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"To study the effects of Homothorax during Drosophila Central Nervous system development"

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Abstract— **Hippo signalling has emerged as a novel tumor suppressor pathway that plays an important role in the regulation of tissue and organ size during development. The Hippo pathway consists of a core kinase cascade in which Hpo phosphorylates the protein kinase Warts. Activated Wts then go and phosphorylate and inactivate the transcriptional coactivator Yorkie (Yki). Wts promotes the association of Yki with 14-3-3 proteins, which helps anchor Yki in the cytoplasm and prevent its transport to the nucleus. Homothorax -a homeodomain transcription factor- in conjunction with Yki has been shown to regulate micro RNA bantam, a positive regulator of growth. Works from various laboratories have shown that down regulation of Homothorax reduced epithelial cell growth in eye discs. Here, we tried to characterize the effects of Homothorax knockdown in glial cells and its effects on CNS growth and development at various stages of Drosophila development. Previous work from our laboratory showed that glia specific knock down of Homothorax does not reduce the glial cell number. However, in the present work, we notice that the mutant larvae displayed wedge shaped CNS phenotype with extended optic lobes. Further, we also studied the effect of Hth RNAi in embryos. In addition to that, we also noticed that Hth RNAi mutant pupae never enclosed into adults. Further, both to confirm the specificity of RNAi and rule out the off target effects of RNAi, we performed rescue experiments using mammalian homologue of Homothorax i.e. UAS Myc Meis. Glia specific expression of UAS Myc Meis completely rescued the Hth RNAi phenotype to normal. This experiment allowed us to study the functional conservation of Homothorax. Further it would be very interesting to study and characterize these phenotypes at both cellular and molecular level.**

Index Terms: - **Hippo signalling, Homothorax, Drosophila, Central nervous system.**

I. INTRODUCTION

Cancer is a disease caused by an uncontrolled division of abnormal cells in a part of the body. It can also be called as a malignant growth or tumour resulting from an uncontrolled division of cells. Normal cells have the ability to reproduce correctly, stop reproducing when necessary, remain in a specific location, and become specialized for specific functions, and self destruct when necessary whereas cancer results from the development of abnormal properties in normal cells that enable them to grow excessively and spread to other locations. This abnormal development can be caused by mutations that occur from factors such as chemicals, radiation, ultraviolet light, and chromosome replication errors. Cancer cells acquire the ability to reproduce uncontrollably. These cells lose the ability to communicate with other cells through chemical signals. They lose the adhesion molecules that keep them bonded to neighbouring cells. There are two types of tumours namely benign which are non cancerous tumours and malignant which are cancerous tumours. Cancer which is also called as malignant tumour causes abnormal cell growth and has the capacity to spread all over the body. They form a subset of neoplasm. A neoplasm or tumour is a group of cells that have undergone unregulated growth, and will often form a mass or lump, but may be distributed diffusely. There are more than 100 different known cancers which affect humans. Cancer is difficult to treat because when cancer cells are removed by surgery or with toxic chemicals or radiation, it is hard to eradicate every single cell. Even if few cancer cells remain, they proliferate to produce the cancerous cell growth again. Abnormal cell growth cannot be checked in cancer cells. (Ref: chealth.canoe.ca)

The errors that cause cancer are self-amplifying and compounding, for example:

- A mutation in the error-correcting machinery of a cell might cause that cell and its progeny to accumulate errors more rapidly.
- A further mutation in an oncogene might cause the cell to reproduce more rapidly and more frequently than its normal counterparts.
- A further mutation may cause loss of a tumor suppressor gene, disrupting the apoptosis signalling pathway and resulting in the cell becoming immortal.

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• A further mutation in signaling machinery of the cell might send error-causing signals to nearby cells. (Bishop, J. M. (1987)

HALLMARKS OF CANCER

Hanahan et.al has recently reviewed decades of literature pertaining to cancer research and hypothesized that a normal cell in order to become cancerous must fulfil or acquire six essential characteristics which are called as Hallmarks of Cancer (shown in Fig 2) . The six hallmarks of cancer which are distinctive and have complementary capabilities enable tumour growth and metastatic dissemination; still continue to provide a solid foundation for understanding the biology of cancer.

