3. Materials and Methods
3.1. Culture and maintenance of earthworm, *Eudrilus eugeniae*:

Earthworm, *Eudrilus eugeniae* was used as a model organism for studying the regeneration process. *E.eugeniae* was cultured and maintained in a tub containing soil, cow dung and leaf litter at an ambient temperature (Viljoen and Reinecke, 1992). Healthy earthworms of similar sizes ranging from 1.0 g to 1.2 g of body weight were carefully selected for experiments. The earthworm bed was changed two month once with the fresh material.

3.2. Dissection of earthworm, *E.eugeniae*:

The earthworm, *E.eugeniae* was fixed with 10% formaldehyde for 20 min in a petri plate. After fixation, the worm was taken out from the petri plate and washed with distilled water and placed on the dissection board for dissection, with a sterile surgical blade (Blade No: 11). The dissected worm was washed with water for clear documentation. Each organ from the dissected worm was taken out separately and documented (Fig.5).

3.3. Amputation of earthworm, *E.eugeniae*:

The earthworm, *E.eugeniae* was maintained in the laboratory and the healthy worms with similar sizes ranging from 1.0 g to 1.2 g body weight were taken and amputated at 11th anterior segment using a sterile surgical blade (Blade. No: 11). The amputated worm was maintained in the tub containing soil and leaf litters, for regeneration studies.

3.4. Lowry’s method of protein estimation:

*Principle:*

Protein can be estimated by different methods, the most popular method was described by Lowry et al., (1951). First the proteins are pre-treated with copper ion in alkali solution. Under alkaline conditions cupric ions (Cu$^{2+}$) chelate with the peptide bonds resulting in reduction of cupric ions (Cu$^{2+}$) to cuprous ions (Cu$^{+}$). The Cuprous ions can also be detected with Folin Ciocalteu reagent. The aromatic amino acids (tyrosine and tryptophan)
in the treated sample reduce the phosphor-molybdatephosphotungustic acid present in the Folin reagent, results in blue purple color complex formation, which has the maximum absorption at 660 nm. The amount of color produced is proportional to the amount of peptide bonds, i.e. size as well as the amount of protein/peptide.

**Materials:**

Clean glass test tubes; Samples; Micro-pipettes; Cuvettes and Spectrophotometer.

**Reagents for Lowry’s Assay:**

**Reagent A:**

2% Sodium Carbonate in 0.1 N Sodium hydroxide.

**Reagent B:**

0.5% Copper Sulphate in 1% Sodium Potassium Tartarate.

**Reagent C:**

50 ml of Reagent A was mixed with 1ml of Reagent B.

**Reagent D:**

Folin - Ciocalteau reagent

Dilute commercial Folin - Ciocalteau reagent (2N) with an equal volume of water.

**Preparation of BSA Standard Solution:**

**Stock Solution:**

50 mg of BSA (Bovine Serum Albumin - Fraction V) was weighed and dissolved in distilled water and make up into 50 ml in a standard measuring flask.

**Working Solution:**

10ml of stock solution was taken and make up into 50 ml with distilled water in a standard measuring flask. 1ml of the solution contains 200 µg proteins.
Methodology:

In a series of test tubes, 20, 40, 60, 80 and 100 μl of the working standard solution (Bovine serum albumin (Fraction V) – from Hi-Media) was added using a micro-pipette. In another two test tubes 5 and 10 μl of the unknown protein sample was added similarly. All the test tubes were making up into 1000 μl volume with sterile distilled water. In a tube with 1ml of water, serves as the blank. 5ml Reagent - C was added to all test tubes including the blank. The tubes were mixed well and allowed to stand for 10 min. After incubation about 500 μl of Reagent D was added and mixed well and incubated in the dark place at room temperature for 30 min, the blue color was developed in the stipulated time period. Optical density value was taken at 660nm in spectrophotometer. Standard graph was plotted against BSA and the amounts of protein in the unknown samples were estimated.

3.5. SDS-PAGE:

Principle:

Electrophoresis is the study of the movement of charge molecules in an electric field. Their rate of movement depends on the strength of the field, net charge, size and shape of the molecules and also the ionic strength, viscosity and temperature of the medium in which the molecules are moving. Polyacrylamide gels are widely used for separating larger molecules like proteins.

Proteins are amphoteric in nature. Hence, the general electrophoresis techniques cannot be used to measure the molecular weight of the protein molecules because the mobility of a substance in the gel is influenced by both charge and size. In order to overcome this, if the protein samples are treated so that they have a uniform charge, electrophoretic mobility that depends primarily on size. The molecular weight of protein was estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a reducing agent β-mercaptoethanol. SDS disrupts the secondary, tertiary and
quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds (both inter-disulfide and intra-disulfide bonds).

**Materials:**

Eppendorfs; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

**Chemical preparation of SDS-PAGE:**

**30% Acrylamide and 0.3% Bis acrylamide:**

Acrylamide - 30 g

N,N’ Methylene Bisacrylamide - 0.3 g

Make up into 100 ml with distilled water.

**4X Separating Buffer:**

Tris HCl - 18.2 g

Distilled water - 60 ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

**4X Stacking Buffer:**

Tris HCl - 6.05 g

Distilled water - 40 ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

**2X Sample Buffer:**

Tris stacking buffer (pH 6.8) - 25 ml

Sodium Dodecyl Sulphate (SDS) - 4 g
20% Glycerol - 20 ml (should be added last and mixed well) Make up into 100 ml with distilled water. During protein sample preparation, 5% β-mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

**10% Ammonium Per Sulphate (APS):**

APS - 100 mg Make up into 1 ml with distilled water.

**1X Running Buffer:**

Tris Hcl - 3.94 g Glycine - 15.014 g SDS - 1 g Make up into 1L with distilled water.

**Staining Solution:**

Coomassie Brilliant Blue R-250 – 200 mg Methanol - 50 ml Glacial Acetic acid - 7 ml Make up into 100 ml with distilled water.

