Disruption of the Pp1-87B Gene Stimulates Tumor Formation in the Eye of Drosophila

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Authors' contributions

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ABSTRACT

Protein phosphatases are a set of enzymes in charge of the dephosphorylation of several proteins and enzymes in a cell. Dephosphorylation process is essential for organizing a huge number of cellular actions. In Drosophila, protein phosphatase 1 at B87 (Pp1-87B) gene encodes one of the four variants of the protein phosphatase 1 catalytic subunit and located on chromosome number three of *Drosophila melanogaster*. In this proposal, Pp1-87B mutation carrying lethal P-element insertions at a third chromosome of *Drosophila melanogaster* was screened to study the possible of this gene as tumor suppressor gene. Disruption of the genetic sequence of Pp1-87B gene can produce mutant phenotypes in the large clone mosaic eyes. The mutant eyes have either a rough or cell lethal phenotype which indicates the disrupted gene is essential for proper eye development. Further analysis of the mechanism by which these disrupted gene function may offer useful information for cancer studies. To study if the obtained set of Drosophila P-element mutations have

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a tumorigenic activity or not, a set of somatic clonal analysis in the whole body or in eye system was used. Thus, the lethal mutations were screened in some mosaic assays using clonal analysis systems. Our results showed that the studied gene may have a significant role in eye development, cell proliferation and could be involved in photoreceptor cell patterning, as well as in ommatidial differentiation and apoptosis. This gene which was determined in the present study could have a real impact on cancer development.

1. INTRODUCTION

Cancer is considered one of the major health problems in the world that need a lot of effort and cost-effective for healing or at least to prevent most of its health adverse consequences. The understanding of mechanisms and genetic pathways which lead to cancer formation would be a useful way to develop new strategies for cancer prevention, treatments and developing new targeted drugs for specific cancers. Proto-oncogenes and tumor suppressor genes are two major classes of genes, which may be required in tumorigenesis [1].

Proto-oncogenes are genes that control cell division positively, but that is altered by mutation and turn into oncogenes [1]. Tumor suppressor genes are negative regulators of cell growth and/or proliferation so that one copy of wild-type gene is enough to exert its functions when both alleles of a given tumor suppressor gene are mutated, loss of this negative control forms a critical step toward tumor progression [2]. Fall under this category of genes, protein phosphatases which regulate most of the cellular functions and therefore might also be predicted to play key roles in the control of many growth processes. Four main protein serine/threonine phosphatase catalytic subunits namely protein phosphatase 1, protein phosphatase 2A, protein phosphatase 2B and protein phosphatase 2C have been identified in eukaryotic cells [3,4]. Moreover, the protein phosphatase 1 (PP1) found to be considerably lower in some human malignancy cells and in human, PP1 reacts with breast cancer susceptibility protein BRCA1 [5]. In the same sense, the okadaic acid which acts as PP1 inhibitor has been informed to work as a tumor promoter and can increase moving and incursion of non-metastatic LLC-C8 cells, signifying that loss of PP1 may result in tumor formation and metastasis [6]. Nevertheless, PP1 regulatory subunits can give a vital component to understand the job of PP1 in tumor development and metastasis.

The use of Drosophila in cancer research has led to the development of many useful strategies and systems that help in the determination of target genes involved in the specific pathways as well as the functions of these genes. In addition, the imaginal disk cells of Drosophila have a cell cycle that is very similar to that of mammalian cells. Data about factors inducing tumors in these cells may directly relate to the risk of these factors for inducing cancer in humans. The *Drosophila melanogaster* eye is a great form system for research of the developmental methods at the cellular and subcellular levels. The eye of Drosophila composed of hundreds of unit eyes that known as ommatidia. Each ommatidium comprises a number of photoreceptor neurons (R cells) and a regular arrangement of non-neuronal accessory cells. R-cellular development starts inside the third-instar larval eye disc and is finished via the end of the third-instar larval stage [7]. Imaginal disks of Drosophila are a good framework to study the impacts of several genetic differences between groups of discrete cells closely related to wild-type neighboring cells, which nearly look like the clonal character of human cancer [6].

