

Understanding the multi-functional role of TCTP in the regeneration process of earthworm, *Perionyx excavatus*

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 2mM 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 2mM 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



Candidate Signature

1 Introduction

The homeostatic process known as ‘regeneration’ directs the recovery of damaged or lost tissues (Carlson 2011). Generally, epimorphosis and morphallaxis are the main principles of regeneration (Morgan 1901, 1910; Agata et al. 2007). There are differences and similarities between the two processes. After wound induction, the primary tissue injury response invites stem cells from the niche (Selvan Christyraj et al. 2020; Sivasubramaniam 2021) and will accumulate at the wound site that depends on signals for healing and regeneration (Owlarn et al. 2017). The cellular mechanism of apoptosis (Pellettieri 2019) and immune responses are essential for the morphallaxis mode of regeneration, and tissue-specific stem cells are not required in this process (Sugiyama and Fujisawa 1978) But epimorphosis requires tissue-specific stem cells, apoptosis, and immune responses (Bergmann and Steller 2010; Abnave and Ghigo 2019). For example, *Hydra vulgaris* follows only morphallaxis (Bosch 2007), limb regeneration of *Xenopus laevis* froglet follows the epimorphosis mode of regeneration (Suzuki et al. 2006), and planarians follow both the regeneration principles (Pellettieri 2019). 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 (Sivasubramaniam 2021).

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 (Bodó et al. 2021). 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 (Liu et al. 2017).

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 ((Huh et al. 2004; Ryoo et al. 2004; Fan and Bergmann 2008; Jung et al. 2010). Apoptotic executioner caspases proceed with Apoptosis Induced Apoptosis (AIA) and AICP to compensate for lost parts (Pérez-Garijo and Steller 2015; Fogarty and Bergmann 2017). Those driving caspases may belong to Feeder cells (Huang et al. 2011; Llamas et al. 2015). 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 (Fujimoto et al. 2002; Oka et al. 2003; Fan and Bergmann 2008; Li et al. 2010; Pellettieri et al. 2010; Chera et al. 2011). In earthworms, apoptotic cells were observed throughout their cell renewal period of regeneration (Bodó et al. 2021). On another side, in juvenile *E. eugeniae* worms, the movement of stem cells from the clitellum to the wound site was observed (Selvan Christyraj et al. 2020). 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 (Paul et al. 2021), silencing a single protein called Translationally Controlled Tumor Protein (TCTP) influences the entire progress of regeneration of *E. eugeniae* regeneration (Subramanian et al. 2017). TCTP has both apoptotic and anti-apoptotic functions (Rho et al. 2011; Koziol and Gurdon 2013) and can decide the fate of cells (Telerman and Amson 2009). TCTP also plays a remarkable role in regeneration (Subramanian et al. 2017) and regulates major cellular mechanisms like proliferation, cell homeostasis, and survival (Chen et al. 2020), maintaining the stability of p53 (Rho et al. 2011). For example, reciprocal repression between TCTP and p53 was observed in knockout mice models (Amson et al. 2012).

Researchers have used chemicals and siRNAs for silencing TCTP, such as Buclizine and Nutlin-3a (Subramanian et al. 2017). Seo and Efferth (2016) 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 (Banik and Chaudhuri 2017). The survival capacity of *P. excavatus* is higher than other earthworm species (Bae et al. 2020). *P. excavatus* is a regeneration animal model for the central nervous system and tissue regeneration (Cho et al. 2009; Bae et al. 2020). Within two weeks of the amputation, *P. excavatus* achieves complete regeneration, including rebuilding vital organs (Bae et al. 2020). Recently, Bae et al. (2020) observed the epimorphosis mode of head regeneration in the earthworm *P. excavatus* (Bae et al. 2020), but the role of morphallaxis in earthworms is vague. Martinez et al. (2005) suggest that the earthworm might follow both epimorphosis and morphallaxis regeneration patterns due to high predatory injury, but clear scientific evidence is unclear.