HIPPO SIGNALLING

The Hippo signalling is a relatively novel tumor suppressor pathway that plays an important role in the regulation of tissue and organ size during development. Mutations in this pathway were first discovered in Drosophila melanogaster, which have consistently demonstrated that dysfunctional Hippo pathway signalling leads to dramatic tissue overgrowth. The deregulation of Hippo signalling leads to a concurrent combination of uncontrolled cellular proliferation and inhibition of apoptosis, which are the two key hallmarks in cancer development. The molecular nature of this pathway was first discovered in Drosophila melanogaster through genetic screens to identify regulators of cell growth and cell division. The pathway is strongly conserved in humans, making Drosophila a suitable and efficient model system to better understand the molecular nature of this pathway.

HOMOTHORAX

Homothorax (hth) are homeobox proteins. Homeobox genes are a large family of similar genes that direct the formation of many body structures during early embryonic development. In humans, the homeobox gene family contains approximately 235 functional genes and 65 pseudo genes. Homeobox genes are present on every human chromosome, and they often appear in clusters.

The aim of current project is to study the role of Hth in Drosophila CNS development.

II. MATERIALS AND METHODS

1. STOCKS:

a. yw hs flp; UAS Hth RNAi/Cyo; MKRS/TM6
b. w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6
c. yw hs flp; UAS Myc-Meis/Cyo; UAS Hth RNAi/MKRS

2. CROSSES:

When we set up a cross, generally female virgins of particular genotypes are collected because female flies store sperms in their body if they have already mated. Hence it becomes difficult to predict the F1 progeny genotype and to carry further studies. In this experiment, we have set up the cross between ywhsflp; UAS HthRNAi/Cyo; MKRS/TM6 males and w; UAS Dicer; repoGal4UASCD8GFP/TM6 virgin females to get third instar larvae and embryos. In this case for larvae collection, from ywhsflp; Hth RNAi/Cyo; MKRS/TM6 stock, we used homozygous flies for HthRNAi in order to have more percentage of desired progeny.

So, for this cross 20 female virgins from w; UAS Dicer; repo Gal4 UASCD8GFP/TM6 and 5 male from yw hs flp; UAS Hth RNAi/Cyo; MKRS/TM6 was taken in the vial and were crossed. Each vial had 10ml of fly food which is the basic requirement of the flies and to lay eggs on the food. w;UAS Dicer;repoGal4UASmCD8GFP/TM6 X ywhsflp; UASHthRNAi /HthRNAi; MKRS/TM6

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F1 generation (50%)

We have also set up a cross between (yw hs flp; UASMyc-Meis/Cyo;Hth RNAi/MKRS and w;UAS Dicer;repoGal4UASmCD8GFP/TM6) to check whether by reintroducing Meis (human homolog of homothorax), it rescue the phenotype of the normal CNS

3. COLLECTION OF CNS (Central Nervous System) FROM LARVAE:

a. Third instar GFP positive larvae of the cross (w; UAS dicer; repoGal4MCD8GFP/TM6 X UAS Hth RNAi/UAS Hth RNAi; MKRS/TM6) were selected
b. It was inverted like a pocket then the posterior part was thrown away which is of no interest to us and anterior part was taken to collect the whole CNS from the larva

4. PLATE PREPARATION FOR COLLECTING EMBRYOS:

For collecting drosophila embryos, simple agar plates were prepared (3% agar and 60mm plates) and yeast paste was also prepared (7gm in 2ml dH2O and kept at 4*C) and put in the middle of the agar plates. The flies were put in a bottle and kept upside down in the plates and allowed to lay eggs. Then, the embryos were collected and followed by the fixation protocol

5. STAGING OF EMBRYOS:

Embryos have been staged for both control (w; UAS dicer; repoGal4 UASmCD8 GFP/TM6) as well as the cross (w; UAS dicer; repoGal4 UASmCD8 GFP/TM6 X ywhsflp; UAS HthRNAi/UAS HthRNAi; MKRS/TM6). Plates has been kept for laying eggs and then taken away after 5 hours and processed the next day because CNS (Central nervous system) in the embryos gets developed in 11-12 hours. This process has been done to get the consistency of results.