Our work aims to examine, for the first time the potential functions of the Drosophila Pp1-87B gene during Drosophila eye development using many innovative genetic experiments. To study if the obtained set of Drosophila P-element mutations have a tumorigenic activity or not, a set of somatic clonal analysis in the whole body or in eye system was used. So, the lethal mutations were screened in some mosaic assays using clonal analysis systems. Clonal analysis (mosaic analysis) refers to the production of genetically homozygous mutant cells in an otherwise heterozygous background and it is very useful in studying lethal mutations especially for later gene functions [8]. Moreover, it has been used successfully to find several novel tumor suppressors in fruit flies, which regulate cell proliferation, cell size and apoptosis [9,10,11,12,13]. FLP/FRT system is designed to

increase the frequency of mitotic recombination in Drosophila. The mosaic techniques using targeted DNA recombination at flippase recombination targets (FRTs) can be driven in flies by the FLP recombinase (flippase) is very efficient and gave a high frequency of somatic clones [14,15].

2. MATERIALS AND METHODS

2.1 Fly Stocks

Standard Drosophila stock maintenance and genetics procedures were followed throughout. Drosophila stocks were maintained on a standard cornmeal and yeast diet at 25°C unless otherwise stated.

Pp1-87B (CG5650) was obtained from the Drosophila Genetic Resource Center (DGRC), Kyoto Stock Center. Stocks were used in the clonal analysis include: (Bloomington stock number in parentheses). Pp1-87B was used for Lethal Phase Analysis; Pp1-87B (Pp1-87B) stock was maintained over *TM6, Tb*, balancers. Stocks were used in the clonal analysis for the induction of somatic closes with the hsFLP FLP/FRT system were obtained from the Bloomington Stock Center (Bloomington, Indiana, USA). The Drosophila stock y[d2] w[1118] P{ry[+t7.2]=ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1; P{ry[+t7.2]=neoFRT}82B was used for induction of small mitotic clones with eyFLP FLP/FRT system. The Drosophila stock (5620) y[d2] w[1118] P{ry[+t7.2]=ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1; P{ry[+t7.2]=neoFRT}82B P{w[+t*] ry[+t*]=white-un1}90E l(3)cl-R3[1]/TM6B, P{y[+t7.7] ry[+t7.2]=Car20y}TPN1, Tb[1] was used for induction of large mitotic clones with eyFLP FLP/FRT system. The Drosophila stock y[1] w[*]; P{w[+m*]=GAL4-ey.H}3-8, P{w[+mC]=UAS-FLP1.D}JD1; P{ry[+t7.2]=neoFRT}82B P{w[+mC]=GMR-hid}SS4, l(3)CL-R[1]/TM2 was used for (EGUF)/*hid* method [16]. All of these stocks were described at FlyBase and obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio. indiana.edu).

2.2 Lethal Phase Determination and Phenotype Analysis

To identify the lethal phase of homozygous mutant Pp1-87B P. element insertion line was balanced over *TM6, Tb.* Flies could lay eggs on apple-juice/agar/yeast plates for 8 h at 25°C. Synchronously developing first instar larvae were hand-collected in groups of 50 animals and placed on standard cornmeal media in Petri dishes. Monitoring the development of the progeny for observing the appearance of homozygotes over the viable developmental stages (egg, larval, pupal and adult). Homozygous mutants were identified by their wild-type body length, in contrast to heterozygotes which have tubby (*Tb*) phenotype. Tracheal observations were made in live animals or heat-killed (by placing larvae on a coverslip and placing it on a 95°C block a few seconds until they stopped moving). The lethality of the homozygous mutants and the time were recorded.

2.3 Clonal Analysis

Loss of function clones was generated using the FLP/FRT system of mitotic recombination [17,18]. Both FLP and FRT sequences were reconstructed in Drosophila in different stocks using Drosophila transformation technique.

2.3.1 Heat shock FLP/FRT somatic clone system

Thirty males of Pp1-87B were crossed to sixty suitable virgin females of the tester strain y w hsFLP; P [ry+; hs-neo; FRT] 82B P{lacW} on separated standard medium bottles. F1 Larvae at 48 - 56 h after egg hatching exposed to heat shock treatment by incubation on 37°C circulating water bath for 60 minutes on two successive days. After each heat shock treatment, F1 larvae were transferred to standard media until adults emerging.

