

Small Substitution in *p¹* of *Drosophila melanogaster* results in Altered Protein Trafficking to Lysosome Related Organelles and Eye Pigmentation Mutants.

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Abstract

The development of eye pigmentation in *Drosophila melanogaster* is caused by the depositing of red and brown pigment precursor to pigment granules. Eye pigmentation mutants' studies have shown a decrease in protein trafficking that alter the biogenesis of lysosome related organelles (LROs). Similar defects in LROs is observed in melanosomes of individuals with Hermansky-Pudlak syndrome (HPS). *Drosophila melanogaster pink* is an orthologue of human *Hermansky- Pudlak syndrome 5(hps5)* gene that causes a decrease of eye pigmentation due to defects in the production of LROs in pigment granules. My colleagues and I have isolated a gene *red blush(rbh)* that causes fruit flies to display a lighter red eye color without a pseudopupil that darkens with age. This study aims to analyze *rbh* through molecular and classical genetics. Through classical genetics, it was discovered that *rbh* is an autosomal recessive mutation located on 3-48 in *Drosophila melanogaster*. To determine if *rbh*, *rosy* and *pink* are mutations on different genes, a complementation test was performed. Results from complementation test indicates that *rbh* is *pink* gene. Using molecular genetics, we found that *pink* mutation is caused by a small substitution in the *p¹* allele. PCR is used to amplify the segment of DNA and analyze its molecular nature. Our PCR product was sequenced, aligned and compared to that of the NCBI database and found that our product is in the protein coding region of the *pink* gene. *pink* and *hsp5* protein show region of highly conserved region in the WD40 domain.

Introduction

Drosophila melanogaster, fruit fly, is a model organism for development, genetics and molecular biology experimental studies. *Drosophila* is often used to study embryonic development because biochemical pathways are highly conserved between species (Jennings, 2011). It has an easy to culture nature, relatively fast generational time and abundant number of offspring that allows for ample genetic research. Since the whole genome of fruit fly has been sequenced it is easy to screen for mutations that have given rise to a mutant phenotype. The small genome of *Drosophila* allows for easier genetic manipulation, and it is because of these many reason that *Drosophila* is a model organism (Robert et al., 2006).

Analysis of biochemical pathways of eye color mutants resulted in most of the knowledge of lysosome- related organelles, LROs (Falcon-Perez et al., 2006). Studies on biosynthetic pathways shows production of eye pigment occurs in several enzymatic steps (Lloyd et al., 1999). Eye pigmentation in *Drosophila* results from the depositing of red and brown pigment, drosopterins and ommochromes, respectively. The first mutation discovered for *Drosophila melanogaster* was the *white* gene which resulted in flies with white instead of red eyes. The *white* gene belongs to a set of ABC transporter family needed for brown and red precursors, guanine or tryptophan, in pigment granules. Other genes included in ABC transporter family of *Drosophila melanogaster* are encoded by *brown(bw)* and *scarlet(st)*. White protein subunit with brown and scarlet subunits forms two different ABC transporters, guanine and tryptophan transporters respectively. White protein with mutations in the cytoplasmic ATP binding domain and transmembrane domain intracellular loops causes a decrease in both guanine and tryptophan transport (Mackenzie et al., 1999). *white* allele interacts with other genes such as *garnet* to ensure that proper intracellular localization of White protein occurs(Lloyd et al., 1999). This shows that abnormal intracellular sorting of White protein can lead to a group of eye pigmentation mutants.

Another group of eye pigment mutants are the granule group that is involved in lysosome-related organelles such as *garnet(g)*. The *garnet* gene, present in all stages of development, causes distinct pigmentation in the eyes and other organs of *Drosophila* (Lloyd et al., 1999). *garnet* mutants result from a mutation in the gene that codes for a putative coat adaptor proteins that produces pigment granules(Ooi, 1997). Garnet protein resembles mammalian AP-3 adaptin complex of trans-Golgi network important for intracellular transporting and sorting of vesicles to lysosome specialized organelles such as pigment granules (Lloyd et al., 1999). Flies with *garnet* have brownish eyes that darken with age because of the reduction in pigment granules as a direct effect of protein delivery to granules. *garnet* gene, highly conserved and seen in developmental expression in vast amount of tissues, has an essential but redundant function needed by all cells because of its absence of other defects besides eye color pigmentation. *pink(p)*, a gene that is enhanced by the presence of *garnet(g)*, is a part of the granule group of eye pigmentation mutants.

Drosophila pink gene, an orthologue of human *hps5* gene, causes hypopigmentation due to the defects in lysosome- related organelles in melanocytes pigment granules (Syrzycka et al., 2017). *Hermansky- Pudlak syndrome(HPS)* is an autosomal recessive disorder that results in defected melanosomes which lead to excessive bleeding and albinism. There is at least 8 different HPS that encodes for one of four protein, AP-3, biogenesis of LROs complex(BLOC) 1, 2 and 3 involved in production of pigment granules(Falcon- Perez et al., 2006). Double mutants for *pink* and *garnet* gene have more reduced red eye pigment than either individual mutant (Falcon-Perez et al., 2006).

