been observed that upon amputation, both apoptosis and stem cell proliferation are concurrently initiated on the 1st, 4th, and 6th days after the amputation event. On the 6th day following the amputation, the blastema initiates the process of differentiation, leading to the development of distinct segments and internal structures. During this stage, the blastema exhibits a significant increase in apoptotic signals, indicating programmed cell death, while the expression of Wnt3a decreases compared to the previous day. The expression of H3 is a marker associated with histone level, transcription regulation, DNA repair, and DNA replication and novel marker for cell proliferation during regeneration. Histone H3 was observed to be elevated on the 1st day following amputation. However, during the blastema stage on the 4th day post-amputation, there was a slight decrease in H3 expression. On the 6th day following the amputation, during the differentiation stage, the expression of H3 was observed to be significantly lower compared to the control worm, as depicted in Fig. 5*A*. It is indicating that the histone H3 increases during proliferation and decreases during differentiation stage. Likely, the expression pattern of caspase-3 exhibited similarities to that of the H2AX protein expression, similarly the expression pattern of PCNA showed resemblances to the H3 protein expression in the in vivo and in vitro regeneration processes. When examining the collective expression patterns of caspase-3, PCNA, Wnt3a, H3, and H2AX in in vivo regenerating worms on the 1st, 4th, and 6th days, it can be observed that these proteins adhere to the principles of AICP.

In contrast, within the context of in vitro conditions, the amputated segments ranging from the 33rd to the 37th segments exhibited a significant presence of caspase-3 and H2AX expression on the initial day. However, during the subsequent regeneration phases on the 4th and 6th days,