2.3.2 Mitotic clonal analysis induction by ey-FLP/FRT system

To generate clones of homozygous lethal tissue in the eye, the ey-FLP/FRT system was used. The Drosophila stock (5620, 5619 and 5253) with the ey-FLP gene inserted in the eye-specific gene on X-chromosome and FRT82B inserted near the centromere of the 3R chromosome was integrated into the crossing scheme to generate a site-specific mitotic recombination in the eye. The ey-FLP gene encodes the Flipase enzyme that induces mitotic recombination at the identical positions of FRT sites on homologous chromosomes after DNA replication. This site-specific recombination event creates the chromosome 3R homozygous for the P-element insertion only in the eye which is screened for a mutant phenotype [19]. Approximately 20 male flies from the mutant of Pp1-87B which contain

FRT82B were crossed with 30 females of stock (5620, 5619 and 5253). This cross was made to induce meiotic recombination which only occurs in the gametes of female progeny and to generate the mosaic eye pattern which is due to the FRT site-specific mitotic recombination.

2.4 Ultrastructure of Somatic Eye Clones

2.4.1 Scanning electron microscopic (SEM)

Scanning EM was performed as described previously [20]. In brief, the eyes were fixed and dehydrated through a graded ethanol series (25, 50, 75, and 100%). The samples were then incubated in hexamethyldisilazane (Sigma-Aldrich), dried under vacuum overnight, mounted on stubs, and imaged with a scanning electron microscope (model SS40; International Scientific Instruments).

2.4.2 Transmission electron microscopy (TEM)

Specimen tissues were prepared for TEM according to the procedure [20]. Section experiments were done by the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. Sections were made of adult eye Pp1-87B mutant. Glass knives for sectioning were prepared immediately before use in order to ensure cleanliness and sharpness. Glass strips were cut into 25-mm squares and then each square cut near diagonally to give two glass knives. The plastic tape was used to form a "boat" at the edge of the glass knife, and then the tape sealed to the glass with dental wax.

Semi-thin sections (1 μm in thickness) were cut using glass knives and the sections floated on water surface were picked up using eyelash and placed on a drop of water on a glass slide. The slide was then heated gently to get rid of the water drop and the sections adhered to the slide. A drop of toluidine blue stain was placed on the sections for 90 sec. then washed with distilled water and the sections were examined under light microscope.

2.5 The Imaginal Disc System

Mutant line larvae were placed into an ice bucket to anesthetize the larvae. Larvae were dissected using fine biology 5 forceps in Ringer's solution onto depression slide, by holding the larvae end and pulling the mouth parts in the opposite direction. The posterior portion of the larva was discarded. The imaginal discs are observed which should look like a bunch of grapes attached to the central nervous system. The eye-antennal disc is characterized by its shape and attached to the brain lobes. The unwanted tissues were discarded, and the eye-antennal discs were transferred to clean and dark microscopic slide and defects of eye imaginal discs are examined under phase contrast and magnification power ranging from 100X to 400X.

2.6 Apoptosis Assay Using Acridine Orange Technique

Eye imaginal discs were dissected from late third instar larvae in Ringer's solution (as described before). Discs were incubated in 1.6×10^{-6}M acridine orange (Aldrich) in Ringer's solution for 3 min. Collected discs were washed three times briefly in Ringer's solution. The discs were mounted on slide glasses with Ringer's solution and view immediately under a fluorescence microscope using the green filter (522 nm) which gives the best sensitivity under magnification power 400X according to [21].

3. RESULTS AND DISCUSSION

3.1 Lethal Phase Determination and Phenotype Analysis

Lethal phase and lethality phenotypes of Pp1-87B gene during Drosophila development. As shown in Fig. (1 A-E). (A) wild-type larva (control). (B) Heterozygous larvae for P. element insertion mutants/TM6.Tb. (C) and magnification in (D), show homozygous Pp1-87B mutant died in third larval stage with a melanotic tumor on a body (black arrows). (E) melanotic tumor on the body.