The *rosy* gene codes for Xanthine dehydrogenase, an enzyme that causes mutants to have a brownish colored eye. Eye color of *rosy(ry)* mutants have reduced amount of red pteridine pigments and normal levels of brown xanthommatin pigments compared to wild type. Adult flies with homozygotes deletion for *ry* gene have a deficient Xanthine dehydrogenase (XDH) that is synthesized in the fat body and transported to the eyes (Reaume et al., 1991). *Drosophila* XDH

is stored in peroxisome of cells and has peroxisomal targeting sequence (PTS) near the C-terminal end of the protein. XDH is transported to peroxisomes by vesicular transport or direct incorporation into the plasma membrane. *rosy* mutation can mimic human metabolic disorders that affect the peroxisome (Beard and Holtzman, 1987). Eye color pigmentation mutation such as *pink* and *garnet* can cause defects in the biogenesis or vesicular transport of LROs.

Eye pigmentation in *Drosophila* is a result of multiple enzymatic steps and can be involved directly and indirectly in the production of pigment granules. Some eye color mutation alters the vesicular transport of proteins to lysosomal related organelles such as the *garnet* and *pink* genes, members of the granule family. *Drosophila pink* alters the biogenesis of lysosome related organelles while *rosy* gene causes a deficiency in Xanthine dehydrogenase. In this study, *red blush* (*rbh*) is studied and analyzed using classical and molecular genetics. Chromosomal mode of inheritance, location, and using PCR DNA sequencing to discover possible DNA mutations.

Materials and Methods

To perform a series of genetic analysis, my colleagues and I maintained separate stocks of dominant markers, *red blush* (*rbh*), Oregon-R wildtypes, *rosy* (*ry*) and *pink* (*p*) flies. All flies were homozygous for their genes but dominant markers *Curly*, *Plum*, *Stubble*, *Dichaete* are placed on balancer chromosome to prevent recombination with genes on female fruit flies. On the second chromosome, double mutation *Curly* and *Plum* are on separate allele while *Dichaete* and *Stubble* double mutations are on the third chromosome. Genetic analysis with these flies is essential to identify *rbh* gene and function by determining mode of inheritance and chromosomal locations. Each strain of flies were maintained in multiple vials up to a period of one month. Each 28 X 95 mm clear polystyrenes vial contained fly food made of 4-24 blue instant cornmeal mixed with about 9 ml of water. Granules of yeast were placed on top of the fly food to prevent contamination. Flies were treated with several drops of water if fly food became dry or antibiotic mixture of

streptomycin and penicillin for bacteria contamination. Flies are housed in either 26-27°C or 18°C incubators. For each genetic cross virgin females were collected about 8-10hrs after enclosure in 26-27°C and about 12-16 hours at 18°C. Virgin females were stored in 18°C after collection and can be used to perform crosses for up to 12 days to ensure that fly culture is healthy. We periodically created subculture or brood vials to increase the amount of progeny. Flies from a vial are transferred to a fresh fly culture when offspring are in the 3rd larvae to early pupa phase. For scoring or manipulation of flies, flies are anesthetized with CO₂ at 5L/ min for no longer than 20 minutes.

Genetic crosses performed:

My colleagues and I, set up a series of complementation cross, discriminant cross 1 and 2(DC1 & DC2) to analyze our mutant gene. For marker DC1 cross, 6 virgin *rbh* females were crossed with at least 3 *Cy/Pm; D/Sb* marker males. 6 virgin *rbh* females were also crossed with at least 3 wildtype males for WT DC1 cross. F1 siblings from WT DC1 cross were crossed with each other to create WT F1 DC1 cross. A WT control cross with 6 virgin *rbh* females and at least 3 *Cy/Pm; D/Sb* males was performed. For marker DC2 cross, *Cy;Sb* DC1 F1 males were backcrossed with virgin *rbh* females. Complementation crosses were performed with 6 virgin *rbh* females with either *rosy(ry)* or *pink(p)* males. F1 progeny were scored for complementation which results in flies that appear wildtype. F1 progeny with pseudopupil and red eyes were scored as wildtype while flies lacking pseudopupil with a mutant eye color pigmentation were scored as mutants. For mapping cross 1(MC1), 6 homozygous *p* virgin females are crossed with double mutant *h,e* marker males to create heterozygotes *h/+*, *+/p*, *e/+* females. For mapping cross 2(MC2) 6 heterozygous *h/+*, *+/p*, *e/+* females were crossed with 4 *h, p, e* triple mutant male flies. Genetic interaction cross is performed to determine is *p* interacts with *garnet(g)*. For this cross, 6

homozygous *p* virgin females were crossed with 4 hemizygous *g* males. F1 progeny for genetic interaction cross were mated together to generate an F2 generation and double mutant *p¹g¹* flies.

Mapping crosses are important in determining the location of *pink* on the third chromosome. For mapping cross 1, homozygous 5 *pink* virgin females are crossed with 5 double mutant *hairy(h)*, and *ebony(e)* marker males to generate heterozygotes *h/+*, *+/p*, *e/+* females. For mapping cross 2(MC2), 6 heterozygotes *h/+*, *+/p*, *e/+* virgin females are crossed with 5 *h*, *p*, *e* males to create a three-point cross. For this cross, 1338 offspring were scored to create a recombination map of *pink* distance on the third chromosome relative to *hairy(h)* and *ebony(e)* genes.