The above mixture was filtered using Whatman No 1 filter paper.

**Destaining Solution:**

Methanol - 30 ml Glacial Acetic acid - 7 ml Make up into 100 ml with distilled water.
**Table.1: Separating gel concentration**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Stock Solution</th>
<th>5%</th>
<th>6%</th>
<th>7%</th>
<th>7.5%</th>
<th>8%</th>
<th>9%</th>
<th>10%</th>
<th>12%</th>
<th>13%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>30% Acrylamide + 0.3% Bis-acrylamide (ml)</td>
<td>5.0</td>
<td>6.0</td>
<td>7.0</td>
<td>7.5</td>
<td>8.0</td>
<td>9.0</td>
<td>10.0</td>
<td>12.0</td>
<td>13.0</td>
<td>15.0</td>
</tr>
<tr>
<td>2.</td>
<td>4X Tris Separating Buffer (ml) pH 8.8</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>3.</td>
<td>Distilled water (ml)</td>
<td>17.5</td>
<td>16.5</td>
<td>15.5</td>
<td>15.0</td>
<td>14.5</td>
<td>13.5</td>
<td>12.5</td>
<td>10.5</td>
<td>9.5</td>
<td>7.5</td>
</tr>
<tr>
<td>4.</td>
<td>10% APS (µl)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>5.</td>
<td>TEMED (µl)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table.2: Stacking gel concentration**

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Stock Solution</th>
<th>5 ml</th>
<th>10 ml</th>
<th>15 ml</th>
<th>20 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>30% Acrylamide + 0.3% Bis-acrylamide (ml)</td>
<td>0.65</td>
<td>2.5</td>
<td>3.75</td>
<td>5.0</td>
</tr>
<tr>
<td>2.</td>
<td>4X Tris Stacking Buffer (ml) pH 6.8</td>
<td>1.25</td>
<td>2.5</td>
<td>3.75</td>
<td>5.0</td>
</tr>
<tr>
<td>3.</td>
<td>Distilled water (ml)</td>
<td>3.05</td>
<td>5.0</td>
<td>7.5</td>
<td>10.0</td>
</tr>
<tr>
<td>4.</td>
<td>10% APS (µl)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5.</td>
<td>TEMED (µl)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
Methodology:

Protein Sample preparation:

In order to identify the protein expression pattern in the regenerated and normal worm, SDS-PAGE was performed. The 6th day regenerated worm tissue, normal worm tissue and their organs were allowed for the protein sample preparation. The tissues were homogenized with 2X protein sample buffer as denoted in the paper (Sudhakar et al., 2008) and boiled in a boiling water bath for 10 min. The samples were then cooled, and stored at -20°C. The proteins present in the samples was estimated using Lowry’s assay and then subjected for protein profile analysis using SDS-PAGE. The prepared protein samples were resolved in the 15% SDS-PAGE.

Preparation of gel:

The glass plates were cleaned assembled so that the longer glass plate laid first, then 2 spacers of 1.5 mm thickness was placed along the sides of the rectangular plate. The notched glass plate was placed on the top of the spacers so that the bottom ends of the spacers and glass plates were aligned and the whole setup was assembled on the stand. 1% agarose was prepared, boiled and it was used to seal the plates.

15% separating gel monomer solution (Table.1) was prepared and carefully poured into the glass plate assembly without forming air bubbles. The gel was overlaid with n-butanol so that it excludes the air. The separating gel was allowed to polymerase for 20 min. The butanol layer was removed and the gel was washed with distilled water.

Stacking gel monomer solution (Table.2) was then prepared and poured over the separating gel. The comb was then inserted and the gel was allowed to solidify for 10 min. After the gel gets solidified, the comb was removed carefully and the wells were washed with
distilled water. The glass plate assembly was then placed in the buffer chamber and running buffer was poured in both upper and lower buffer chamber.

**Sample loading and electrophoresis:**

Protein sample of 80 µg and the appropriate molecular markers were loaded in the wells. The apparatus was connected to the power pack with proper polarity and 5 to 6 v/cm was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulls away from the gel.

**Staining and destaining the gel:**

The gel was carefully taken out from the glass plate and the stacking gel was removed. Separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the commassie brilliant blue R-250. The gel should be fully submerged in the staining solution for 2 hours by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to destaining solution until protein bands are visualized clearly. The gel was then documented (Lark gel documentation system). For immunoblotting experiments, the small SDS-PAGE apparatus set up was used.

**3.6. Mass-spectrometry analysis:**

In order to identify the protein responsible for down regulation during the process of regeneration, the mass-spectrometry was carried out and the data were analyzed. The down regulated protein during the process of regeneration was sliced out from the SDS PAGE gel and stored at 1% acetic acid and subjected to Mass spectrometry analysis. The Mass spectrometry analysis was performed in Proteomics Group of the Core Facility, Integrated Functional Genomics, Inter-disciplinary Center for Clinical Research IZKF Medical Faculty, University of Munster, Germany. Nearly, 124 peptides were obtained and the each peptide
was subjected to BLAST analysis, to identify the target protein. The identified protein was further aligned from higher to lower organisms using the Align-X software.

3.7. Immunoblotting:

The resolved protein samples in SDS-PAGE gel was transferred to Transfer buffer and incubated for 15 minutes. The PVDF membrane was activated by dipping it in methanol and transferred to Transfer buffer. Whatman filter paper (soaked in Transfer buffer) was placed one by one on the transferring unit. The PVDF membrane was placed over the filter paper and then the gel was carefully placed on the PVDF membrane. The Whatman filter paper was again stacked over it, forming the Whatman filter paper – gel – PVDF membrane – Whatman filter paper sandwich. The transferring unit was set to 20 V for 20 minutes to allow the transfer of protein bands from the gel to the PVDF membrane. The transferred PVDF membrane was blocked with 4% BSA and latter the membrane was incubated with primary antibody (TCTP primary antibody and Actin primary antibody with the dilution of 1:1500 and 1:3000 in 2% BSA, respectively) for overnight at 4°C. The non specific binding of primary antibody was washed out with 1X TBST buffer. The secondary antibody conjugated with HRP was used in the dilution concentration of 1:10000. The washed membrane was developed (as per manufacturer's instruction) with the substrate DAB/H₂O₂ (from Amresco) to get the brown colored product, which appeared on the membranes.