3.2 Clonal Analysis

3.2.1 FLP/FRT heat shock system

Appropriate cell number and organ size in a multicellular organism was determined by coordinated cell growth, proliferation, and apoptosis. Disruption of these processes can cause cancer. Tumor phenotypes of Pp1-87B gene in a somatic clone were induced using FLP/FRT heat shock system are shown in Fig. (2). Some flies exhibited an undifferentiated mass of wing (B) in addition, deformed and a melanotic tumor on the legs (C) and (D). The adult eye was severely disorganized to give rough eye with a tumor.
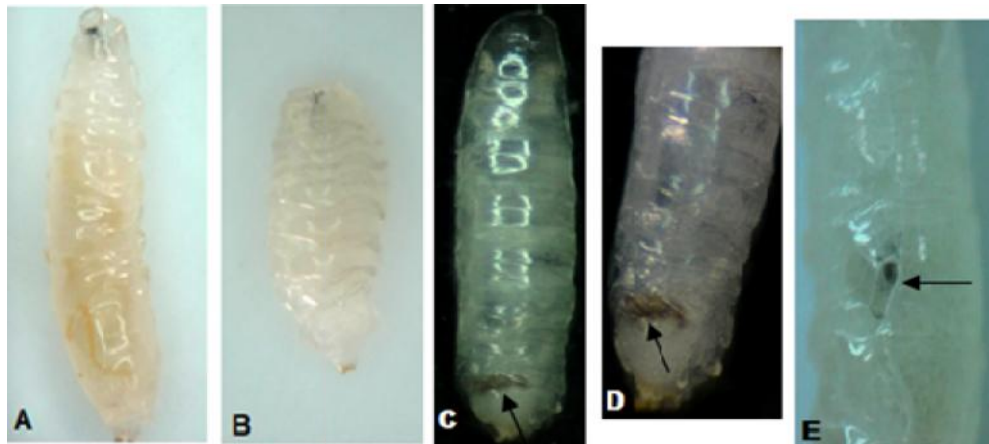
Fig. 1. Lethal phase and lethality phenotypes of Pp1-87B gene during *Drosophila* development. (A) wild type larva (control). (B) Heterozygous larvae for P. element insertion mutants/TM6.Tb. (C) and magnification in (D), show homozygous Pp1-87B mutant died in third larval stage with melanotic tumor on body (black arrows). (E) melanotic tumor on the body
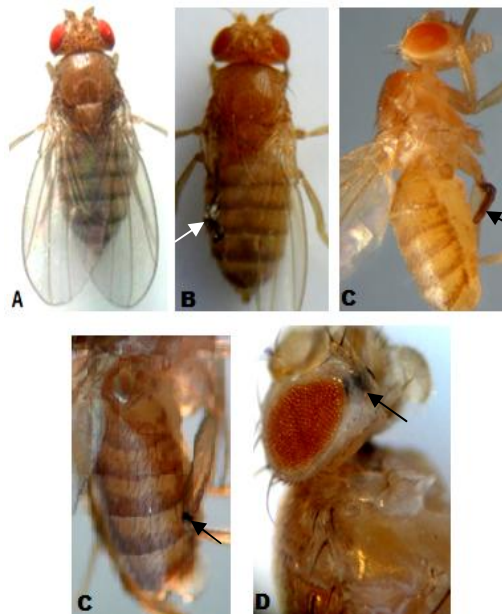

Fig. 2. Induced homozygous somatic clone phenotypes of P-element recessive lethal insertion mutations on the right arm of the third chromosome (82B), after heat shock treatment of heterozygous larvae using FLP/FRT system: homozygous mutation clones showed either one or several phenotypes as following: (A) wild-type fly. (B) Deformed wing and tumor. (C) Show melanotic tumor on the leg of third pair. (D) Melanotic tumor on head and reduced eye

3.2.2 Pp1-87B rescues the eye small-eye phenotype

The ey-Flp/FRT system [18] was used to produce Pp1-87B gene homozygous mutant clones by site-specific mitotic recombination in the developing visual system and examined induced eye clone phenotypes due to homozygous loss of this gene. Since the entire homozygous fly resides on recessive lethal chromosomes, clones of cells homozygous for these mutations were produced by mitotic recombination. To investigate whether Pp1-87B gene could play a role in the developing visual system, mosaic animals were generated using clonal analysis in the eye because of adult

Homozygous wild-type (white)

Homozygous mutant (dark orange)

Non-recombinant (light orange)
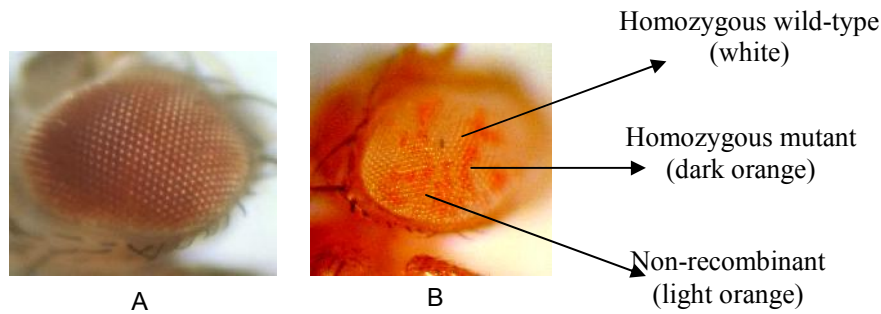
A   B

Fig. 3. Eye phenotype of small clone for P-element insertion lines. Bright field microscopy images of the adult eyes of the original heterozygous insertion lines are shown in (A) Initial Stock, and (B) The mosaic-eyed flies (eye color variegation) produced by the small clone, which are white, dark orange, and, light orange respectively