A cross of MC1 virgin females and *h*, *p*, *e* marker males results in 8 phenotypic classes: WT, hpe, he, hp, pe, p, e, and h. Progeny of MC2 are scored for sex and the four phenotypic classes. Sex of fruit fly is determined by the presence of penis, genital arch and secondary sex characteristics such as sex combs. Females are scored using lack sex combs and presence have a vaginal plate. Wildtype flies show no mutant phenotype with bright red eyes and pseudopupil. Flies with ebony phenotype have a darker black body color than wildtype. MC2 progeny with lighter red/ pink eyes with no pseudopupil are scored as pink. *hairy* displays a phenotype of a dark spot at the base of the scutum.

Scoring progeny:

Progeny of crosses were scored for traits Curly, Plum, Stubble, Dichaete , wildtype, and Rbh and sex of the flies. Under the microscope, males and females are scored using their genital or secondary sex characteristics. Males are sorted by presence of epandrium and sex combs and females have ovipositor. Flies with Curly trait have wings that curly up while flies with Dichaete have wings that extend 45 degrees away from their body at an elevated position. Plum causes flies to have light brownish eyes with no pseudopupil and flies with Stubble had shorter and thicker

hair bristles compared to wildtype flies. *rbh* has light bright eyes with lack of pseudopupil while wildtype (Oregon-R) has bright red eyes with pseudopupil. For Discriminant crosses 1, we scored for *rbh* and for dominant markers *Cy*, *Pm*, *Sb*, *D*, and sex. We scored for sex, *rbh*, *Cy*, and *Sb* in marker DC2 progeny. Complementation cross progenies were scored for presence of *rbh* or wildtype phenotype. For all crosses, a minimum of at least 100 progenies were scored to analyze our gene. For genetic interaction cross, progenies were scored for pink, garnet , and possible double mutants. Flies with *pink* mutation are scored for lighter red eyes with no pseudopupil while *garnet* mutants were scored for brownish eyes with no pseudopupil.

Chi Square Test Analysis:

Chi square goodness of fit test is used as a statistical analysis to determine high correlation between observed and expected data sets. Data set comparisons were performed at level of significance $p=0.05$ at appropriate degrees of freedom. For $p \leq 0.05$, comparison of data sets is statistically significant and if $p > 0.05$ then comparison is not statistically significant. Males and females are scored under the same phenotypical class except in the case of sex linked mode of inheritance where only one sex is present. In the latter case, males and females are scored as separate phenotypic class.

DNA Extraction

pink, *rosy* and wildtype flies were each placed in 1.7ml Eppendorf tubes. To lysis the cell, a mixture of 10mm EDTA and 250uL of 50mM Tris-HCl at pH 8 were put in each tube. After lysis of the cell, 250ul of 1% SDS and 20mM NaOH is added to the solution to denature DNA and protein and to dissolve the lipids in the cell. To precipitate lipids, proteins and SDS so that it can be isolated from the DNA sample, 350uL of potassium acetate is used. DNA is precipitated with 800ul of cold 100% isopropanol then centrifuged at 13,000 rpm for 10 mins. After centrifugation,

DNA pellet is isolated and dried at 65°C for 5 mins then vortexed with 150ul of deionized sterile water for PCR amplification.

PCR amplification and purification:

To amplify the isolated DNA from *pink*, *rosy*, and WT flies, 15uL of PCR mixture containing forward and reverse primers, sterile H₂O, dNTP, Tris-HCl buffer, KCl, MgCL2, Taq DNA polymerase and DNA sample. DNA is denatured for 5 minutes at 94°C and then for 10 seconds at 96°C to ensure that all DNA is rendered single stranded. Annealing of primer and Taq polymerase was performed at 57°C for 30 seconds to ensure optimal annealing. Taq polymerase adds nucleotide to elongate the strand at a maximum temperature of 72°C for 2 minutes. This process was repeated 30 times to create ample DNA before the final elongation step at 72°C for 5 minutes. For each of the DNA sample, different forward and reverse primer were used for *p*, *ry* and *rbh*. The forward and reverse primer for *p* used for amplification is (5'-GTCCTTGAACGGCAGAGAAC-3') and (5'-TGATCCAGAGCGATGTTGAG-3') respectively. To amplify *rosy(ry)* gene forward primer (5'-CGAACTTCCCAACTGATGGT-3') and reverse primer(5'-GGACTACCCTCGATTGTGA -3') is used. This is used amplify the pink and rosy locus respectively. Our PCR product is purified with QIAGEN QIAquick PCR purification kit following the manufacturers' protocol. After purifying our product, quantification is necessary before sequencing DNA.

Quantification using Agarose Gel Electrophoresis:

PCR is quantified using 1.5% agarose gel electrophoresis using 1X tris-acetate-EDTA(TAE) buffer and agarose. To prepare our PCR product for electrophoresis, 5uL of 2X loading buffer is mixed with 5uL of DNA sample. This mixture is loaded into the wells and ran for ~40 mins at 120 V. Each sample contained three negatively charged tracking dye: Xylene

cyanole, Bromophenol blue and Orange G. To visualize bands, gel is viewed on a UV light box and analyzed by comparison to marker ladder.

BLAST ANALYSIS:

BLAST program is used to sequence and analyze DNA of *pink*, *rbh* and *rosy*. Forward and reverse primers are used to locate the DNA segment that was amplified using PCR. To identify the mutation that resulted in mutant phenotype, wildtype DNA sequences is compared to the DNA sequences of *pink* and *rosy*. Furthermore, *pink* DNA is searched on NCBI BLAST to confirm DNA homology and determine protein produced by gene amplified. Significance level of DNA scores using BLAST is at E value of 10^{-6} or less. This ensures that sequences added by Taq is accurate and less due to error since Taq lacks 3'-5' exonuclease activity.