**Preparation of 10X PBS:**

- **NaCl** – 80.0 g
- **KCl** – 2.0 g
- **Na₂HPO₄** – 14.4 g
- **K₂HPO₄** – 2.4 g
- Distilled water – 800 ml
- pH was adjusted to 7.4 with HCl
The solution was make up into 1000 ml with distilled water, autoclaved and stored at room temperature.

1X PBS:

100 ml of 10X PBS and makes up into 1000 ml with sterile distilled water

1XTBST:

In 1000 ml of 1XPBS, 1 ml of Tween 20 was added and mixed well.

Transfer Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>4.55 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>21.625 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>150 ml</td>
</tr>
</tbody>
</table>

Make up to 1000 ml with distilled water.

4% BSA solution:

0.4 g of Bovine Serum Albumin (BSA) was dissolved in 10 ml of 1XTBST.

2% BSA solution:

0.2 g of Bovine Serum Albumin (BSA) was dissolved in 10 ml of 1XTBST.

3.8. Histology:

Principle:

Histology is the technique used to study complete architecture of the tissue patterns by means of examination and analysis of cell/tissue physiology & morphology at the microscopic level. The principle behind the histological technique is to preserve microscopic anatomy of tissue and make them hard, so that very thin section (5 to 7 microns) can be made. After staining, the section should represent the anatomy of the tissue as close to as possible to their structure in life. This is achieved by passing the total as selected part of the tissue through a series of process described below.
Materials:

Earthworm tissues (Normal Head Segments, 3\textsuperscript{rd} day Regenerated Head segments, Normal clitellum segments and 3\textsuperscript{rd} day Regenerated clitellum segments); Screw-cap bottles; Clean Albumenized glass slides; Coupling jars; Cover slips and Forceps.

Reagents used for Histology:

Fixation:

10\% Formaldehyde

Dehydration:

Different grades of isopropyl alcohol were used for dehydrating the tissues such as, 60\% isopropyl alcohol; 70\% isopropyl alcohol; 80\% isopropyl alcohol; 90\% isopropyl alcohol; 100\% isopropyl alcohol and again with 100\% isopropyl alcohol.

Clearing:

Xylene

Impregnation with wax:

Paraffin wax – 1; Paraffin wax – 2; Paraffin wax – 3

Reagents used for processing earthworm tissue sections:

Dewaxing:

Xylene

Rehydration:

100\% isopropyl alcohol

Staining agents:

Basic dye Haematoxylin and Acidic dye Eosin.
**Acid alcohol:**

For preparing the acid alcohol, 1 ml of concentrated Hydrochloric acid was mixed with 99 ml of 70% isopropyl alcohol.

**Decolourizer:**

100% isopropyl alcohol

**Methodology:**

**Fixation:**

The earthworm tissues were dissected and fixed in 10% formaldehyde for 24 hours. Fixation preserves the structure and morphology of the specimen throughout the harsh conditions of dehydration, clearing, embedding, sectioning and staining. After fixation the tissues were washed in distilled water.

**Dehydration:**

The next step of tissue processing was dehydration. The tissues were dehydrated using increasing strength of isopropyl alcohol such as 60%, 70%, 80%, 90% and 100%. The tissues were dehydrated in 60, 70 and 80% isopropyl alcohol for one hour, overnight dehydration in 90% isopropyl alcohol and finally one hour in 100% isopropyl alcohol followed by one more time with fresh 100% isopropyl alcohol for one hour.

**Clearing:**

During dehydration water in tissue has been replaced by alcohol. The next step after dehydration was clearing, which was an intermediate step before wax impregnation; alcohol should be replaced by paraffin wax. As paraffin wax was insoluble in alcohol, it should be replaced with a substance in which wax was soluble. This step was called as clearing. Tissues were cleared using xylene for 45min. All the above steps were carried out in slide warmer at 60°C.
**Impregnation of tissues in wax and embedding:**

The next step was the impregnation of tissues with paraffin wax. The melting point of the paraffin wax was 58-60°C. The tissues were maintained in three waxes at 58-60°C. The steps were tabulated in Table.3. After impregnation the processed tissues were embedded in paraffin wax which provides sufficient external support during sectioning. Fresh melted wax was poured in mould and the impregnated tissues were placed carefully in it using forceps and then allow to settle and solidify. After the block was completely cooled it was cut into individual blocks and trimmed.

**Table.3: Steps followed in histology**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation - 10% formaldehyde</td>
<td>50°C</td>
<td>24 hours</td>
</tr>
<tr>
<td>Dehydration- 60% isopropyl alcohol</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Dehydration- 70% isopropyl alcohol</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Dehydration- 80% isopropyl alcohol</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Dehydration- 90% isopropyl alcohol</td>
<td>50°C</td>
<td>Overnight</td>
</tr>
<tr>
<td>Dehydration- 100% isopropyl alcohol</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Dehydration- 100% isopropyl alcohol</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Clearing – Xylene</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Wax impregnation – Paraffin Wax 1</td>
<td>58-60°C</td>
<td>Overnight</td>
</tr>
<tr>
<td>Wax impregnation – Paraffin Wax 2</td>
<td>58-60°C</td>
<td>3 hours</td>
</tr>
<tr>
<td>Wax impregnation – Paraffin Wax 3</td>
<td>58-60°C</td>
<td>3 hours</td>
</tr>
</tbody>
</table>
**Processing and staining the thin tissue sections:**