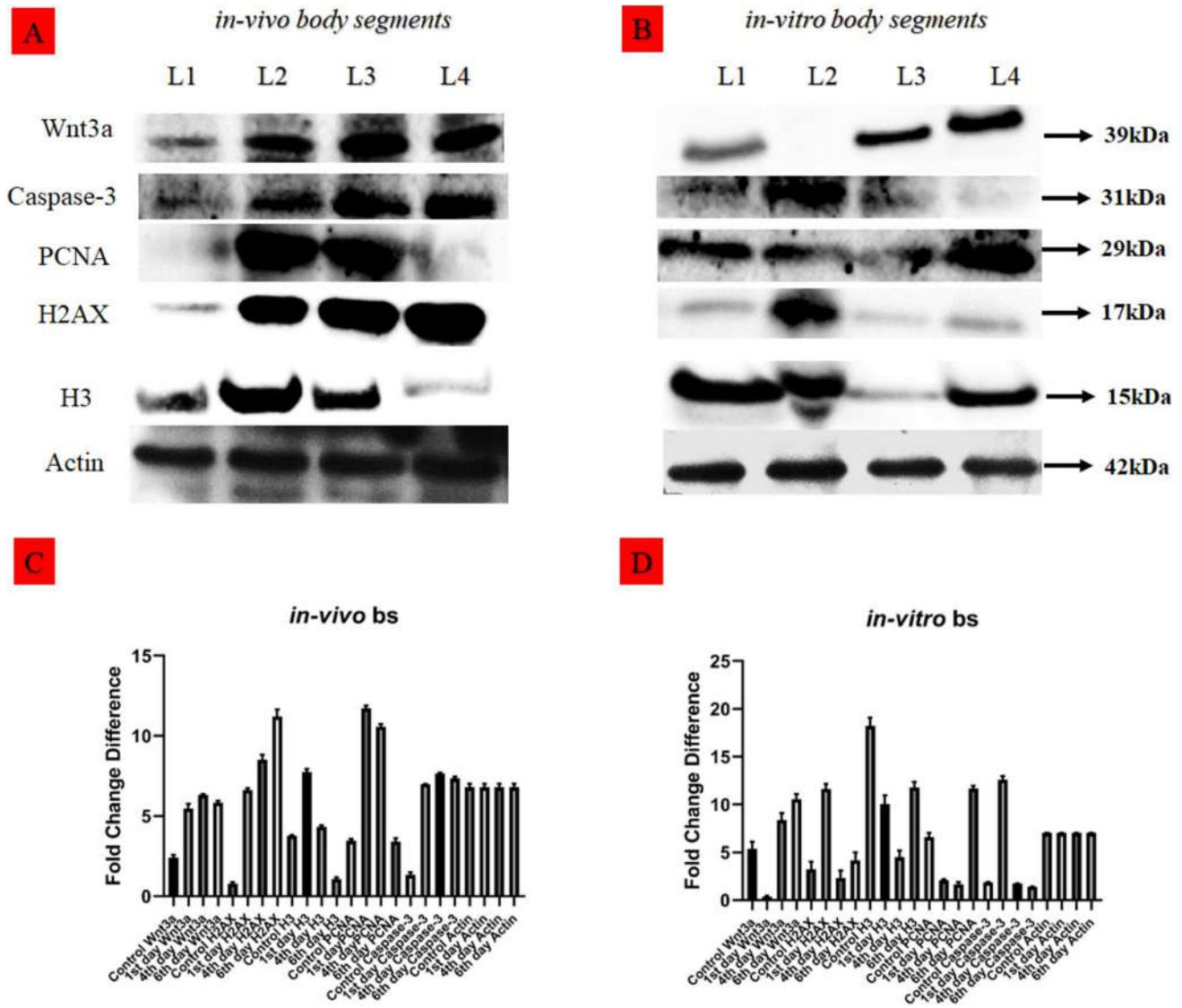


**Figure 5.** Comparing the AICP principles in in vivo (normal) and in vitro (abnormal) regenerating worms: (*A*) H2AX, Wnt3a, H3, and β-actin expression were analyzed using Western blotting. Protein samples were obtained from in vivo control and in vivo regenerating worms. (*A*) L1, protein samples from control; in vivo non-regenerating body segments, L2, after 24 h (1st day) of post-amputation; L3, after 56 h (4th day) of post-amputation; L4, after 144 h (6th day) of post-amputation. Simultaneous increased expression of caspase-3, H2AX, and Wnt3a were observed on 4th and 6th days of post-amputation whereas PCNA and H3 expression are gradually decreasing on succeeding days of regeneration. (*B*) L1, control in vitro body segments; L2, amputated in vitro body segments following 24 h (1st day); L3, after 56 h (4th day) of post-amputation; L4, after 144 h (6th day) of post-amputation. On the 1st day of post-amputation, caspase-3, H2AX, and H3 expression are higher but lacking the Wnt3a signals. On the 4th and 6th days of post-amputation, caspase-3 and H2AX expression are lost but show Wnt3a signals. PCNA and H3 expression are less in the 4th day but increased in the 6th day of post-amputation which collectively shows lack of AICP pattern. For loading control β-actin was used. (*C*, *D*) Quantification of protein expression was calculated based on the band intensity using ImageJ software and that is represented in a bar diagram. The data were acquired and examined utilizing the statistical technique of one-way analysis of variance (ANOVA) by GraphPad Prism 8.0.1 software. To analyze the statistical significance, experiments were carried out three times, and their values are shown as mean ± SD. Statistical significance was determined by $p$-values $< 0.05$. bs, body segments.

there was a noticeable decrease in caspase-3 and H2AX expression. This observation suggests that the apoptotic processes were not effectively transmitting appropriate signals to facilitate proper regeneration in the worms that were maintained in an in vitro environment. In support of our findings, the expression of Wnt3a was observed to be fully inhibited on the first day of in vitro culture of amputated segments. However, over the course of the 4th and 6th days, there was a gradual increase in Wnt3a expression, with no concurrent presence of caspase-3 and H2AX signals.

Furthermore, the observed reciprocal repression between PCNA, H3, and Wnt3a suggests that cellular proliferation is not under the regulation of AICP signals, as indicated in Fig. 5*B*. During the early phases of in vitro preservation, there was an observed upregulation of Wnt3a, PCNA, and H3 in comparison to the naturally regenerating worm in its in vivo state (Fig. 5*A*, *B*). The quantification and graphical representation of the western blotting data can be observed in Fig. 5*C* and *D*, respectively.