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 (SC et al. 2012; Gopi Daisy et al. 2016; Niranjana et al. 2018). 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, 2mM 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 15cm 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 140µg, 160µg, and 200µg, 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 & 3. Control and Buclizine treatment for TCTP silencing, 4 & 5. Control and Thymidine treatment for cell arrest analysis, and 6. Buclizine and thymidine treatment for combinatorial toxicology. 7 & 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 hours, 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 5µg/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 blastema 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 hours. 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 5µm sections were made using a microtome (Subramanian et al. 2017). 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 (80µg) of protein samples was loaded onto SDS page gel electrophoresis and applied to run at 60 volts for 2 hours 30 minutes 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, 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, USA; the band intensity was analyzed using ImageJ analysis software (NIH, 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), 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 (60Å X 60Å X 60Å) 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 hours, as shown in Fig. 1C-G. Following 24 hours 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 hours of post-amputation (Fig. 1D). The worm starts to restore their lost tissue by forming a pre-blastemal structure following 72 hours of post-amputation (Fig. 1E) and with increased size in 96 hours (Fig. 1F). In proceeding days, the blastema starts to elongate further and differentiate to form the new lost segments following 120 hours 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 2mM 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-2J). Also, in the control worm, the functional mouth is formed along with a well-developed prostomium, but in 2mM thymidine-treated worms, it initiates the formation of the intestine without forming a functional mouth and prostomium (Fig. 2I-2J).

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 (Seo and Efferth 2016). Buclizine was injected into the worm in different concentrations of 140µg, 160µg and 200µg and observed for the regeneration ability to identify the role of TCTP in *P. excavatus* regeneration, as shown in Fig. 3A-3L. 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 140µg 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 (19kDa) and β-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 β-actin is used as a positive control, and its molecular weight is 42kDa. 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 stereochemical assessment, the local errors of unbounded atomic interactions were assessed by the ERRAT server (Fig. 3P). The ERRAT analysis showed an 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 3.6Å and 3.5Å (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 2mM 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 Figure 4A-4I. Well-differentiated blastema was observed in the control group of worms on the 8th day, as depicted in Figure 4A-4C. The combinational injection of Buclizine and Thymidine completely inhibited the regeneration process for eight days post-amputation (Fig. 4D-4F). 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 2mM Thymidine or Buclizine, their physiological functions shut down, leading to their death within six 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 Figure 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-4I).

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 μ g 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 μ g) injected worm samples were taken for analysis. Comparative histology of the regenerative bud tip between control (Fig. 5B) and 9 μ g 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 (Banik and Chaudhuri, 2017). Regeneration studies show that the earthworm, *P. excavatus*, has an enormous regeneration ability. The worms 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 (Selvan Christyraj et al. 2020). The

pre-blastema was observed within 48 hours, and within another 24 hours, 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* (Bae et al. 2020), 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 (Yokoyama et al. 2007). From histology, it confirms that the regenerative ability of the earthworm is suppressed in 2mM 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 (Michalopoulos 2020).

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 (Acunzo et al. 2014). 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 (Bommer and Kawakami 2021). 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 (Seo et al. 2017). 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 (Seo et al. 2017). TCTP is a multi-functional protein that plays a crucial role in cell proliferation, cell growth, and apoptosis by interacting with many regulatory proteins (Chen et al. 2020). TCTP's part is also well documented in regenerative models involving epimorphosis or cell proliferation (Subramanian et al. 2017; Chen et al. 2020), but its role is not revealed in the aspect of morphallaxis. The combinatorial injection of 2mM 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 2mM 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 (Feroz and Sheikh 2020). 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 (Seo and Efferth 2016; Kumar et al. 2017). 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 (Bommer and Telerman 2020).