Results:

***rbh* results in lighter eye mutants with no pseudopupil.**

My colleagues and I have discovered a mutation that causes *Drosophila melanogaster* to have a lighter eye pigmentation lacking a pseudopupil (Figure 1). I called this gene *red blush*(*rbh*) based on the eye color the mutant flies exhibits. *rbh* causes newly enclosed flies to have bright orange eyes that are easily distinguishable <2 hours after eclosion but darken slightly with age. The only variation noted in *rbh* mutants is the slightly darkening of eye color that results from aging. The eye color variation of this mutation is very low and darkening of the eye pigment of 2-3 day old flies is observed. This mutation has very high penetrance with every mutant progeny displaying the phenotype for bright orange eyes. Mutant males and females showed the same level of expression of *rbh* and there was no notable phenotypical difference between them. *rbh* mutant flies showed no changes in the length of life cycle compared to wild type flies. Wildtype *Drosophila* and *rbh* mutants show many phenotypic similarities such as presence of antenna, arista, and ocelli. *rbh* mutants showed expected life cycle, response to CO₂ and recovery from

anesthetization like wild type flies. This mutation does not cause any other behavioral and phenotypic difference except eye color change and lack of pseudopupil in fruit fly.

Discriminant Cross 1:

Discriminant cross 1(DC1) is performed to determine if *rbh* allele by Mendelian genetics is a dominant or recessive. The three discriminant crosses used are marker DC1, WT DC1 and marker DC1 control because each cross help determine how the *rbh* allele is inherited. For marker DC1, if *Cy/Pm; D/ Sb* marker males are crossed with homozygous *rbh* virgin females, pink phenotype will show up on all F1 progeny if dominant and will be masked if autosomal recessive regardless the chromosomal location of pink. If *rbh* was sex linked recessive, all F1 male progeny will have *rbh* phenotype since they inherit one X chromosome from their mother. F1 females should be unaffected since they inherit two copies of X chromosome, one from each parent. Therefore, *rbh* phenotype will be masked. Performing marker DC1 will determine if the *rbh* allele is dominant, autosomal recessive or sex linked recessive.

Cy/Pm; D/Sb marker flies contain dominant markers with recessive mutant alleles that can interfere with the analysis of marker DC1 cross. Therefore, WT DC1 cross is necessary to observe proper Mendelian segregation of alleles. In WT DC1 cross, *rbh* virgin females are crossed with WT males. If mutant allele is autosomal recessive then all F1 progeny would be wildtype while flies will display the *rbh* phenotype if its allele is dominant. F1 progeny from WT DC1 cross are used to generate an F2 progeny to show classic phenotypic ratios such as 75% WT and 25% mutant phenotype for an autosomal recessive allele. This cross can be used to observed expressivity for a phenotypic trait due to inbreeding. When WT virgin females are crossed with *Cy/Pm; D/Sb* marker males for the DC1 control cross, if Mendelian segregation of allele is applied there should be a 1:1:1:1 ratio of F1 progenies similarly to marker DC1. For the four phenotypic classes each dominant marker will appear on F1 progenies. Since both *Cy* and *Pm* are on the

second chromosome they cannot be passed down to the same progeny similarly with *D* and *Sb* on the third chromosome.

***rbh* Shows Autosomal Recessive Characteristics.**

For marker DC1, *rbh* mutant allele is masked in all progeny. This is an indication of an autosomal recessive allele(Figure 2). Progeny for F1 displayed four different phenotypic classes: Cy; Sb, Cy; D, Pm;D and Pm; Sb. Because *rbh* is autosomal recessive there was no phenotypic difference between males and females. The expected ratio for this cross is 1:1:1:1 assuming there is no interference between *rbh* and recessive alleles on dominant marker. Chi square of $p \leq 0.05$ is needed to prove statistical significance. This is furthermore confirmed in WT DC1 because all progeny is wildtype as expected(Figure 3). WT DC1 F1 were crossed for observation of classic phenotypic ratios. This cross produced F2, about 25% of progeny displayed *rbh* phenotype as expected(Figure 6). Although after completion of DC1, we can conclude that *rbh* allele is autosomal recessive, discriminant cross 2 is necessary to discover more about the chromosomal nature of *rbh*. For DC2, *rbh*;Cy;Sb DC1 males were selected for this cross because both dominant marker phenotypes will not interfere with the eye color pigmentation of *rbh* mutants.

Discriminant Cross 2:

The goal of discriminant cross 2 is to determine the chromosomal location of *rbh*. For DC2, *rbh*;Cy; Sb DC1 males were crossed with homozygous *rbh* females. For this study, inheritance of *rbh* allele with Cy dominant marker means that *rbh* is on third chromosome. If *rbh* phenotype is seen with *Sb* dominant marker then the allele is on the second chromosome. However, if *rbh* phenotype is seen with both Cy and *Sb* phenotype then the allele is on the fourth chromosome. In DC2 cross, F1 progeny of *rbh*;Cy; Sb will have four phenotypic classes in a 1:1:1:1 if Mendelian's law of independent assortment occurs.