## Discussion

The regenerative capacity varies among distinct animal species, and comprehending its underlying mechanism remains a complex and unresolved matter to this day. The presence of stem cells that are localized within the specialized microenvironment of an organism plays a crucial role in dictating the destiny of cells, their ability to adapt and change, and their potential for regenerative processes (Chacón-Martínez *et al.* 2018). *P. excavatus*, a species of earthworm, exhibits an epigeic lifestyle, meaning it resides on the uppermost layer of the soil (Gajalakshmi *et al.* 2001). Consequently, it frequently encounters physical harm due to the presence of predators such as avian species and amphibians, including toads. The amputation studies have revealed that the *P. excavatus* species exhibits a noteworthy capacity for regeneration in their posterior segments. This regenerative ability may evolve as a response to the frequent physical injuries inflicted by predators. The anterior segments (head and clitellum region), despite housing essential organs like the brain, heart, seminal vesicles, testis, and ovary, do not possess the ability to regenerate. This is due to their limited exposure to potential injuries caused by predators, as they are primarily buried in the soil and only the posterior segments are exposed. While it is true that the anterior segments of worms do not possess the capacity for regeneration, it is noteworthy that the posterior amputated segments exhibit a greater inclination towards anterior regeneration as opposed to posterior regeneration. The initial 14 segments exhibit prompt regeneration, as evidenced on the 12th day following amputation. This observation affirms that for amputated earthworm segments to maintain survival and functionality as earthworms, they must regenerate all essential organs including the prostomium, brain, heart, seminal vesicles, testis, ovary, and other accessory glands typically found in the anterior segments (head and clitellum region) of these organisms. The data additionally indicates that *P. excavatus* serves as an exceptional model organism for investigating the process of post-embryonic organogenesis, specifically in relation to the regeneration of amputated adult body segments.

The regenerative capacity primarily relies on the activation of stem cells within their specialized microenvironment, known as the niche, and subsequent functional adaptations in response to external cues (Wagers 2012; Selvan Christyraj *et al.* 2020). The presence of a clitellum in earthworms has been extensively studied, and it has been observed that the stem cell niche is located within the segments of the clitellum. During the process of regeneration, stem cells have been observed to migrate from the neighboring segment to the blastema, as reported by Christyraj *et al.* (2019) and Johnson Retnaraj Samuel *et al.* (2012) (Johnson Retnaraj Samuel *et al.* 2012; Selvan Christyraj *et al.* 2020). However, there is currently no available information regarding the stem cell niche of earthworms that does not require clitellum for their regeneration. The investigations on *P. excavatus* have substantiated its status as a clitellum-independent organism, exhibiting a noteworthy capacity for regeneration in its posterior segments (regrowth of tail portion) while lacking the same regenerative ability in the amputated anterior segments (regrowth of head portion). Based on the regenerative capacity, a notable presence of elevated ALP signals was detected in the posterior segments, commencing from the 16th segment. However, these intense signals were notably absent in the anterior segments (head and clitellum portion), specifically the first 15 segments. The earthworm exhibits a simple brain structure situated within its anterior segments. Additionally, the nerve cord is positioned along the ventral side of the worm, displaying exclusively positive signals for alkaline phosphatase (ALP). In the case of *Enchytraeus japonensis*, it has been documented that the nervous system plays a crucial role in governing both the regeneration of the anterior and posterior regions (Yoshida-Noro and Tochinai 2010). By comparing this with the observations made in *P. excavatus*, it was noted that the ALP signals were exclusively observed in the nerve cord of the anterior segments. However, in the posterior segments, these signals were observed not only in the nerve cord but also throughout the segments. This suggests that *P. excavatus* possesses an additional control mechanism to determine its regeneration ability. Recent investigations involving planarian organisms have revealed intriguing findings regarding the behavior of pluripotent stem cells. These specialized cells have demonstrated the remarkable ability to perceive injuries within the organism and subsequently exhibit a heightened proliferation of specific progenitor cells. This response appears to be contingent upon the specific context of the missing tissue, as elucidated by Bohr *et al.* in their 2021 study. In the current investigations, the presence of numerous ALP positive cells in the posterior segments has been observed, indicating their significant role in determining the regenerative capacity. However, it is noteworthy that the anterior ALP-positive signals, which are exclusively found in the nerve cord, do not possess this regenerative ability.

Despite the presence of abundant alkaline phosphatase (ALP) signals and the remarkable regenerative capacity observed in the posterior segments, the ability to regenerate is compromised when the number of amputated segments is fewer than 16. The data unambiguously substantiates the correlation between the number of posterior segments and the regenerative capacity of amputated worms, as exemplified in the case of *Eisenia fetida* (Xiao *et al.* 2011). Based on our investigation, it was observed that the worm exhibited a failure to sustain its segmental integrity, resulting in the expulsion of its intestinal tract. Furthermore, there was a loss of coelomic fluid circulation between the segments, ultimately leading to the demise of the organism, despite the presence of stem cells within the segment. However, in contrast, the anterior segments from the 1st to the 17th, despite their undisturbed intestinal tract and ability to maintain segmental integrity, and exhibit an inability to survive and regenerate. This can be attributed to the absence of segmental stem cells, which are typically found in the posterior segments. In addition to stem cells, various factors that impact the potential for regeneration include injury type, the process of ageing, the composition of the extracellular matrix, physiological adaptation, immune response, neurogenic capability, and angiogenic capability (Iismaa *et al.* 2018). It is imperative to conduct thorough investigations to further understand the intricacies of regeneration potential in relation to these factors.