lethality in pre-stages. Homozygous mutation of Pp1-87B gene results in a mosaic phenotype in the Small Somatic Clones of the Adult Drosophila Eye (above Fig. 3 A and B). The mutant ommatidia contained three different cell types of Pp1-87B +/+, Pp1-87B +/-, or Pp1-87B -/- cell types. The different cell types could be recognized by eye color (white, light orange, and orange, respectively), which corresponded to an increased copy number of the mini-white gene in the insertions.

These results agreed with [18] when generated mosaics using ey FLP. Flies are a mosaic of pigmented (heterozygous or homozygous for the w+ marker) and unpigmented (lacking the marker) tissue. Also, agreed with [22] who used FLP-mediated mitotic recombination to render many cells in the developing eye. Mosaic eye in a *boiC1* heterozygous and homozygous cells are pigmented orange, while the dark red patches mark ommatidia was in *ihogDC1* heterozygous cells.

3.3 Large Clone

To increase the size of clones of homozygous lethal tissues in the eye, the Minute mutation was merged into the ey-FLP/FRT method. The Minute mutation prevents cell development when heterozygous but is recessive lethal. This would give an advantage for the homozygous clone to be larger. Thus, in the twin spot, cells that are homozygous for the Minute mutation perish, and cells that are heterozygous for the Minute mutation develop tardily. Thus, enormous clones of homozygous P-element mutations can be produced. The eye phenotypes of large clones were examined to verify whether the mutated gene was crucial for the eye growth [18]. The reduction in eye size and black tumors (black arrows) with undifferentiated mass white tumors (blue arrows) and mosaic/rough eye (red arrow) with irregular shape eye, were observed illustrated in (Fig. 4 A-C).

Fig. 4. Induced large homozygous clones of P-element recessive lethal insertion mutations on the right arm of the third chromosome (82B) in *Drosophila* eyes using ey-FLP/FRT system: (A) wild-type eye. (B) and (C) Pp1-87B mutant: reduced in eye size and black tumors (black arrows) with undifferentiated mass white tumors (blue arrows) and mosaic/rough eye (red arrow) with irregular shape eye

3.4 Specific Eye Clones Using EGUF-GMR Hid Analysis

In this system used GAL4/UAS with FLP/FRT construct. The GAL4 protein with increases of flippase enzyme which lead to high mitotic recombination, tumors formation in the eye and different phenotypes on the eyes such as reduction, outgrowth, apoptosis and so on. However, same results were obtained using EUGF system but with stronger phenotypes due to increasing the frequencies of mitotic recombination as shown in Fig. (5 A-C). Strongly reduced eye size with outgrowth tumor with white tumor (circles), irregular reduced eye phenotype with glossy (black arrows). The sizes and the shapes of this mutant cells were very irregular. Mitotic recombination clones of these genes indicated that these genes might play rules during eye development, in cell proliferation and could be required for photoreceptor cell determination patterning, as well as in ommatidial differentiation.

3.5 Scanning Electron Micrographs (SEM) of Eye Tumors

Scanning electron microscopy was used to examine the phenotypes of mutant eyes with clones. The Pp1-87B mutant is able to generate tumors in various parts of the eye Fig. (6 A- F), these tumors were generated using (FLP/FRT) system. (A) wild-type eye and its magnification in (B) shows an organized ommatidial architecture with a hexagonal shape and typical orientation pattern of bristles, (C) shows irregular eye in shape and size. (D) Shows smaller eye with tumor clone (red arrow). (F) Magnification of (E) show missing parts of ommatidia structure and irregular bristles form, with some patches,

appeared smooth in texture, lacking ommatidia (red circle).

The results of transmission electron micrographs as shown in Fig. 7, agreed with [23], who found that the Bifocal (*bif*) is a putative gene controlling PP1, which act as interacting protein, that mediates normal photoreceptor morphology in Drosophila. Further, the same authors stated that the tasks of *bif* and PP1-87B in either stabilizing R-cell morphology (for *bif*) or controlling the cell cycle (for PP1-87B) can be detached from their job in visual axon object. Nevertheless, the axon objecting phenotypes are observed in both PP1-87B mutants and PP1-87B overexpression studies, suggesting that an optimal PP1 activity may be required for normal axon targeting.