The block was placed in microtome (Besto) and sliced into thin sections (7 micron in thickness). The thin sections were placed in egg albumenized slides (egg white was mixed with equal volume of glycerol and spread over the slides). The slides were placed in slide warmer and the temperature was maintained at 60°C. Little amount of water was sprinkled over the slides and the thin sections were placed in the slide containing water. As the slide was on the slide warmer, the wax get melts at that time the water was removed and the slide was again placed in the slide warmer for about 10 min. Then the slides were dewaxed by dipping in xylene for 5 min followed by dipping in 100% isopropyl alcohol for 3 min to remove the xylene. Then the slides were washed in running tap water for 5 min and placed in basic dye haematoxylin for 7 min. The slides were washed in running tap water gently, for 10 min followed by acid wash for 2 seconds to remove the excess stain. After acid wash the slides were blooming in running tap water for overnight and then dipped in acidic dye eosin for 30 sec and the slides were washed in water. Then the slides were dipped in decolourizer to remove the excess stain for about 2-3 min. Finally, the sections were cleared using xylene and mounted with DPX and then viewed under Nikon Ti-S microscope. Solvents used for histology were purchased from Merck Specialities Private Limited, India.

**3.9. Autofluorescence in the earthworm, *E.eugeniae* tissue sections:**

In order to study autofluorescence in the worm, histology was performed as described above. The 7 micron thin tissue sections were placed in egg albumenized slides (egg white was mixed with equal volume of glycerol and spread over the slides). The slides were placed in slide warmer and the temperature was maintained at 60°C. Little amount of water was sprinkled over the slides and the thin sections were placed in the slide containing water. As the slide was on the slide warmer, the wax get melts at that time the water was removed and
the slide was again placed in the slide warmer for about 10 min. Then the slides were
dewaxed by dipping in xylene for 5 min followed by dipping in 100% isopropyl alcohol for 3
min, washed with water and viewed under a Nikon Ti-S inverted fluorescent microscope for
autofluorescence without any staining pattern, after which the slides were stained with a
haematoxylin – Eosin combination for further documented.

3.10. Autofluorescence in coelomic fluid cells:

To observe the autofluorescence of coelomic fluid cells, coelomic fluid was collected
from the mature worm by giving 4.5 volts. Due to electric shock, the earthworms were
starting releasing the ceolomic fluid, it was collected in the eppendorf tube and smeared on a
clean glass slide. The smear was fixed with 4% Paraformaldehyde; briefly washed with 1X
PBS; and it was examined under a Nikon Ti-S inverted fluorescence microscope, after which
the slides were stained with a haematoxylin – eosin combination for further documented.

3.11. Sample preparation for thin-layer chromatography:

Earthworm tissues were weighed and taken in a sterile centrifuge tube and
homogenized with a Homogenizer (Micro-pestle from Helini Biomolecules) by adding an
equal volume of sterile distilled water. The homogenates were centrifuged at 5000 rpm for 10
min in a mini centrifuge (Eppendorf Mini Spin Centrifuge), and the supernatant was resolved
by thin-layer chromatography.

3.12. Thin-layer chromatography:

*Principle:*

Thin Layer Chromatography (TLC) is based on the principle of separation. The
separation depends on the relative affinity of compounds towards stationary and mobile
phase. The compounds under the influence of mobile phase (driven by capillary action) travel
over the surface of the stationary phase. During this movement the compounds with higher
affinity to stationary phase travel slowly while the others travel faster. Thus the separation of
components in the mixture is achieved. Once separation occurs individual components are visualized as spots at respective level of travel on the plate. Their nature or character is identified by means of suitable detection techniques.

**Materials:**

Glass plates (30x24cm); Silica gel G for TLC; Chromatography chamber; Capillary tube; Earthworm, *E. eugeniae* tissue lysate; Mobile phase (solvent system) - Chloroform : Benzene 1:1; Ninhydrin Solution for visualizing the amino acids and UV lamp.

**Ninhydrin Solution:**

1.5 g of Ninhydrin was dissolved in 100 ml of n-Butanol followed by the addition of 3 ml of Acetic Acid.

**Methodology:**

**Preparation of preparative TLC plates:**

The slurry was prepared by mixing silica gel with water in the ratio 3:2. The slurry was coated onto the glass plate at a thickness of about 0.25mm and the plates were allowed to dry at room temperature for 15-30 min. Then the plates were kept in hot air oven at 100-120°C for 2 hours (Sadasivam and Manikam, 1996) to remove the moisture and to activate the adsorbent on the plate.

**Sample loading:**

A line was drawn on the plate about 1.5-2.0 cm from the bottom. The samples were spotted on the plate using capillary tube. All the samples should place at equal distance from one end of the plate. The spots were allowed to air dry and spotting can be done repeatedly for a more concentrated sample spot.

**Developing chromatogram:**

The solvent mixture (Butanol (7): Chloroform (4): Acetic acid (5): Ammonia (1): Water (1)) was poured into the tank to a depth of about 1.5 cm. The tank was covered with a
glass plate and it was allowed to stand for an hour to ensure that the atmosphere within the tank becomes saturated with solvent vapour. This process is called equilibration. After equilibration, the plate was placed vertically in the tank so that it stands in the solvent with the spotted end dipping in the solvent. The solvent moves upwards due to capillary action and the compounds get separated. As soon as the solvent reached the top of the plate it was removed from the tank, dried and proceeds for the identification of the separated compounds.

**Identification of compounds:**

The chromatogram was allowed to dry and visualized under UV light. The TLC plate was further sprayed with Ninhydrin Solution to track the presence of protein, which shows the purple color in the chromatogram. The spot visualized under UV was marked and it was scraped off using a surgical blade and the scraped material was transferred to a microfuge tube. The Rf values of the spots were calculated using the formulae

\[
R_f = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}
\]

**3.13. Elution of fluorophore and riboflavin by TLC:**

For eluting the fluorophore and riboflavin from the silica gel, the scraped flurophore was taken from the chromatogram (TLC) along with the Silica gel in a tube and equal amount of distilled water was added and it was mixed well. The tube was then centrifuged at 10,000 rpm for 10 min, and the supernatant was used for further analysis such as thin-layer chromatography and spectrofluorometric analysis. Solvents used for the TLC were purchased from Merck Specialities Private Limited, India.