In the worm that was maintained in an in vivo setting, it was observed that segments 22 to 38 exhibited the ability to undergo wound healing within 24 h following amputation. Furthermore, these segments initiated the formation of a visible regenerative bud by the 4th day. The short-amputated segments (specifically segments 33 to 37) that are being maintained in an in vitro condition have demonstrated successful survival when exposed to L15 medium supplemented with 10% fetal bovine serum (FBS). The data unambiguously depicts an aseptic habitat wherein the presence of essential nutrients and growth factors augments the organism's capacity for survival, wound repair, and regenerative capabilities, as previously documented (Rajagopalan *et al.* 2022). The intriguing phenomenon observed in this investigation is the occurrence of anomalous multiple bud formation at a distance from the site of amputation. This peculiar event can be attributed to the high abundance of stem cells found in the posterior segments of *P. excavatus* worms, which are known to be influenced by the presence of fetal bovine serum (FBS) in the culture medium. Posterior segments that have undergone amputation, specifically those consisting of fewer than 15 segments, exhibit an inability to sustain life and are unsuccessful in their regenerative efforts under in vivo circumstances. To gain insights into the viability and regenerative capabilities of segments with a count below 15, we exclusively preserve the amputated posterior five segments (specifically, segments 33 to 37) under controlled in vitro circumstances. Under in vitro conditions, the amputated posterior five segments exhibit the remarkable ability to sustain their survival and initiate the formation of multiple buds. In the absence of FBS, the amputated segments exhibit the capacity for survival; however, they demonstrate an inability to undergo wound healing and consequently fail to initiate the formation of multiple buds. FBS contains crucial components such as growth factors, hormones, fatty acids, carrier proteins for lipids, factors that aid in spreading, enzymes, macromolecules, low-molecular weight nutrients, factors that promote attachment, carbohydrates, and trace elements (Brunner *et al.* 2010). Notably, FBS plays a significant role in cellular reprogramming (Kwon *et al.* 2016), which initiates the development of multiple buds.

In a medium devoid of L15 nutrients, the amputated segments (specifically segments 33rd to 37th) exhibit viability but exhibit an inability to undergo wound healing and initiate the formation of multiple buds. Despite the ample presence of stem cells, the worm exhibits a decision to abstain from regenerating in the event of severe injury. However, in the presence of FBS, it enhances the proliferation of stem cells, leading to the initiation of multiple buds. Conversely, in the absence of FBS, the short-amputated segments are able to survive in an in vitro environment. This survival may be attributed to the presence of essential nutrients that are available in L15 medium.

The comprehensive comprehension of the regenerative process shall be achieved through the comparative analysis of the customary and anomalous regenerative phenomena within the framework of apoptosis-induced compensatory proliferation, as expounded by Mollereau *et al.* (2013). Proliferation, a process facilitated by apoptotic cells, is believed to have a pivotal role in compensatory proliferation, as suggested by Fan and Bergmann (2008). In the context of an in vivo environment, it is observed that the levels of apoptosis signal exhibit a consistent increase during the 1st, 4th, and 6th days of regeneration in a typical regenerating worm. This trend is in contrast to the control non-regenerating worm, where such an increase is not observed. In conjunction with the presence of caspase-3 and H2AX, we also noted the consistent expression of Wnt3a during the regenerative stages spanning the 1st, 4th, and 6th days. The data unambiguously demonstrate that apoptotic cells transiently release Wnt3a to initiate the process of successful regeneration. This simultaneous occurrence of apoptosis and Wnt activation has been extensively documented in the head regeneration process of *Hydra*, as reported by Chera *et al.* (2009, 2011). In addition to caspase-3, H2AX, and Wnt3a, the PCNA and H3 protein, which is involved in cell proliferation, also exhibits regulated expression patterns. Specifically, it demonstrates increased expression during the initial stages of blastemal formation on the 1st and 4th days. However, during the subsequent blastemal differentiation process, its expression is downregulated,