6. FIXATION:

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Embryo fixation:

a. Embryos were collected from fly cross on juice-agar plates.

b. Little water onto the plate was squirted, and paintbrush was used to dislodge the embryos from the agar. The embryos were suspended into the water.

c. Water/embryo mixture was poured into an embryo collection basket. Yeast chunks were placed into the embryo basket. Multiple rinses were given to get all of the embryos.

d. The squirt bottle was used to rinse the embryos (in the basket) well and also to dissolve any yeast chunks in the basket.

e. The basket was placed into a petri dish filled with 2:1 bleach.

f. The embryos were transferred into fixative: heptane (1:1 ratio)

g. The embryos were fixed for 20-25 mins, shaken vigorously and placed on a rotator. The bottom layer was removed and methanol was added.

h. The embryos were transferred into methanol: heptane and shaken vigorously.

i. The tube was capped and vortexed for 2' minutes on full. This step dechorionated the embryos, which allowed them to sink to the bottom of the methanol. The tube was examined. There were a number of embryos at the bottom of the tube, and some embryos trapped at the methanol: heptane interface. The embryos at the bottom are properly dechorionated- these are what we want to keep.

j. The methanol: heptane liquid was removed, taking care to not remove the embryos at the bottom. This methanol: heptane waste was put in the"Methanol: Heptane" waste bottle and 1.6 mL of fresh methanol was added to the tube.

k. The tube was stored in the -20℃ freezer.

Larval fixation:

a. Larvae were collected from the food and inverted like a pocket

b. The inverted larvae were fixed using 4% PFA and kept in a rotator for 20 mins

c. They were rinsed using ringers solution (3times)

7. IMMUNOSTAINING:

a. The embryos/larvae were firstly rinsed and then washed with PBST (2 times, 20 min each)

b. Then 400 µL PBST + 5% serum was added to the embryos and kept for 30 mins in a rotator.

c. Primary 1* antibody (Repo+Elav) was put on the eppendorf with embryos along with 400 µL PBST and serum and kept at 4*C overnight.

d. They were again washed 4X over 1 hr with PBST and washed in PBST and serum for 30 mins.

e. They were then incubated with 2* antibody (Cy3+Cy5) and kept at RT for 2hrs.

f. They were again washed 4X over 1 hr with PBST.g. They were mounted on the slides.

h. Samples from the cross as well as control were compared.

8. MOUNTING AND CONFOCAL IMAGING:

Larvae CNS were finally dissected and mounted on a slide using vecta shield and beads and then confocal images of larvae were taken at different zoom. Embryos were directly mounted on slide using 90% glycerol and a cover slip was put and observed under confocal microscope.

III. RESULTS

Here we study the role of Hth in Drosophila CNS development using UAS Hth RNAi and glia specific Gal4 line- repo Gal4 mCD8GFP, in which glial cell bodies are labelled with GFP. We planned to study the effect at 3 different stages of Drosophila development that is : embryonic stage, third instar larval stage and adult stage.

The crosses of (yw hs flp; UAS Hth RNAi/Cyo; MKRS/TM6) X (w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6) were made and kept till the adult flies emerge to check their phenotype.

They were checked every day; no flies emerged from the pupal case after 10 days, hence pupal lethality was confirmed.