**3.14. Estimation of riboflavin:**

The known concentration of riboflavin was resolved using thin-layer chromatography and visualized under UV light. By using imaging software NIS-Elements BR 3.1, the
grayscale intensity was measured. From the data, a standard graph was generated and the concentration of riboflavin in the different samples of the worm was determined.

A solution of riboflavin in distilled water was scanned using a spectrophotometer and the absorbance maxima were determined. A standard graph was then plotted by taking different concentrations of riboflavin. Using the standard graph, unknown concentrations of riboflavin in worm samples were calculated.

3.15. Spectrofluorometric analysis:

Spectrofluorometric analyses were performed using the lysate of earthworm. Earthworm tissues were homogenized with sterile distilled water and the lysate obtained was centrifuged at 5000 rpm for 10 min (Eppendorf Mini Spin Centrifuge). The supernatant was collected, and it was subjected to spectrofluorometric analyses with standard riboflavin, FMN (Flavin mononucleotide) and FAD (Flavin adenine dinucleotide) (purchased from HiMedia Laboratories Private Limited, India).

3.16. BrdU labeling retention assay:

Principle:

Bromo-deoxy-uridine (5-Bromo-2'-deoxy-uridine, BrdU - purchased from Sisco Research Laboratories Private Limited, India) is an analog of the DNA precursor ‘Thymidine’. Injection of BrdU into the earthworm, labels all the cells (including the adult stem cells). After a prolonged period of incubation (commonly known as chasing) the BrdU concentrations in all proliferating cells became reduced and sloughed off. But, the incorporated BrdU in the adult stem cells became retained, because stem cells have a slow proliferation rate other than normal cells. They can divide only if they have a tendency to renew or to restore the damaged organs or body segments. The amount of BrdU in the DNA of cells can be detected with specific monoclonal anti-BrdU antibody (from Sigma). Thus, labeling retention assay is used to identify the location of stem cells and its niche.
Materials:

Screw cap bottles; Clean Albumenized glass slides; Coupling jars and Cover slips.

BrdU stock solution

BrdU stock solution (10 µg/ml)

BrdU of 10 mg was dissolved in 1 ml of 1X PBS.

Preparation of 10X PBS:

NaCl – 80.0 g
KCl – 2.0 g
Na₂HPO₄ – 14.4 g
K₂HPO₄ – 2.4 g
Distilled water – 800 ml

pH was adjusted to 7.4 with HCl

The solution was make up into 1000 ml with distilled water, autoclaved and stored at room temperature.

1X PBS:

100 ml of 10X PBS and make up into 1000 ml with sterile distilled water

1XTBST:

In 1000 ml of 1XPBS, 1 ml of Tween 20 was added and mixed well.

Fixation:

10% Formaldehyde was used for fixation.

Dehydration:

Different grades of isopropyl alcohol were used for dehydrating the tissues, such as, 60% isopropyl alcohol; 70% isopropyl alcohol; 80% isopropyl alcohol; 90% isopropyl alcohol; 100% isopropyl alcohol and again with 100% isopropyl alcohol.
**Clearing:**

Xylene

**Impregnation with wax:**

Paraffin wax – 1; Paraffin wax – 2 and Paraffin wax – 3

**Dewaxing:**

Xylene

**Dehydration:**

100% isopropyl alcohol

**Permeabilization:**

0.25% Triton X 100

**Endogenous peroxidase blocking:**

H$_2$O$_2$ - 10 ml

Methanol - 10 ml

Make up into 100 ml with 1X PBS

**Denaturation:**

2N HCl

**Blocking:**

2% Bovine Serum Albumin in 1X TBST

**Primary Antibody:**

Mouse monoclonal anti-BrdU antibody (from Sigma) at the dilution of 1: 200 in 1XTBST containing 2% BSA.

**Secondary antibody:**

Goat anti mouse IgG HRP at the dilution of 1: 500 in 1XTBST containing 2% BSA.

**Developing using Diaminobenzidine (DAB) kit (from Amresco):**

Dilution buffer - 2 ml
H$_2$O$_2$ - 2 µl
DAB - 40 µl

**Counter stain:**
Erhlich Haematoxylin

**Acid alcohol:**
For preparing the acid alcohol, 1ml of concentrated HCl was mixed with 99ml of 70% isopropyl alcohol.

**Decolourizer:**
100% isopropyl alcohol

**Methodology:**
The BrdU stock solution was prepared at a concentration of 10mg/ml in 1X PBS and each worm was injected with 10µl of BrdU from the 20$^{th}$ segment to the 24$^{th}$ segments. The worm injected with BrdU was maintained for 30 days for chasing. On the 30$^{th}$ day the worm was amputated at the 11$^{th}$ segment and allowed to regenerate the lost part. The regeneration blastema appeared on the 4$^{th}$ day was formalin-fixed, paraffin-embedded, sectioned and allowed to stain with anti-BrdU antibody.

**3.17. Immunohistochemistry:**
To visualize BrdU incorporation, paraffin-embedded earthworm tissue sections (6 µm) were de-paraffinized with xylene and hydrated. Endogenous peroxidase was inhibited by incubation of the sections for 30 min with freshly prepared 10% H$_2$O$_2$ and 10% Methanol in 1X PBS. The sections were then treated with 0.1% Trypsin in 0.1% CaCl$_2$ at 37°C for 10 min. DNA was denatured by incubating the section with 2N HCL at 37°C for 45 min. Non-specific staining was blocked by treatment with 2% BSA for 1 hour at room temperature. The sections were then incubated overnight at 4°C with mouse monoclonal anti-BrdU antibody (Clone BU-33, Sigma) at a dilution of 1:200 in 2% BSA. After incubation with primary
antibody, tissue sections were washed and incubated for 1 hour with goat anti-mouse IgG conjugated with horseradish peroxidase (Lot No: 062100; GeNei) at a dilution of 1:500. Staining was developed with Diaminobenzidine (DAB Kit; Amresco) substrate and the sections were counterstained with Ehrlich haematoxylin. The prepared slides were mounted with DPX and observed under a Nikon Ti-S microscope.