indicating a controlled modulation of cell proliferation. The H3 protein is primarily utilized for quantifying total histone levels. However, in the context of regeneration, we observed an upregulation of the PCNA and H3 protein during the proliferation phases, followed by a downregulation during the differentiation stages. Similar alterations in protein expression have been documented in numerous studies. Notably, our research contributes novelty by reporting these expression changes in earthworms, distinct from the previously studied PHH3 protein (Ladstein *et al.* 2010; Habberstad *et al.* 2011; Villani *et al.* 2016; Elmaci *et al.* 2018). In order to achieve successful regeneration, it is crucial to have proper coordination of signals originating from the extracellular, intracellular, and intercellular environments. Any erroneous or misleading information can lead to excessive cell proliferation, which can ultimately result in the development of cancer (Diwanji and Bergmann 2017).

In the context of an in vitro experiment involving a worm with abnormal regeneration, it has been observed that during the early stages of in vitro maintenance, there is a significant increase in the expression of Wnt3a, PCNA, and H3. This heightened expression indicates that the smaller segments of the amputated worm are experiencing considerable stress. To cope with this stress and ensure their survival, these segments rely on the essential nutrients and growth factors present in a L15 medium with 10% FBS. This medium serves as a trigger for regenerative signals, albeit not following a pattern of apoptosis-induced compensatory proliferation (AICP) during the subsequent days of regeneration. The presence of multiple buds that develop at a distance from the amputation sites is indicative of an abnormal regeneration mechanism. However, in order to ensure survival, the organism expresses H2AX, Wnt3a, and H3 in an unsynchronized manner, which deviates from the typical pattern observed in the process known as AICP. The precise regulation and coordinated control of the Wnt signalling cascade are necessary for maintaining a delicate equilibrium between cellular proliferation and differentiation. The lack of signalling support from the organism may play a role in the interference of regulatory mechanisms that govern the proliferation and differentiation of stem cells, as well as programmed cell death. Given that in vitro amputated small segments do not adhere to the principles of apical dominance and instead exhibit the formation of multiple buds, it is plausible to consider their potential application in the field of cancer research.

In order to gain a deeper understanding of the regeneration patterns within the context of AICP (apoptosis-induced compensatory proliferation), it is imperative to conduct comprehensive analyses of studies pertaining to epimorphosis, morphallaxis, and proliferation without dedifferentiation. These investigations will provide valuable insights into the correlation between normal and abnormal regeneration processes.

## Conclusion

The species *P. excavatus* exhibits a remarkable capacity for regeneration in their posterior segments (after clitellum portion), while this regenerative ability is absent in their anterior segments (head and clitellum portion). During the process of regeneration, the worm exhibits a greater inclination towards anterior regeneration (regrowth of head portion) as opposed to posterior regeneration (regrowth of tail portion). This preference can be attributed to the fact that the anterior region encompasses a multitude of vital organs that are essential for the organism to carry out its normal physiological functions. The stem cell microenvironment of *P. excavatus* is primarily localized within the posterior segments (after clitellum portion), while being absent in the anterior segments (head and clitellum portion), thereby accounting for the observed variations in regeneration capabilities. The process of normal regeneration adheres to the principles of apoptosis-induced compensatory proliferation (AICP), which play a functional role in regulating the regenerative process and culminate in the restoration of specific structures. In the context of in vitro maintenance of amputated small posterior segments, it is observed that the typical pattern of AICP is not adhered to. Consequently, this deviation leads to the development of abnormal multiple buds, which originate at a distance from the site of amputation. In summary, our findings indicate that the presence of stem cells alone is insufficient for their effective activation. Instead, it is the presence of specific physiological conditions that ultimately determines the stem cells' ability to regenerate tissue effectively.