3.6 The Pp1-87B Phenotype Results from Effects on Imaginal Discs

The imaginal discs of Drosophila are the larval precursors of the adult organs such as eyes, wings, legs, etc., provide a convenient experimental model for the process of pattern formation in development. The imaginal discs have double epithelial layers which are determined during zygotic development. As the disc cells proliferate in normal development, a complex spatial pattern of cell specification is somehow elaborated by the end of the larval stage [24]. In addition, [25] mentioned that cells within the imaginal discs are very similar in terms of cell cycle regulation to mammalian cells making them a good model. The imaginal discs of the Drosophila larva have quite a while ago acted as an excellent example to comprehend how to control organ size. The size of the imaginal disc at the initiation of pupation is a prime determinant of the size of the grown-up
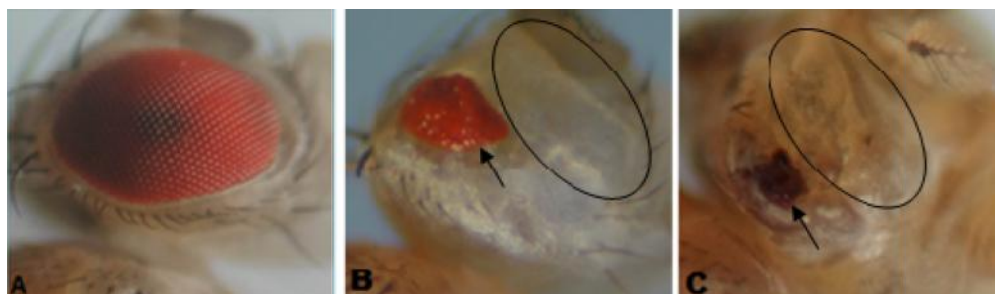

Fig. 5. Induced large homozygous clones of P-element recessive lethal insertion mutations on the right arm of the third chromosome (82B) in *Drosophila* eyes using *EGUF-GMR hid* system: (A) wild type eye. (B) and (C) Pp1-87B mutant: Strongly reduced eye size with outgrowth tumor with white tumor (circles), irregular reduced eye phenotype with glossy (black arrows)
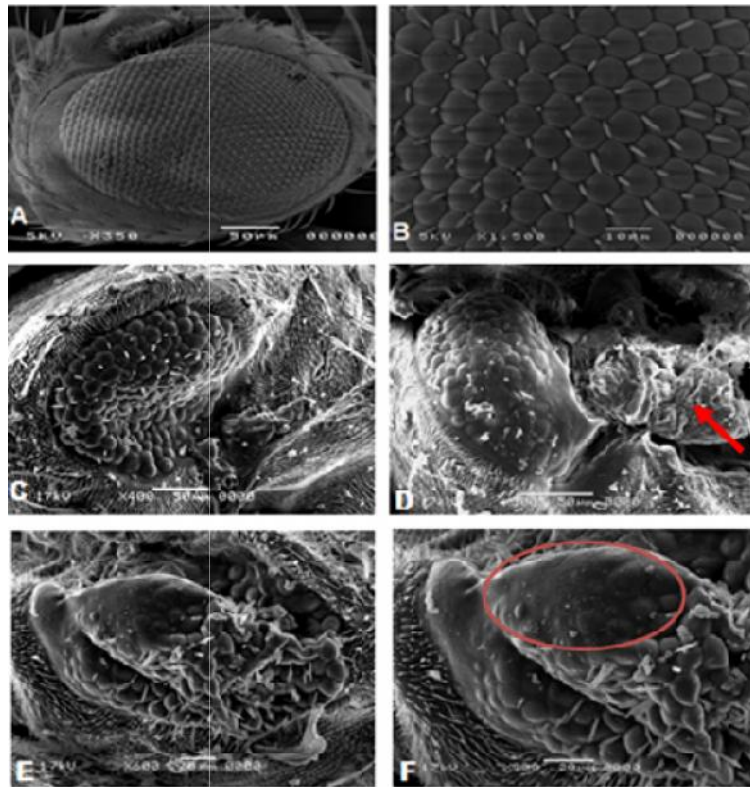
Fig. 6. Scanning electron micrographs of wild-type *Drosophila* eye comparing with induced large homozygous eye clones of tested P. element mutations (Pp1-87B) consulting eye FLP/FRT system. (A) wild-type eye and its magnification in B shows an organized ommatidial architecture with hexagonal shape and typical orientation pattern of bristles, (C) shows irregular eye in shape and size. (D) Shows smaller eye with tumor clone (red arrow). (F) Magnification of (E) show missing parts of ommatidia structure and irregular bristles form, with some patches appeared smooth in texture, lacking ommatidia (red circle)

organ after metamorphosis. Nonetheless, there is a correlation between the size of the organ and its physiological functions. Hence, it is imperative to have developmental controls to achieve an adequate growth but not extreme of the imaginal discs [26].