Unlike buclizine, the inhibitory effect of Nutlin-3a in targeting TCTP is well documented in many *in-vivo* models (Zuber et al. 2011; Subramanian et al. 2017; Kang et al. 2020). 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 (Ryoo and Bergmann 2012), stem cell migration (Sahu et al. 2021) play a significant role in determining the regeneration potential. TCTP determines cell fate by regulating major cellular functions like apoptosis and proliferation (Hsu et al. 2007; Rho et al. 2011; Telerman and Amson 2017). Notably, the TCTP protein is known for its anti-apoptotic role (Bommer and Thiele 2004; Rho et al. 2011; Lee et al. 2022) and also act as an apoptotic protein in some context of abrogate DNA repair (Omabe 2022). Like TCTP, Wnt3a is also known for its pleiotropic cellular functions regulating cell proliferation, cell renewal, cellular differentiation, apoptosis, and motility (He et al. 2015). Compared to other WNTs, Wnt3a is remarkably important in determining regeneration potential in *in-vitro*, *ex-vivo*, and *in-vivo* conditions (Chang et al. 2020). 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 (Gu et al. 2014), 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 (Halder and Johnson 2011). YAP/TAZ complex also have a role in determining the cell fate by controlling the genes related with cell proliferation and apoptosis (Varelas 2014).

5 Conclusion

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.

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 suffering to the experimental animals.

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IMPACT OF THE RESEARCH IN THE ADVANCEMENT OF KNOWLEDGE OR BENEFIT TO MANKIND.

The research on the multi-functional role of Translationally Controlled Tumor Protein (TCTP) in the regeneration process of the earthworm *Perionyx excavatus* offers several potential impacts and benefits:

1. Advancement in Regenerative Medicine

- **Understanding Regeneration Mechanisms:** By exploring TCTP's role in earthworm regeneration, the study contributes to the broader understanding of cellular and molecular mechanisms underlying regeneration. Earthworms are known for their impressive regenerative abilities, and insights gained from their regeneration processes can inform regenerative medicine in humans.
- **Potential Applications in Human Medicine:** Discoveries about how TCTP influences regeneration in earthworms might lead to new strategies or therapeutic targets for enhancing or inducing regenerative processes in humans, potentially benefiting those with injuries or degenerative diseases.

2. Basic Biological Research

- **Insight into Protein Functions:** TCTP is involved in various cellular processes, including cell growth, survival, and stress responses. This research provides detailed information about how TCTP functions specifically in the context of regeneration, which can enhance our general understanding of protein function in biological systems.
- **Model Organisms for Study:** Using *Perionyx excavatus* as a model organism can reveal conserved biological pathways that might be relevant across different species, including humans. Such comparative studies can deepen our grasp of fundamental biological processes.

3. Environmental and Ecological Benefits

- **Soil Health and Ecosystem Services:** Earthworms play a crucial role in soil health and nutrient cycling. Understanding their regenerative abilities might have indirect benefits for agriculture and environmental management by improving our knowledge of how to maintain or enhance earthworm populations, which are vital for soil ecosystems.

4. Biotechnology and Agriculture

- **Genetic Engineering:** Insights from this research might contribute to biotechnological applications, such as developing genetically engineered organisms with enhanced regenerative capabilities or improved traits for agricultural use.
- **Pest Management:** Improved understanding of earthworm biology and regeneration could potentially inform pest management strategies, ensuring that earthworm populations are not adversely affected by agricultural practices.

5. Educational and Research Tools

- **Developing Research Models:** The findings can serve as a basis for developing new experimental models and tools in biological research. This might help scientists investigate regeneration and related processes in other organisms, including humans.

6. Cancer Research

- Cell fate determinants like TCTP/p53 may also play a vital role in Apoptosis Induced Compensatory Proliferaiton (AICP). Meanwhile, only few reports have reported that TCTP and p53 can regulate the caspase-3. In many animal models, TCTP/p53 reports have been revealed, but the aspect of AICP is not discussed. Meanwhile, TCTP/p53 are in a reciprocal repression relationship and, most importantly, TCTP can also regulate p53. Interestingly, TCTP can also be used as a regulator in the p35-undead cell model of AICP as it can regulate p35. Therefore, analysing the role of TCTP/p53 in AICP studies will help us to comprehend the AICP studies as well as in the development of therapeutic approaches in cancer and regenerative medicines.

Overall, this research not only advances scientific knowledge about TCTP and regeneration but also holds potential practical benefits across medicine, biotechnology, and environmental management.