***rbh* is on the Third Chromosome.**

Marker DC2 cross produces four phenotypic class; rbh; Cy, Cy; Sb, rbh and Sb(Table 6). Because rbh phenotype is displayed in flies with Cy phenotype only, *rbh* is on the third chromosome (Figure 5). Chi square test of marker DC2 is analyzed at a significance level of $p=0.05$. if $p \leq 0.05$, statistical significance is indicated and that *rbh* is on chromosome hypothesized. A p value greater than 0.05 indicates that data is not statistically significant due to underlying factors. Chi-square analysis of observed data of marker DC2 shows that data is not statistically significant(Table 7)

***rbh* is the *pink* gene**

A complementation test cross was performed with *rbh* virgin females and *pink(p)* and *rosy(ry)* autosomal recessive eye pigmentation mutants(Figure 7). F1 progeny are scored based on presence or absence of complementation. If F1 progeny complemented or appeared wild type then the two mutations are on different genes because *rbh* mutants would lack *pink* or *rosy* on corresponding locus. If the progeny appeared to have a mutant phenotype then the two mutation are on the same gene.

rbh and *ry* mutants complemented each other(Figure 8). Progeny of *rbh* females and *ry* mutants appeared wildtype with bright red eye and a pseudopupil. Because *rbh* and *ry* complemented each other they are most likely on different genes. *pink* mutants and *rbh* mutants failed to complement each other and resulted in progenies with lighter eyes and no pseudopupil. This results shows that *pink* and *rbh* are most likely on the same chromosome. Because of the results from the complementation test, *rbh* will be referred as *pink(p)* from now on.

***pink* allele is located on 3-48**

Because *p* is an autosomal recessive allele on the third chromosome, we used males with autosomal recessive allele *hairy(h)* and *ebony(e)* on the third chromosome for the mapping cross. This will be used to create a recombination map to determine the chromosomal location of *p*. Since the chromosomal locations of *h* and *e* is 3-26 and 3-71 respectively, recombination

frequency can be used to determine the location of *p*. Mapping cross 1(MC1) and mapping cross 2(MC2) were performed to achieve this. For mapping cross 1(MC1), homozygous *p* virgins females were crossed with *h*, *p*, *e* marker males to generate heterozygous wildtype *h*/*+*, *+/p*, *e*/*+* females. For MC2, *h*/*+*, *+/p*, *e*/*+* virgin females from MC1 are crossed with *h*,*p*,*e* marker males to create a three-point cross. Due to meiotic recombination of the third chromosome of female drosophila, there are eight phenotypic classes of progenies: WT, hpe, hp, he, pe, p, e and h. Parental progenies will display *p* or *he* phenotypes(Figure 10). Single recombinant progenies are *hp*, *pe*, *e* and *h* while rare double recombinant will show *WT* and *hpe* phenotype. Since the location of *p* is known from precedent experiment, the expected frequency of parental, one single recombinant, second single recombinant and double recombinant phenotype is insert expected %.

Although our parental class is the largest class of progeny as expected, 394 and 376 flies were counted for *h*, *e* and *p* phenotypes respectively instead of expected an expected amount of 402. For rare double recombinant class, 34 progeny are expected for each phenotype but 35 and 22 *hpe* flies were counted. Small deviation is observed in both single recombinant progeny class. For the first recombination event, 126 and 118 progenies were observed instead of expected values of 113 for *h* *p* and *e* phenotypic class respectively. For the second recombination event, 137 and 130 progenies were observed instead of an expected 120 progenies for each *p* *e* and *h* phenotypic class respectively. For the Chi-square analysis, male and females were scored as one phenotypic class. At 7 degrees of freedoms (dfs), calculate chi-square value is $11.06 < 14.07$ at *p*= 0.05 indicating that observed data is statistically significant. Chi-square analysis of recombination frequency between *h*,*p*, and *e*. Data shows that observed frequency between *h*-*p*, *p*-*e* and *h*-*e* are 22.5%, 24.22% and 38.19% respectively. At 2 degrees of freedom (dfs), chi square data calculation of $0.40 < 5.99$ at *p*=0.05 indicates that recombination frequency data are statistically significant and that *p* is located at 3-48.(Table 10)

***Pink* shows enhancing interaction with *garnet*¹(*g*¹)**

To identify genetic interaction with *p*, six *p* virgin females were crossed with four *garnet*(*g*¹) males for the parental cross. Since *g*¹ is a recessive sex linked mutation, all progenies are expected to be wildtype due to masking of the recessive allele. However, F1 progenies appeared both wildtype and mutant. Deviation from Mendelian expectation indicates that there is some interaction or linkage involved with *p*¹ and *g*¹. F1 generation are crossed and mate to generate an F2 with double mutants. Double mutant *p*¹/*g*¹ display appear lighter than both *p* and *g* (Figure 9). Observation of new phenotype is indicative that there is genetic interaction between *pink* and *garnet*.

Analysis of PCR using Agarose Gel Electrophoresis shows that *p* is caused by a small-scale mutation.

DNA amplified with PCR were analyzed using gel electrophoresis to estimate wildtype, *rosy*(*ry*), and *p* band length. *pink* and *rosy* specific allelic primers were used to amplify DNA in wildtype, *p* and *ry* genome for comparison. Analyze of gel allows to determine what mutation is present in *p* such as mutation substitution, insertion, deletion or evidence of transposons. Because flies with *pink* phenotype show no large scale behavioral or phenotypic defects compared to wildtype such as infertility or increased motility, we concluded that its mutation is due to a small mutation such as insertion, deletions or substitution. Bands of gels of *pink* DNA should show little to no difference in band length when compared to WT. Amplified region for *pink* and *rosy* is 841bp and 754bp respectively. *pink* and *rosy* are on different loci therefore, *pink* is wildtype at *rosy* and vice versa. *pink* DNA amplified by *rosy* primers will appear wildtype and same is true for *rosy* DNA amplified with *pink* primers. Wildtype DNA fragment amplified for both case above should be approximately the same base pair in length.