Eye-antennal imaginal disc mosaics can be obtained utilizing the ey-FLP/FRT technique, in which mitotic recombination is occurred in the eye imaginal disc by placing the FLP recombinase gene under the driving of the *eyeless* enhancer [18]. These methodologies, especially that have surveyed overabundance or more fast development of mutant tissue in the resultant grown-up eyes, have effectively recognized a large number of the tumor suppressor genes [27].

In eye-antennal disc, the antennal portion of the disc is on the top and the eye portion is on the bottom (Fig. 8A). Eye discs mutant for PP1-87B showed the reducing in the eye part of the disc and abnormal folding (white arrow) (B). Abnormal in shape and size with abnormal eye portion and folding (C) and (D).

3.7 Detection of Cell Death (Apoptosis) Using Acridine Orange

To assess cell death (apoptosis) of Pp1-87B gene we stained eye-antennal imaginal disc of late third instar with the vital dye Acridine orange [28]. Acridine orange staining that highlights dead cells. Eye discs were dissected from FLP-FRT system. A low level of cell death is normally observed in wild-type eye discs, mainly in the region just posterior lateral fold (Fig. 9A). In contrast, Pp1-87B in late third instar larvae an increased number of dying cells is detected posterior to the morphogenetic furrow in the mutant disc (white arrows) (B) and (C).
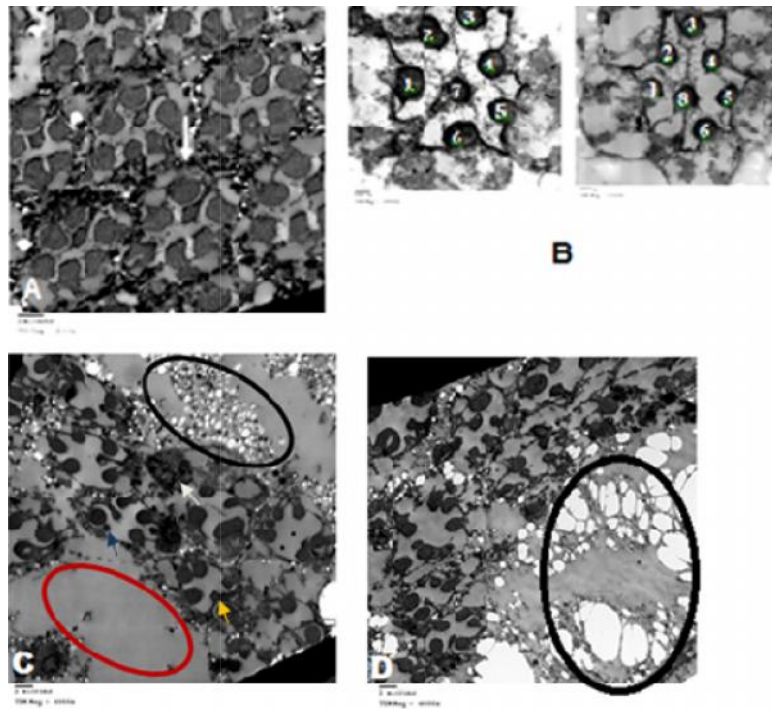
Fig. 7. Transmission electron micrographs of wild-type *Drosophila* eye compared with induced large homozygous eye clones of tested P. element mutations Pp1-87B consulting eye FLP/FRT system. (A and magnify in B) wild-type eye section, shows ommatidia is precisely organized into hexagonal shape by interommatidial cells (white arrow) and ommatidial rows, each ommatidia contains seven visible R-cells. (C) Gaps with absence of all photoreceptors (red circle) with necrotic (white arrow) with abnormal and decreased size of R7 (orange arrow) with fused R5/R6 with lack of R7 (white arrow). (D) Shows increase of phagosomes (black circle). Scale bars, (A) 6000X; (B) 20000X; (C) and (D) 4000X
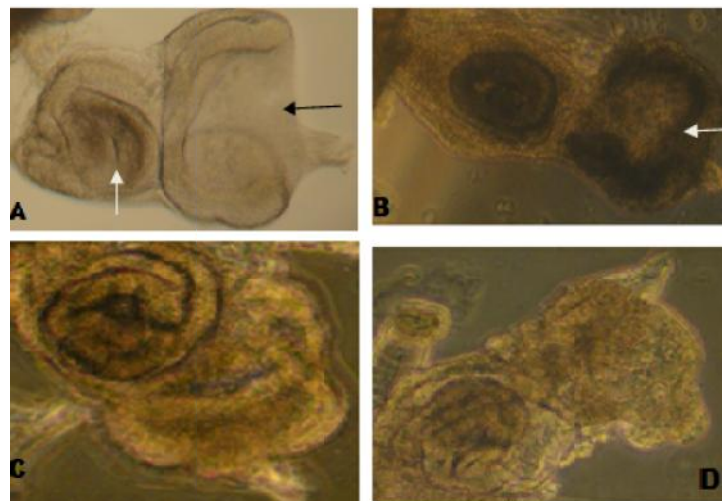