A handwritten signature in black ink, appearing to be 'P. H.' followed by a stylized flourish.

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Figure legend

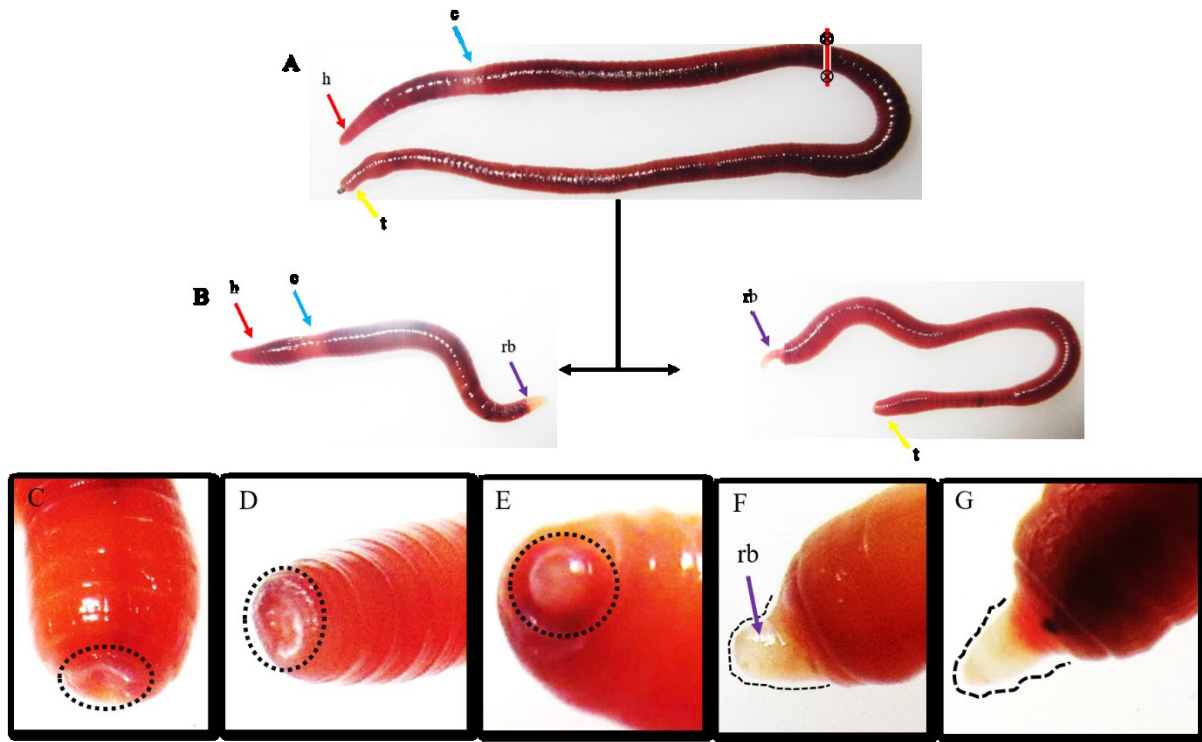


Fig. 1 Regeneration dynamics in clitellum independent earthworm, *P. excavatus*: A. The earthworm, *P. excavatus*, appears red-brown and possesses an anterior head followed by clitellum, intestine, and anus. B. amputation of earthworm at post-clitellum segments (50th segments), resulting in the development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