Gel electrophoresis suggests that *p* allele is a small-scale mutation. *Pink* forward and reverse primer are used to amplify DNA in lane 3,5, and 7. Forward and reverse *rosy* primers are

used to amplify DNA in lane 2, 4, and 6. Using pink primer in lane 3, wildtype DNA is amplified at the pink locus resulting in wildtype sequences of ~ 800 bp. In lane 5, *rosy* DNA is amplified using *pink* primer giving the similar results as wildtype DNA. *pink* DNA amplified using pink primers in lane 7 resulted in *pink* DNA sequences of ~800 bp. Because *pink* DNA, wildtype DNA and *rosy* DNA amplified with *pink* primers are approximately the same size using gel electrophoresis, this indicates that a small mutation is present in the *p* locus of *Drosophila melanogaster*. Wildtype DNA amplified with *rosy* primer in lane 2 resulted in wildtype sequence of approximately 750 bp at the *rosy* locus. In lane 4, *rosy* DNA amplified with *rosy* primer resulted in *rosy* sequences ~ 650 bp. *Pink* DNA amplified with *rosy* primers in lane 6 resulted in wildtype sequence ~750 bp long indicating that *p* is wildtype at the *rosy* locus. To determine the allelic nature of *pink* mutation, comparison of DNA sequences of PCR product is necessary. (Figure 11)

***pink* mutation is a ATG-> TT substitution in *p*¹ allele**

Analysis of gel electrophoresis is used to quantify DNA fragments however further analysis is needed to detect the nature of mutation of *pink*. DNA amplified with PCR is sequenced and compared to wildtype sequence using NCBI BLAST database. This method allows to determine what mutation is within the *pink* loci and its allelic identity. Gel analysis indicates that *pink* allele is caused by a small mutation such as point substitution, insertion or deletion in the *pink* locus resulting in a band approximately 800 bp in length (Figure 11). Comparison of DNA sequenced from PCR product and wildtype sequence on BLAST suggests a substitution of nucleotides TT for ATG on the *pink* locus (Figure 12). E-value of 0 and score of 767 means that sequence identity, amount of sequences matched, is statistically significant. This substitution is absent in wildtype and *rosy* DNA amplified with *pink* primers indicating that *p* is WT at *rosy* locus. A substitution of ATG-> TT is shown on mutant *p* gene chromatogram of *pink* DNA sequenced with *p* primers. Chromatogram of wildtype *p* gene using *ry* DNA and *p* primer shows wt ATG nucleotides (Figure 14 and 15). Alignment of *ry*¹gene with wildtype shows a 42 bp deletion in the *rosy* locus (figure 16)

16). WT DNA and pink DNA amplified with rosy primer resulted in WT sequences without the deletion (figure 17). To analyze molecular aspect of complementation test, *rosy* DNA amplified with *pink* primers and vice versa resulted in wildtype sequence. E-value and score for *ry* gene alignment is $8e^{-40}$ and 647 respectively.

***pink* gene is human orthologue *Hermansky-Pudlack syndrome 5(hps5)* gene.**

Previous studies show that *p* is an orthologue of *hps5* gene in humans which is responsible for pigmentation, we hypothesized that there are some highly-conserved sequences similarly found in some other organisms (Figure 18). Using BLAST, alignment of amino acid sequences of *p* and *hps5* shows that there are regions of homology between both sequences. *Drosophila* *pink* and human *hps5* gene show a sequence identity of 33%, which indicates the amount of identical amino acid between human *hps5* and *drosophila* *pink* amino acid sequence. Including 72 additional similar but not identical amino acid sequences shows a positive value of 182/334 or 54%. Amino acid sequences show an E value and score of $3e10^{-44}$ and 176 respectively. This suggests sequence homology between *pink* and *hps5* protein is significant.

Discussion:

The purpose of this study is to identify isolated gene *red blush(rbh)* using classical and molecular genetics. Discriminants crosses were used to determine that *rbh* is an autosomal recessive gene on the third chromosome. Complementation test indicated *rbh* is *p*, a gene known to affect protein trafficking to lysosome related organelles(LROs) resulting in reduced red and brown pigment in the eyes. Genetic interaction cross shows that *pink* interacts with *garnet* resulting in a more severe phenotype. Analysis of PCR data shows that *p*¹ is unknown *p* allele and is caused by a small substitution that alters the open reading frame(ORF) resulting in an altered protein. BLAST protein sequence alignment shows that *p* is an orthologue of Hermansky-Pudlak syndrome 5(HPS5) with high levels of conservation in the WD40 protein domain family. This homology can be used as a basis for a deeper understanding on the mechanisms of protein trafficking to LROs in higher eukaryotes