Fig. 8. Eye-antennal discs phenotypes of Pp1-87B using the ey-FLP/FRT system. (A) wild-type eye-antennal imaginal discs, shows Antenna precursor (white arrow) and eye precursor (Black arrow). (B) Reduced in the eye part of disc and abnormal folding (white arrow). (C) and (D) abnormal in shape and size with abnormal eye portion and folding
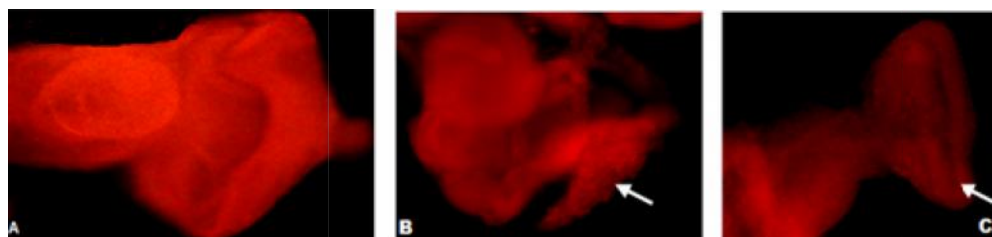
Fig. 9. Detection of cell death in eye-antennal imaginal discs of Pp1-87B in late third instar larvae. Eye discs were dissected from ey-FLP/FRT system. (A) In wild-type, no dead cells. (B) and (C) An increased number of dying cells is detected posterior to the morphogenetic furrow in the mutant disc (white arrows)

In Drosophila, apoptosis is induced by three genes, *reaper*, *grim* and *head involution defective* (*hid*), located in the 75C1-C2 region of the Drosophila third chromosome [29]. *Jak/STAT* signaling is known to promote proliferation during eye development, and is required for the morphogenetic furrow (MF) initiation; a loss of *Jak/STAT* function results in reduced eyes [30]. Thus, *Jak/STAT* signaling plays positive roles in eye development. Further, cell-cycle kinases exemplify a group of catalysts administering the cell division cycle [31]. In the same sense, in Drosophila, Jun N-terminal kinase (JNK), which can be inactivated by PP1, is involved in cell death mechanisms and regulates epithelial morphogenesis [32,6].

PP1 forms a noteworthy class and is profoundly preserved among all eukaryotes inspected to date. PP1 is included in the control of numerous cellular functions, including glycogen metabolism, muscle contraction, and mitosis [33]. In Drosophila, four genes code for the catalytic subunit of PP1 (PP1c), three of which belong to the PP1a subtype. PP1b9C (*flapwing*) encodes the fourth PP1c gene and has a specific and nonredundant function as a non-muscle myosin phosphatase. PP1a87B is the major form and contributes 80% of the total PP1 activity. The PP1 activity is required for mitotic progression and that the other loci cannot supply sufficient activity to complement the loss of expression of the PP1-87B gene. Alternatively, the PP1-87B product may have a distinct specialized function in mitosis [34].

4. CONCLUSION

In conclusion we hope that our study of PP1-87B gene in Drosophila will provide a new vision for understanding the mechanisms and genetic pathways which lead to cancer formation and would add a valuable approach to develop new strategies for cancer prevention, treatments and developing new targeted drugs for specific cancers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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