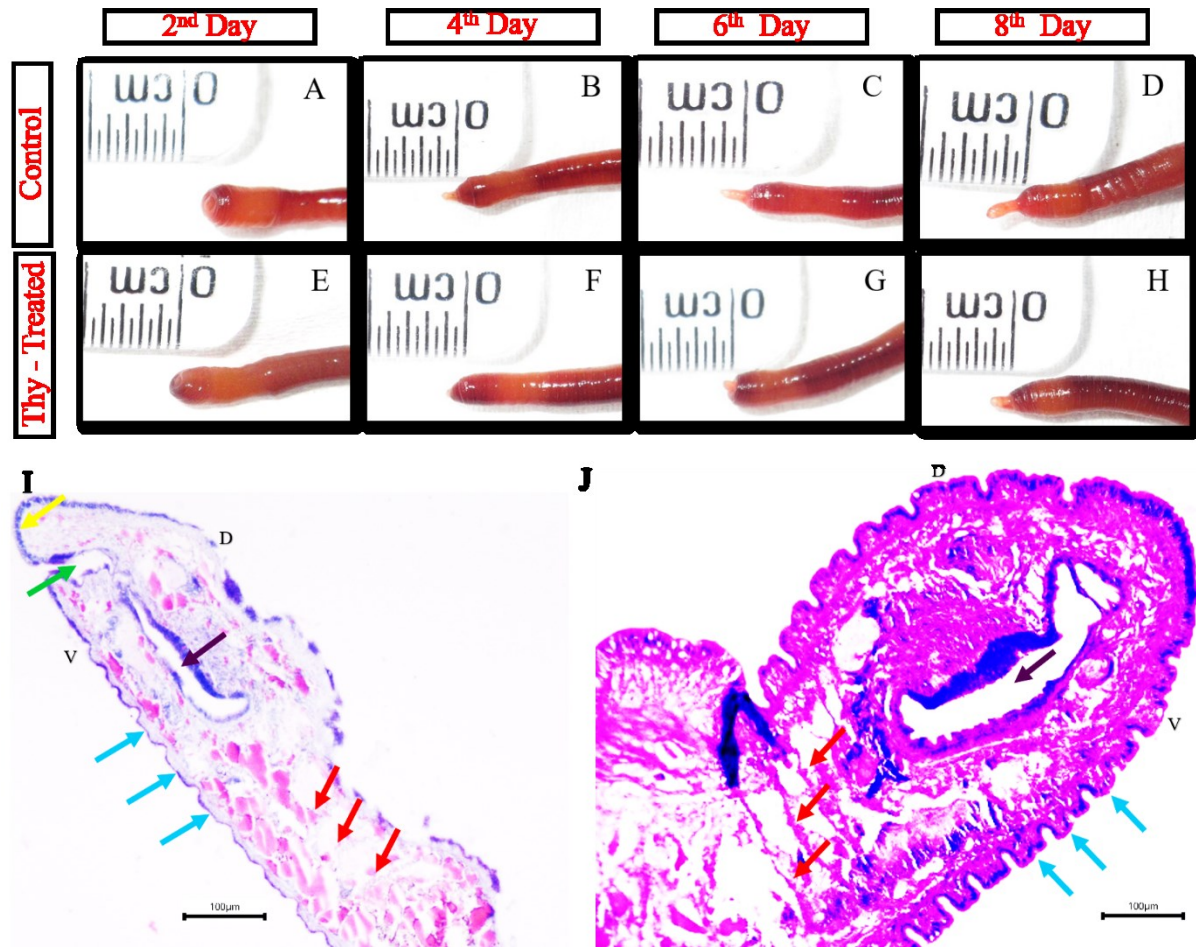
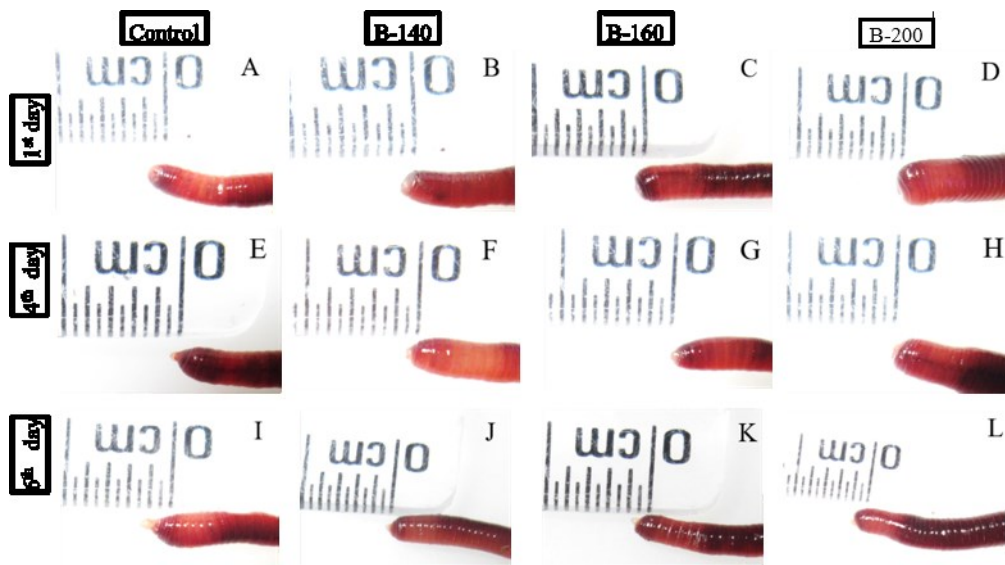
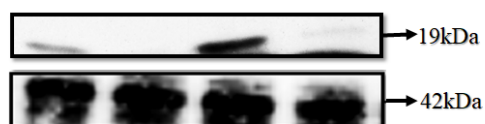