3.18. Histochemistry for alkaline phosphatase:

The regenerated blastema of *E. eugeniae* were fixed with 100% methanol for 15 min and washed with TBS and stained with BCIP/NBT solution (from Amresco) (which is a substrate for alkaline phosphatase) for 90 min at room temperature. After the incubation in the BCIP/NBT solution, the worm tissues were washed with TBS, fixed with AGE fixative (Acetic acid (4): Glycerol (1): Ethanol (2)) for 15 min and whole mounted with DPX. The prepared slides were observed under a Nikon Ti-S microscope for Alkaline Phosphatase (ALP) activity.

3.19. Counting of cells:

The BrdU positive cells stained with anti-BrdU antibody and developed with DAB shows dark brown colour nucleus and the other cells were stained with haematoxylin. The slides were carefully observed under the Nikon Ti-S microscope and the BrdU positive cells were counted. The counting was repeated thrice to verify the data and also for the statistical analysis. Similarly, autofluorescent cell has been counted under the Nikon Ti-S fluorescent microscope.

3.20. Designing of siRNA:

In order to design the siRNA, the mRNA sequence of the TCTP and GFP were retrieved from the NCBI website. By specialized software based on siRNA design guidelines first described by Tuschl *et al.*, (1999), the siRNA was designed using the mRNA sequence of TCTP and GFP. The designed siRNA was shown below in the Table.8. The siRNA was
tagged with the fluorescein at the 3’ end of the sense strand and two molecules of deoxy thymidine at the 3’ end of both sense and anti-sense strand respectively. The designed siRNA was synthesized commercially from Sigma as per the below configuration along with the control siRNA (Table.8).

3.21. Resuspension of siRNA:

The synthesized dried TCTP siRNA was re-suspended in sterile nuclease free water as per the guidelines in the technical data sheet provided by Sigma. The re-suspended stock was stored at -20°C for long time use and stored at 4°C for short term use. The final Stock concentration of the re-suspended siRNA was 100pmol/µl. The injection buffer used for the *in vivo* knockdown of TCTP siRNA was 1X Phosphate buffered saline (1X PBS) pH 7.4. The siRNA is tagged with a fluorescent compound which is pH dependent, so 1X PBS was used as an injection buffer instead of using other buffers.

3.22. Injection of siRNA into earthworm, *E.eugeniae*:

To study the knockdown efficiency of TCTP siRNA, healthy earthworms of similar sizes ranging from 1.0 g to 1.2 g body weight were carefully selected for experiments and amputated at the 11\(^{th}\) segment with a sterile surgical blade (Blade. No: 11). The amputated worms were maintained in the laboratory in a specialized medium containing leaf litters and soil with appropriate moisture. First injection of TCTP siRNA was injected at the ventral side of 20\(^{th}\) to the 24th segment of the worm with 30 gauge needle. After 48 hrs of the first injection, a second injection was administrated at the ventral side of the worm at 20\(^{th}\) to 24\(^{th}\) segment. Similar experiments were done by injection of control siRNA as a positive control and without injection of anything in 1X PBS as a negative control.

3.23. Injection of compounds to the earthworm, *E.eugeniae*:

To demonstrate the augmentation kinetics of riboflavin in the process of regeneration, earthworms were injected with riboflavin (Hi-Media), mixture of antibiotics (Penicillin,
Amphotericin B and Streptomycin – from Hi-Media) and both. Healthy earthworms of similar sizes ranging from 1.00 g to 1.2 g body weight were carefully selected for experiments. Riboflavin, mixture of antibiotics and both were injected to the earthworm, *E. eugeniae*, (different experimental setup as discussed in the results and discussion) at the ventral side of 20th to the 24th segment of the worm with 30 gauge needle. Similar types of injection were performed with sterile water for control purpose.

3.24. TUNEL assay:

**Principle:**

TUNEL assay is the useful technique to detect the apoptotic cell through the detection of DNA double strand break. The principle behind the TUNEL assay is that, apoptotic cells have a double strand break in its genomic DNA and the end-labeling with BrdU by terminal transferase (TdT) at the free 3’OH ends of genomic DNA. Apoptotic cell has more double strand break in the genomic DNA and also it has more free 3’OH ends of genomic DNA, which is not in the case of normal cells. After the incorporation of BrdU by terminal transferase, it can be stained with anti-BrdU antibody and visualized by secondary antibody conjugate.

**Materials:**

- Screw cap bottles; Clean Albumenized glass slides; Coupling jars and Cover slips.

**BrdU stock solution:**

- BrdU stock solution (10 µg/ml)
  
  BrdU of 10 mg was dissolved in 1 ml of 1X PBS.

**Fixation:**

- 10% Formaldehyde was used for fixation.
**Dehydration:**
Different grades of isopropyl alcohol were used for dehydrating the tissues such as, 60% isopropyl alcohol; 70% isopropyl alcohol; 80% isopropyl alcohol; 90% isopropyl alcohol; 100% isopropyl alcohol and again with fresh 100% isopropyl alcohol;

**Clearing:**
Xylene

**Impregnation with wax:**
Paraffin wax – 1; Paraffin wax – 2 and Paraffin wax – 3.

**Dewaxing:**
Xylene

**Dehydration:**
100% isopropyl alcohol

**Endogenous peroxidase blocking:**

\[
\text{H}_2\text{O}_2 \quad -10 \text{ ml}
\]

Methanol - 10 ml

Make up into 100 ml with 1X PBS

**Protease treatment:**
0.1% Protease (GeNei) in 1X PBS

**Permeabilization:**
0.25% Triton X 100

**TUNEL Reaction Buffers:**

**Equilibration Buffer – 45 µl**

1X TE – 32 µl

5X Terminal Transferase Buffer – 10 µl

25 mM CoCl2 – 3 µl
Nucleotide Mix – 5 µl

1 mM BrdU – 0.5 µl
1 mM ATP – 0.5 µl
1X TE – 4 µl

TUNEL Reaction Mix:

Equilibration Buffer – 45 µl
Nucleotide Mix – 5 µl
Terminal Transferase enzyme (Fermentas) – 1 µl (20u/ µl)

20X SSC Buffer:

NaCl of 17.5 g and 8.82 g of Sodium Citrate was dissolved in 80 ml of sterile distilled water. The pH of the solution was adjusted to 7.0 with a few drops of 14 N HCl. The solution was making up into 100 ml with sterile distilled water, autoclaved and stored at room temperature.