For this study, genetic analysis was performed with discriminant cross1, 2 and complementation crosses. Based on the data collected for marker DC1 and WT DC1, we concluded that *p* is an autosomal recessive mutation. In marker DC1, *p* allele has recessive characteristics and is masked by wildtype or dominant marker allele. At 3 degrees of freedom, the chi square value of 7.82 at p=0.05. Marker DC1 has a chi square value of 11.38>7.82 which indicates that null hypothesis *rbh* is autosomal recessive is not statistically significant. One factor that can cause the deviation in expected chi square is the limited amount of progeny scored. The larger the progeny analyzed the more likely to observe expected Mendelian ratio. Marker DC1 control aims to determine if dominant markers independently assort. At 3 dfs, marker DC1 control data sets have a chi square value of 7.74<7.82 at p=0.05 indicating that data is statistically significant. This suggests that dominant marker alleles segregate independently of each other. WT DC1 control cross had all wildtype flies as expected because *p* is masked by the wildtype allele. DC2 is important to determine the chromosomal location of *p* gene.

For the discriminant cross 2, Pink phenotype was seen with chromosome 2 dominant marker only, *Curly*, meaning that *pink* is on the third chromosome. At 3 dfs, marker DC2 cross has a chi square value of $8.77 > 7.82$ at $p=0.05$. Since $p>0.05$, data set comparison is not statistically significant. Expected phenotypic ratios for marker DC2 is 1:1:1:1 and deviation is due to small data number analyzed. WT DC1 F2 control observes progeny for classic Mendelian ratios to further determine the mode of chromosomal inheritance of *pink* gene. Expected ratio of F2 generation is 3:1 wildtype to mutants for an autosomal recessive gene.

Before classical Mendelian crosses were performed, *rbh* is hypothesized to several genes that display similar phenotype such as *pink*, *rosy*, *light*, *carmine*, and *garnet*. However after DC1 and DC2, it is known that *rbh* is an autosomal recessive allele on the third chromosome. Since *light* is on the left arm of chromosome 2 and *carmine* and *garnet* are on the X chromosome, they are excluded from further analysis in this studies. Although both *pink* and *rosy* are on the third chromosome, when complementation crosses were performed with *rbh* and *rosy* or *pink*, *pink* did not complement with *rbh* indicating the *rbh* is *pink*.

Since *pink* is an autosomal recessive allele, we used males with three autosomal recessive alleles *hairy(h)*, and *ebony(e)* to determine *p* genetic map location. Using the known location for *h* and *e*, 3-26 and 3-71 respectively, *p* location can be calculated using recombination frequency. To perform this cross, heterozygotes *h*/*+*, *+/p*, *e*/*+* virgin females were crossed with homozygous triple mutant *h,p,e* males. If *p* was an autosomal dominant mutation, for the first mapping cross which generates heterozygote progenies, recessive marker alleles is used. When creating heterozygotes for autosomal recessive, sex linked dominant and sex linked recessive, either dominant or recessive allele can be used. For mapping of sex linked gene, virgin females are not necessary. Since males will display mutant phenotypes and females will appear wildtype, heterozygous females and heterozygous males are crossed for sex linked three-point cross. An alternative mating scheme can be performed for sex linked recessive by using males with three

mutant alleles, two marker genes and your gene of interest. For three-point mapping cross, our result showed small deviation to expected but distribution of phenotypic classes of progenies were as expected. The largest phenotypic class were the parental class, *he* and *p* and the least were double recombinants, wildtype and *hpe*. Deviation to expected amount of double recombinant frequency is often observed due to crossover interference, a phenomenon where one single crossover events decreases the likelihood of another occurring. Single recombinant progenies, *hp*, *pe*, *h* and *e*. Although deviations from expected values are present, chi square analysis of $11.06 < 14.99$ at $p=0.5$ indicates that observed data is statistically significant (Table 10). Observed recombination frequency between *h* and *p* is 22.50%. The observed frequency for *p-e* and *h-e* is 24.22% and 38.19% respectively. Chi square analysis of recombination frequency of gene pairs are calculated (Table 11). At 2 degrees of freedom, chi-square value of $0.40 < 5.99$ at $p=0.05$ indicating that *p* is located at 3-48.

Gel electrophoresis is used to quantify *p*, *ry* and wildtype DNA amplified by PCR. Using pink primers with wildtype, *ry* and *p* DNA showed little to no changes in base pair of region amplified. This indicates that a small-scale mutation in *p*¹ such as substitution, insertion, deletions. Gel analysis of wildtype, *p*, and *ry* DNA amplified with *ry* primer indicates that *ry*¹ is an approximately 100bp deletion. This deletion is not seen in wildtype and *p* DNA which results in a larger DNA fragment size. However, sequences of *p* DNA using *ry* primers shows a deletion of 42 bp at *ry* locus.

Using Gel Electrophoresis, band length suggests that *p* is caused by a small-scale mutation due to the little to no discrepancy between wildtype, *p* and *ry* DNA amplified with pink primers. Further analysis of DNA sequences of PCR products indicates that *p*¹ is caused by a substitution of TT for ATG in the coding region. This results in a mutation that causes an abnormal reading frame of 1-648 amino acids followed by an addition 39 amino acid and premature stop codon(Falcon-Perez et al). The consequences of this mutation are changes in the conserved

proteins in the C- terminus (Syrzycka et al). High conservation of C- terminus sequence across evolution correlates with the loss of function characteristics seen with altered C- terminus. Highly conserved regions may indicate protein homology therefore protein alignment is necessary.