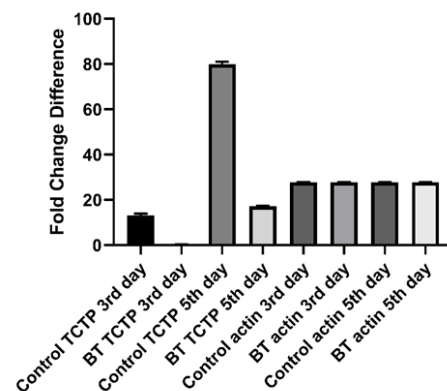
Fig. 2 Regeneration following 2mM Thymidine (cell cycle inhibitor) injection: A. Control worm heals wound on 2nd day of post-amputation. B. Blastema size of 0.2 mm was observed on the 4th day after post-amputation. C. regenerative bud elongated to 0.3 mm on the 6th day. D. blastemal size almost doubled (0.5mm) 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 2mM 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 intestinal tract; 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.



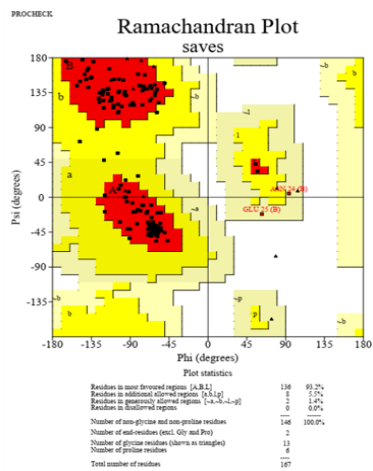
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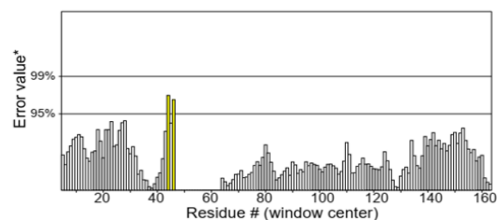


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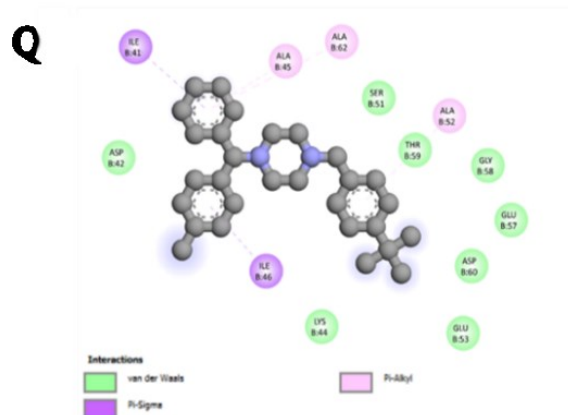


Fig. 3 Pharmacological suppression of TCTP using buclizine and its effects on regeneration: A-D represents control worm (1X PBS), 140 μ g buclizine, 160 μ g buclizine, and 200 μ g buclizine injected worms respectively following day-1 of post-amputation. E-H represents the 4th day post amputated worms correspondingly from the control worm (1X PBS), 140 μ g buclizine, 160 μ g buclizine, and 200 μ g 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 μ g buclizine, 160 μ g buclizine, and 200 μ g buclizine-injected worms, respectively. In all the buclizine-injected worms, regeneration is 1/5th 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.