1X SSC Buffer:

5 ml of 20X SSC was taken in a 100 ml screw cap bottle and 95 ml of sterile distilled water was added.

Blocking:

2% Bovine Serum Albumin (BSA) in 1XTBST

Primary Antibody:

Mouse monoclonal anti-BrdU antibody (from Sigma) at the dilution of 1: 200 in 1XTBST containing 2% BSA.

Secondary antibody:

Goat anti mouse Ig-G HRP at the dilution of 1: 500 in 1XTBST containing 2% BSA.
Developing using Diaminobenzidine (DAB) kit (from Amresco):

- Dilution buffer: 2 ml
- H$_2$O$_2$: 2 µl
- DAB: 40 µl

Counter stain:

- Erhlich Haematoxylin

Acid alcohol:

For preparing the acid alcohol, 1 ml of concentrated HCl was mixed with 99 ml of 70% isopropyl alcohol.

Decolourizer:

- 100% isopropyl alcohol

Methodology:

Healthy earthworms of similar sizes ranging from 1.0 g to 1.2 g body weight were carefully selected for experiments and amputated at the 11$^{\text{th}}$ segment with a sterile surgical blade (Blade No: 11) and allowed to regenerate the lost part. The regeneration blastema appeared on the 4$^{\text{th}}$ day and its adjacent segments with clitellum were formalin-fixed, paraffin-embedded, sectioned and allowed for TUNEL assay.

The paraffin-embedded earthworm tissue sections (6 µm) were de-paraffinized with xylene and hydrated. Endogenous peroxidase was inhibited by incubation of the sections in freshly prepared 10% H$_2$O$_2$ and 10% Methanol in 1X PBS for 30 min. The sections were then treated with 0.1% protease in 1X PBS at 37°C for 10 min. The sections were permeabilized with 0.25% Triton-X 100 for 2 min on ice. After permeabilization, tissue sections were blocked with Equilibration Buffer at room temperature (50 µl/section) for 10 min. After Blocking, 50 µl of Reaction Buffer were added to each section and incubated at 37°C for 60 - 90 min. Control experiments were performed without the addition of Terminal
Transferase enzyme in the reaction Buffer. The slides were soaked in 1X SSC Buffer for 15 min at room temp to stop the reaction followed by washing the slides with 1X PBS.

Non-specific staining was blocked by treatment with 2% BSA for 1 hour at ambient temperature. The sections were then incubated overnight at 4°C with mouse monoclonal anti-BrdU antibody (Clone BU-33, Sigma) at a dilution of 1:200 in 2% BSA. After incubation with primary antibody, tissue sections were washed and incubated for 1 hour with goat anti-mouse IgG conjugated with horseradish peroxidase (Lot No: 062100; GeNei) at a dilution of 1:500. Staining was developed with Diaminobenzidine (DAB Kit; Amresco) substrate and the sections were counterstained with Ehrlich haematoxylin. The prepared slides were mounted with DPX and observed under a Nikon Ti-S microscope.

3.25. Total RNA isolation:

Total RNA isolation from the earthworm tissues were carried out using the QIAGEN – RNeasy Mini Kit (Cat. No: 74104) as per the manufacturer protocol and guidelines. The total RNA was isolated from the 6th day anterior regenerated worm blastema and segments from 1 to 11 of control worms. The isolated total RNA was re-suspended with the appropriate amount of DEPC treated water and stored at -70°C deep freezer for further experiments.

3.26. Purification of poly A⁺ mRNA from the total RNA:

Purification of poly A⁺ mRNA from the total RNA was carried out using the QIAGEN - Oligotex mRNA Mini Kit (Cat. No: 70022) as per the manufacturer protocol and guidelines. The isolated poly A⁺ mRNA was stored at -70°C deep freezer for further experiments.
3.27. Quantification of RNA:

The concentration of RNA can be determined by measuring the absorbance at 260 and 280 nm in NanoDrop. The quality of RNA was quantified using the A260 / A280 ratio. 1 µl of RNA sample was used to quantify the RNA.

3.28. cDNA Synthesis and identification of regeneration specific candidate genes using cDNA subtraction kit:

The purified mRNA was used for cDNA synthesis by SMART Scribe™ Reverse transcriptase (Cat. No. 637401 from Clontech Laboratories). The synthesized cDNA from the regenerated blastemal segments, which contains specific (differentially expressed) transcripts as a tester, and the for the reference, cDNA from the normal segments act as a driver. Tester and driver cDNAs were hybridized, and the hybrid sequences were removed. Consequently, the remaining unhybridized cDNAs represent genes that were expressed in the tester yet absent from the driver mRNA were specifically amplified using PCR. The PCR-Select cDNA Subtraction Kit (Cat. No. 637401 from Clontech Laboratories) selectively amplifies the differentially expressed sequences.