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## Declarations

**Conflict of interest** The authors declare no competing interests.

## References


Bergmann A, Steller H (2010) Apoptosis, stem cells, and tissue regeneration. Sci Signal 3:re8–re8

Bodó K, Kellermayer Z, László Z *et al* (2021) Injury-induced innate immune response during segment regeneration of the earthworm. Eisenia Andrei Int J Mol Sci 22:2363

Brunner D, Frank J, Appl H *et al* (2010) The serum-free media interactive online database. ALTEX-Altern Anim Exp 27:53–62

Cebrià F, Adell T, Saló E (2018) Rebuilding a planarian: from early signaling to final shape. Int J Dev Biol 62:537–550

Chacón-Martínez CA, Koester J, Wickström SA (2018) Signaling in the stem cell niche: regulating cell fate, function and plasticity. Development 145:dev165399

Chellathurai Vasantha N, Rajagopalan K, Selvan Christyraj JD *et al* (2019) Heat-inactivated coelomic fluid of the earthworm Perionyx excavatus is a possible alternative source for fetal bovine serum in animal cell culture. Biotechnol Prog 35:e2817

Chera S, Ghila L, Dobretz K *et al* (2009) Apoptotic cells provide an unexpected source of Wnt3 signaling to drive hydra head regeneration. Dev Cell 17:279–289

Chera S, Ghila L, Wenger Y, Galliot B (2011) Injury-induced activation of the MAPK/CREB pathway triggers apoptosis-induced compensatory proliferation in hydra head regeneration. Dev Growth Differ 53:186–201. https://doi.org/10.1111/j.1440-169X.2011.01250.x

Cho S-J, Koh KS, Lee E, Park SC (2009) Differential expression of three labial genes during earthworm head regeneration. Biosci Biotechnol Biochem 73:2609–2614

Choi K-W, Hsu Y-C (2007) To cease or to proliferate: new insights into TCTP function from a Drosophila study. Cell Adh Migr 1:129–130

Diwanji N, Bergmann A (2017) The beneficial role of extracellular reactive oxygen species in apoptosis-induced compensatory proliferation. Fly 11:46–52

Dotto GP (2009) Crosstalk of Notch with p53 and p63 in cancer growth control. Nat Rev Cancer 9:587–595

Elmaci I, Altinoz MA, Sari R, Bolukbasi FH (2018) Phosphorylated histone H3 (PHH3) as a novel cell proliferation marker and prognosticator for meningeal tumors: a short review. Appl Immunohistochem Mol Morphol 26:627–631

Elston R, Inman GJ (2012) Crosstalk between p53 and TGF-β Signalling. J Signal Transduct 2012:

Fan Y, Bergmann A (2008) Apoptosis-induced compensatory proliferation. The cell is dead. Long live the cell! Trends Cell Biol 18:467–473

Fogarty CE, Bergmann A (2017) Killers creating new life: caspases drive apoptosis-induced proliferation in tissue repair and disease. Cell Death Differ 24:1390–1400

Furth N, Aylon Y, Oren M (2018) p53 shades of Hippo. Cell Death Differ 25:81–92

Gajalakshmi S, Ramasamy EV, Abbasi SA (2001) Potential of two epigeic and two anecic earthworm species in vermicomposting of water hyacinth. Bioresour Technol 76:177–181

Gopi Daisy N, Subramanian ER, Selvan Christyraj JD *et al* (2016) Studies on regeneration of central nervous system and social ability of the earthworm Eudrilus eugeniae. Invertebr Neurosci 16:1–13

Goyal H, Chachoua I, Pecquet C *et al* (2020) A p53-JAK-STAT connection involved in myeloproliferative neoplasm pathogenesis and progression to secondary acute myeloid leukemia. Blood Rev 42:100712

Gu X, Yao L, Ma G *et al* (2014) TCTP promotes glioma cell proliferation in vitro and in vivo via enhanced β-catenin/TCF-4 transcription. Neuro Oncol 16:217–227

Gupta S (2016) Animal models: Unlock your inner salamander. Nature 540:S58–S59

Habberstad AH, Gulati S, Torp SH (2011) Evaluation of the proliferation markers Ki-67/MIB-1, mitosin, survivin, pHH3, and DNA topoisomerase IIα in human anaplastic astrocytomas-an immunohistochemical study. Diagn Pathol 6:1–8

Ho L, Alman B (2010) Protecting the hedgerow: p53 and hedgehog pathway interactions. Cell Cycle 9:506–511

Iismaa SE, Kaidonis X, Nicks AM *et al* (2018) Comparative regenerative mechanisms across different mammalian tissues. NPJ Regen Med 3:1–20

Johnson Retnaraj Samuel SC, Elaiya Raja S, Beryl Vedha Y *et al* (2012) Autofluorescence in BrdU-positive cells and augmentation of regeneration kinetics by riboflavin. Stem Cells Dev 21:2071–2083

Koide Y, Kiyota T, Tonganunt M *et al* (2009) Embryonic lethality of fortilin-null mutant mice by BMP-pathway overactivation. Biochimica et Biophysica Acta (BBA) - Gen Subj 1790:326–338