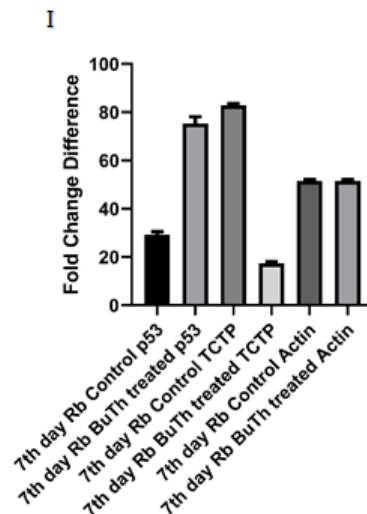
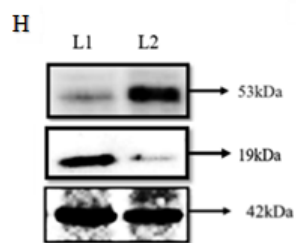
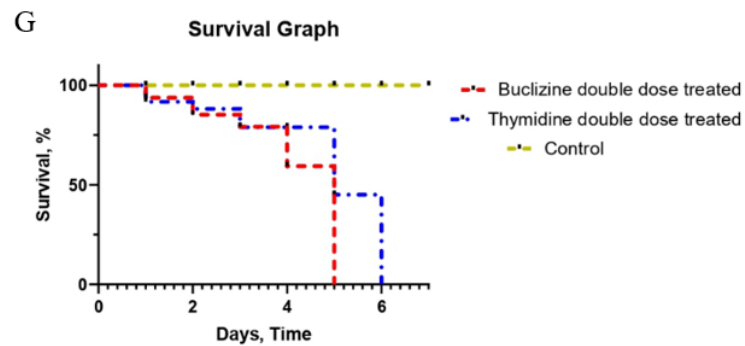
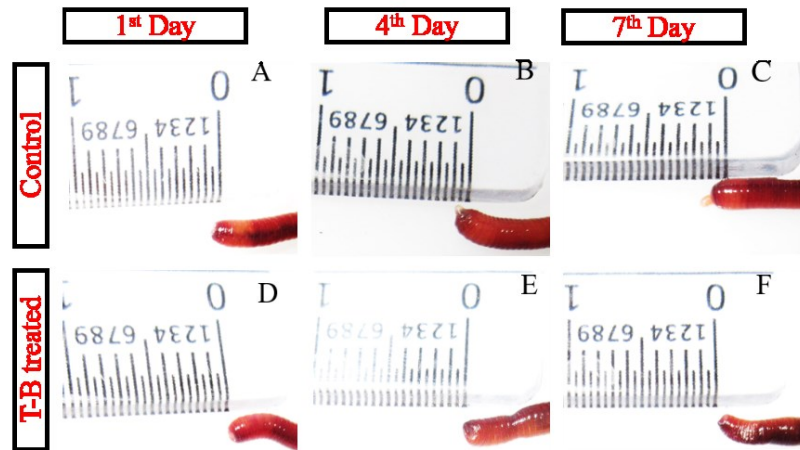


Fig. 4 Combinatorial injection of Buclizine and Thymidine: A. control worm following day-1 post-amputation at 10th segment. B. the amputated worms form blastema on the 4th day. C. on the 7th day, the amputated worm forms elongated blastema. D. Buclizine and Thymidine injected worm following 1st day of

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 - 53kDa; TCTP - 19kDa; Actin - 42kDa). 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.