3.29. Cloning and sequencing of differentially expressed genes:

The obtained product from the PCR was cloned into the Bluescript vector (pBSK-) and sequenced. In order to clone the PCR amplified differential expressed genes, pBSK vector was restricted digested with Sma I restriction enzyme and dephosphorylated with shrimp Alkaline phosphatase and ligated with the amplified target. The ligated vector was transformed into XL1 Blue bacterial strain and the plated in a IPTG/X-gal coated LB plate with ampicillin. The white colonies (positive clones) were picked and colony PCR was performed using pBSK universal forward and reverse primers. The sequence data have been BLAST with the Nucleotide and EST database by performing the sequence alignment, the regeneration specific genes have been identified.
3.30. Designing of primers for Actin:

β-Actin mRNA sequences from Homo sapiens to c.elegans was retrieved from the NCBI website as FASTA format and aligned using AlignX software. Based upon the homology, primers were picked for amplification of β-actin in the earthworm, E.eugeniae.
Forward Primer - 5’ GATGATCATCTTCAAGGACGTT 3’
Reverse Primer - 5’ GGTTAGAAGCACTTCCTATGGAC 3’.

3.31. Agarose Gel Electrophoresis:

**Principle:**

Agarose gel electrophoresis is a technique used to separate the DNA molecules based upon the charge and mass ration. In an electric field, DNA migrates towards the anode due to the presence of negatively charge phosphates in the backbone. The migration of DNA molecules is determined by their size; however, the relationship between the fragment size and rate of migration is nonlinear, since larger fragments have a greater frictional drag and are less efficient at migrating through the gel.

**Materials:**

DNA samples; Electrophoresis-grade agarose; Microwave oven; DNA markers; Horizontal gel electrophoresis apparatus; Gel casting platform; Gel comb and DC power supply.

**Reagents required:**

- 0.5 M EDTA (pH 8.0); 50X TAE Buffer ; 1X TAE Buffer; 10X TE Buffer; 1X TE Buffer; Ethidium Bromide and 6X Gel Loading Dye.

**Preparation of reagents:**

**0.5 M EDTA (pH 8.0):**

EDTA of 14.6125 g was added to 80 ml of sterile distilled water. EDTA will not dissolve in water until the pH of the solution was adjusted to pH 8.0. The pH was adjusted by
means of 2N NaOH solution. Finally the solution was making up into 100 ml with sterile distilled water autoclaved and stored at room temperature.

**50X TAE Buffer (Stock Solution):**

Tris base of 242 g was taken in a 1 L screw cap bottle and 57.1 ml of glacial acetic acid was added followed by the addition of 100 ml of 0.5 M EDTA (pH 8.0) and make up into 1000 ml with sterile distilled water.

**1X TAE Buffer (Working Solution):**

Working solution – 1X TAE Buffer from the stock solution – 50X TAE Buffer can be calculated by the simple formula. If we need 1L 1X TAE Buffer, it can be easily applied to formula as demonstrated below.

\[
\frac{\text{Required Volume} \times \text{Required Concentration}}{\text{Available Concentration}} = \frac{1000 \text{ml} \times \text{1X TAE Buffer}}{50 \text{X TAE Buffer}} = 20 \text{ ml}
\]

Then, 20 ml of 50X TAE was taken and make up into 1000 ml with sterile distilled water. The resultant is the 1X TAE Buffer.

**10X TE Buffer (Stock Solution):**

10 ml of 1 M Tris HCl (pH 8.0) was taken in a 100 ml screw cap bottle and 2 ml of 0.5 M EDTA (pH 8.0) was added and make up into 100 ml by adding 88 ml of sterile distilled water. The prepared 10X TE Buffer was autoclaved and stored at 4°C.

**1X TE Buffer (Working Solution):**

1 ml of 10X TE Buffer was taken and make up into 10 ml with sterile distilled water in a 15 ml screw cap tube and stored at 4°C.
**Ethidium bromide solution 10 µg/ml:**

**Stock solution:**

10 mg of Ethidium Bromide was dissolved in 1ml of Nuclease free water.

**Working solution:**

100 µl of Ethidium Bromide was taken from the stock solution and make up into 1000 µl with Nuclease free water (1mg/ml). Finally 10 µl was taken from the working solution and make up into 1000 µl with Nuclease free water (10 µg/ml).

**6X Gel loading Buffer:**

30% sterile glycerol (in sterile distilled water); 0.25% Bromophenol blue and 0.025% Xylene cyanol.

For preparation of 10 ml 6X Gel loading Buffer, the following reagents were added, mixed well and stored at 4°C.

- Sterile glycerol - 3 ml
- Sterile distilled water - 7 ml
- Bromophenol blue - 0.025 g
- Xylene cyanol - 0.025 g

**Methodology:**

**Preparation of agarose gel:**

For preparation of 1% Agarose, 250 mg of Agarose was dissolved in 25 ml of 1X TAE and it was boiled in microwave oven and cooled to 60°C. At this time, 1 µl of EtBr was added to the melted agarose, mixed well and poured without forming air bubbles in the gel casting platform containing the comb. After the gel gets solidified, the gel casting platform along with the solidified gel was placed in the electrophoresis apparatus. The comb was removed gently and the sufficient 1X TAE buffer was added so that the wells get submerged in the tank.
*Electrophoresis:*

The DNA samples were loaded into the well of agarose gel along with 6X loading dye and DNA markers were also loaded in the appropriate lane. The apparatus was connected to the DC power pack and 5 to 6 v/cm was applied to start the electrophoresis. The progress of the separation was monitored by the migration of the dyes in the loading buffer. After electrophoresis, the DNA bands were visualized under UV gel documentation system.

3.32. **Percentage of cells in S-phase:**

To calculate the Percentage of cells in S-phase, the PCNA positive cells and the total number of cells in blastema were counted in the control and TCTP knockdown sections, respectively. The Percentage of cells in S-phase was calculated based upon the following formula.

\[
\text{The percentage of cells in S-phase} = \frac{\text{Number of PCNA positive cells}}{\text{Total number of cells}} \times 100
\]

3.33. **Statistical analysis:**

Statistical analysis such as standard deviation, *p*-value for BrdU-positive cells versus a positive control, and regeneration growth kinetics were performed using Microsoft Office Excel 2007.