EVALUATION OF LARVAL PHENOTYPE:

We looked at the Central nervous system development in the larvae upon Homothorax knockdown ((yw hs flp; UAS Hth RNAi/Cyo; MKRS/TM6) X (w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6). Along with these experiments we also used control wild type larvae with genotype: w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6. The markers used for this experiment is repo which is a glial specific marker particularly used for staining the glial cells and elav, a neuro specific marker which particularly stains the neurons. We got the confocal images of Central nervous system and brain lobes of (w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6) third instars wild type larvae. We could see the normal phenotype of Central nervous system with two brain lobes and one ventral cord intact stained with antibody (Primary: repo and Elav) (Secondary: Cy3 and Cy5) as shown in figure (8.1a,b,c,d)&(8.2a,b,c,d) respectively. We also observed the images of the whole Central nervous system and brain lobes from the mutant flies (yw hs flp; UAS HthRNAi/Hth RNAi; MKRS/TM6 X w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6) which lead to a different phenotype (wedge shaped) of the central nervous system unlike the normal central nervous system as shown in the figure (8.1e,f,g,h)&(8.2e,f,g,h) respectively. To address the specificity of RNAi, we did rescue experiments using UAS Myc-Meis of human origin to check whether by reintroducing

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"Meis" which is a human homologue of homothorax, it rescues the HthRNAi phenotype of the central nervous system. We could find out that "Meis" rescues the HthRNAi phenotype as shown in figure (8.3a,b,c,d). We also observed the brain lobes as shown in figure (8.3e,f,g,h) This rescue suggests that RNAi is very specific to Hth and there are no off target effects. Hence, Hth/Meis function is conserved

EVALUATION OF EMBRYONIC PHENOTYPE:

We looked at the Central nervous system development in the embryos upon Homothorax knockdown in the wild type (w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6) as well as mutant ((yw hs flp; UAS Hth RNAi/Cyo; MKRS/TM6) X (w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6). The marker used for this experiment is elav, a neuro specific marker which particularly stains the neurons. We got the confocal images of w; UAS Dicer; repoGal4mCD8GFP/TM6, the wild type embryos by antibody staining (Primary: mouse Elav) (Secondary: anti mouse Cy3) to check the central nervous system development. This showed separate brain lobes from the ventral cord. We also observed the images of the mutant embryos (yw hs flp; UAS HthRNAi/Hth RNAi; MKRS/TM6 X w; UAS Dicer; repo Gal4 UASmCD8GFP/TM6) with antibody staining (Primary: mouse Elav) (Secondary: anti mouse Cy3). This experiment lead to a different phenotype of the central nervous system i.e. all part of the brain lobes and ventral cord are fused together, unlike the normal one just the same as we observed in the larval central nervous system development.

IV. DISCUSSION

Earlier studies established that Hth along with Yki regulates epithelia tissue growth. Previous work from our laboratory showed that glia specific knock down of Homothorax does not reduce the glial cell number. However, in the present work we notice that the mutant larvae displayed wedge shaped CNS phenotype with extended optic lobes. In wild type larvae, CNS has two brain lobes that are distinctly away from ventral nerve cord (Fig:8a). In contrast to wild type phenotype, the mutant larval showed wedge shaped CNS with two brain lobes fused to ventral nerve cord (Fig:8b). Upon close examination we found that mutant CNS has extended optic lobes that fused to ventral nerve cord leading to wedge shaped phenotype. Further, we also studied the effect of Hth RNAi in embryos. Similar phenotypes as that of larvae were observed in mutant embryos. In addition to that, we also noticed that Hth RNAi mutant pupae never enclosed into adults. Further, both to confirm the specificity of RNAi and rule out the off target effects of RNAi, we performed rescue experiments using mammalian homologue of Homothorax i.e. UAS Myc Meis. Glia specific expression of UAS Myc Meis completely rescued the Hth RNAi phenotype to normal. This experiment allowed us to study the functional conservation of Homothorax. Future it would be very interesting to study and characterize these phenotypes at both cellular and molecular level.

V. CONCLUSION

From these studies, we conclude that though we see a wedge shaped phenotype of the Central nervous system in mutant larvae upon Homothorax knockdown, we could not see any changes in glial cell number. Further, both to confirm the specificity of RNAi and rule out the off target effects of RNAi, we performed rescue experiments using mammalian homologue of Homothorax. Expression of UAS Myc Meis completely rescued the Hth RNAi phenotype to normal. Hence, Hth/Meis function is conserved and therefore, can be used to study mammalian cells. It would further be interesting to study and characterize these phenotypes at both cellular and molecular level.

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