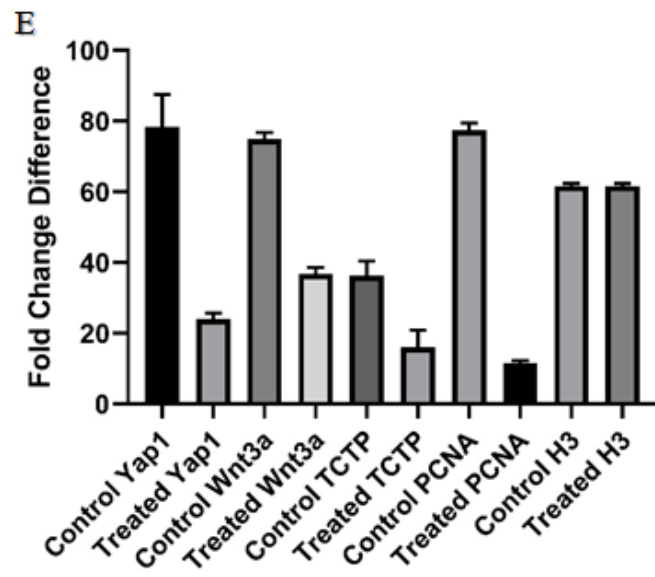
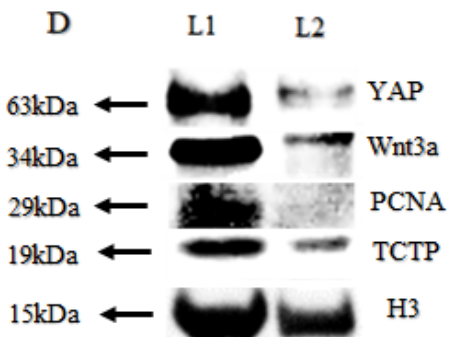
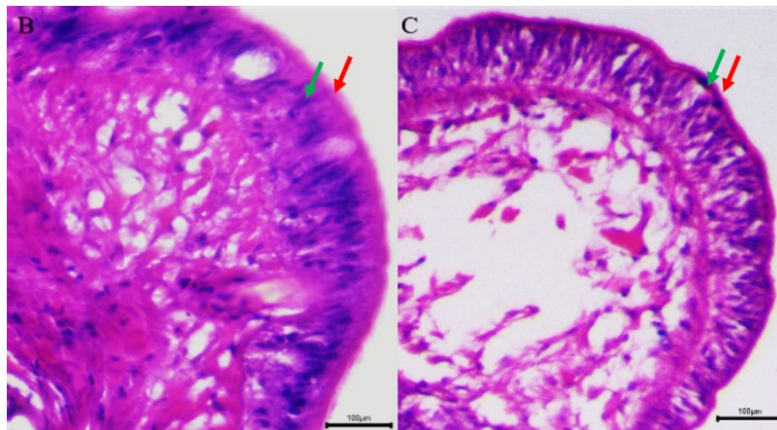
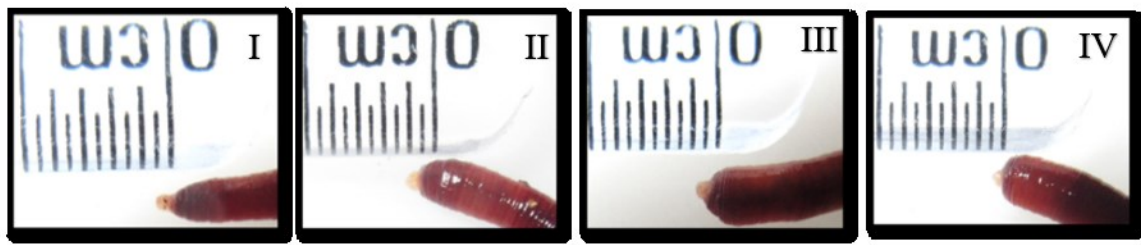
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Fig. 5 TCTP and its connecting link with regenerative protein: A (I). Control amputated worm with 5th day bud. II, III and IV represent progressive regeneration suppression in Nutlin-3a injected

worms with 5, 7, and 9 μ g, 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 \pm 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.”



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