Kwon D, Kim J-S, Cha B-H *et al* (2016) The effect of fetal bovine serum (FBS) on efficacy of cellular reprogramming for induced pluripotent stem cell (iPSC) generation. Cell Transplant 25:1025–1042

Ladstein RG, Bachmann IM, Straume O, Akslen LA (2010) Ki-67 expression is superior to mitotic count and novel proliferation markers PHH3, MCM4 and mitosin as a prognostic factor in thick cutaneous melanoma. BMC Cancer 10:1–15

Lee K-H, Li M, Michalowski AM *et al* (2010) A genomewide study identifies the Wnt signaling pathway as a major target of p53 in murine embryonic stem cells. Proc Natl Acad Sci 107:69–74

Liu Q, Chan AKN, Chang W-H *et al* (2022) 3-Ketodihydrosphingosine reductase maintains ER homeostasis and unfolded protein response in leukemia. Leukemia 36:100–110

Mishra DK, Srivastava P, Sharma A *et al* (2018) Translationally controlled tumor protein (TCTP) is required for TGF-β1 induced epithelial to mesenchymal transition and influences cytoskeletal reorganization. Biochim Biophys Acta (BBA)-Mol Cell Res 1865:67–75

Mollereau B, Perez-Garijo A, Bergmann A *et al* (2013) Compensatory proliferation and apoptosis-induced proliferation: a need for clarification. Cell Death Differ 20:181

Paul S, Balakrishnan S, Arumugaperumal A *et al* (2021) The transcriptome of anterior regeneration in earthworm Eudrilus eugeniae. Mol Biol Rep 48:259–283

Rajagopalan K, Christyraj JDS, Chelladurai KS *et al* (2022) Comparative analysis of the survival and regeneration potential of juvenile and matured earthworm, Eudrilus eugeniae, upon in vivo and in vitro maintenance. Vitr Cell Dev Biol 58:587–598

Rodriguez-Enfedaque A, Bouleau S, Laurent M *et al* (2009) FGF1 nuclear translocation is required for both its neurotrophic activity and its p53-dependent apoptosis protection. Biochim Biophys Acta (BBA)-Mol Cell Res 1793:1719–1727

Selvan Christyraj JD, Azhagesan A, Ganesan M *et al* (2020) Understanding the role of the clitellum in the regeneration events of the earthworm Eudrilus eugeniae. Cells Tissues Organs 208:134–141

Subbiahanadar Chelladurai K, Selvan Christyraj JD, Azhagesan A *et al* (2020) Exploring the effect of UV-C radiation on earthworm and understanding its genomic integrity in the context of H2AX expression. Sci Rep 10:21005

Villani V, Mahadevan KK, Ligorio M *et al* (2016) Phosphorylated histone H3 (PHH3) is a superior proliferation marker for prognosis of pancreatic neuroendocrine tumors. Ann Surg Oncol 23:609–617

Vogg MC, Galliot B, Tsiairis CD (2019) Model systems for regeneration: Hydra. Development 146:dev177212

Voorneveld PW, Kodach LL, Jacobs RJ *et al* (2015) The BMP pathway either enhances or inhibits the Wnt pathway depending on the SMAD4 and p53 status in CRC. Br J Cancer 112:122–130

Wagers AJ (2012) The stem cell niche in regenerative medicine. Cell Stem Cell 10:362–369

Waidmann S, Petutschnig E, Rozhon W *et al* (2022) GSK3-mediated phosphorylation of DEK3 regulates chromatin accessibility and stress tolerance in Arabidopsis. FEBS J 289:473–493

Xiao N, Ge F, Edwards CA (2011) The regeneration capacity of an earthworm, Eisenia fetida, in relation to the site of amputation along the body. Acta Ecol Sin 31:197–204

Yang L-H, Xu H-T, Han Y *et al* (2010) Axin downregulates TCF-4 transcription via β-catenin, but not p53, and inhibits the proliferation and invasion of lung cancer cells. Mol Cancer 9:1–14

Yoshida-Noro C, Tochinai S (2010) Stem cell system in asexual and sexual reproduction of Enchytraeus japonensis (Oligochaeta, Annelida). Dev Growth Differ 52:43–55

Zhao A, Qin H, Fu X (2016) What determines the regenerative capacity in animals? Bioscience 66:735–746