Alignment of protein sequences using BLAST suggests that *pink* shares amino acid sequence homology to human *hps5* gene. Precedent studies show that *hps5* and *pink* protein are possible orthologues. High sequence similarity is seen in the conserved WD40 protein domain family. WD40 proteins are one of the most abundant protein family and have diverse functions many which can cause or affect diseases. WD40 protein domains have diverse functions in conserved protein sequences. WD40 domain is found in many eukaryotic protein and function in regulation cytoskeleton assembly, RNA processing, gene expression/ epigenetic regulation, cellular processes, protein coupled receptor and signal transduction. Because of its regulatory functions, many WD40 protein domain are targets for cancer therapy and regenerative medicine. Studies of WD40 protein domain show structural scaffold ability that can interact with other proteins or nucleic acids since WD40 domains have no enzymatic activity (stirnimann et al)

pink causes Drosophila to have pink or light red eye color pigmentation with lack of pseudopupil that darken with age. Previous studies show a significant decrease in the amount of red and brown pigments that results in abnormal eye pigmentation observed with pink phenotype(perez). Human orthologue of *Drosophila pink(p)*, Hermansky-Pudlak Syndrome 5(HPS5) BLOC-2 subunit, is a member of the granule group. It is an autosomal recessive disorder that causes defects of melanosomes causing albinisms and in severe cases prolonged bleeding (Syrzycka et al). Phenotypic defects are due to the altering of protein trafficking to lysosome related organelles in blood cells and melanocytes. Secretory lysosomes, intracellular components important for biogenesis and protein trafficking, are seen in melanosomes of melanocyte cells of mammals (Falcon-Perez et al., 2006). If protein transportation to these organelles are reduced it results in eye color pigmentation mutants such as *pink*. Previous studies using

immunoprecipitation, confirmed stable protein interaction between HPS3, HPS5 and HPS6 and velocity analysis of BLOC-2 and HPS-3 (Di Pietro et al.) This suggests that HPS 3, HPS5 and HPS6 assemble into multi-subunit complex to form mammalian BLOC-2 complex. These complex are expressed ubiquitously but regulated through posttranslational modifications and interactions with lysosome related organelles(LROs) protein trafficking machinery(Mark et al). They act to support trafficking pathways therefore loss or decrease of function impacts development of LROs. Although most of the molecular function of BLOC-2 remains unknown, it is involved in the pathway of melanosomes development in mice (Bultema et al.,2012).

ruby-eye 2(ru2), a gene that encodes mouse orthologs in human HPS5 gene, causes several lysosome related organelles defects. There is an increase in immature or unpigmented skin melanosomes in *ru2* mutants which causes hypopigmentation in mouse coat and eyes (Nguyen et al.,2002). Studies show that although lysosomal structure is normal, there is a decrease in secretion of lysosomal enzyme in urine. (Zhang et al.,2003). Low secretion of lysosomal enzymes in urine indicates an accumulation of lysosomal enzymes in the kidney. In mice, a similar result of HPS5 like mutation is inadequate or abnormal platelet dense granules which produce abnormal platelets and prolonged bleeding. (Zhang et al.,2003). *ru2* mutation can cause retinal pigment epithelium melanosomes defects and significantly decreases the number of melanosomes present. The molecular processes in which this mutation affects secretion of lysosomal enzyme and abnormal platelet granules is not fully understood.

A genetic interaction is performed between *pink* and *garnet* a severe phenotype is seen. Double mutants *p¹/g¹* appear to have lighter eyes than *p* and *g* alone. Previous genetic interaction studies on Drosophila show a significant decrease in red pigments compared to both single mutants (Falcon-Perez et al.,2006). Drosophila garnet protein is the mammalian homologue of Delta subunit of the Adapton complex AP-3. AP-3 complex forms tetramers that function as intermediates between protein molecules and vesicle coats in the transporting pathways from

trans-golgi to lysosomes or endosomes (Lloyd et al., 1999). In mice, AP-3 and BLOC-2(a complex contains HPS3, HPS5, and HPS6 proteins) single mutants displayed relative hypopigmentation while AP-3/BLOC-2 double mutants resulted in severe phenotype in coat color (Bultema et al., 2012). Genetic interaction in multiple organism suggests that protein product of *pink* is made but misfolding of proteins alters its function in the BLOC-2 complex. A misfolded protein may decrease the interaction of the protein with others in the complex.

Hermansky-Pudlak syndrome(HPS) is a rare autosomal recessive disease that can affect several organs in the body. Oculocutaneous albinism, bleeding problems due to and storage of abnormal fat can be seen in individuals with HPS. This disease can be often debilitating since multiple platelet transfusions is require for prolonged bleeding and risk of lung disease is fatal (Zhang et al., 2003). Platelets of a three-year-old Turkish boy with HPS type 5 showed moderately decrease in platelet count and reduced function. Decrease in aggregation response to ADP and secretion of ATP as a response to collagen is observed (Zhang et al., 2003) Although several genes in multiple organism have been identified to be involved in HPS, the molecular processes of how these protein function is not fully understood. *pink* is among one of the orthologue of human HPS5 gene therefore further studies on *p* can be beneficial. Although molecular process of how BLOC-2 is not known, future studies on *p* can help determine what mechanisms BLOC-2 uses to regulate lysosome biogenesis. The molecular processes in which *p* gene gives rise to mutant phenotype is not fully understood. This study shows suggests that a small substitution ATG -> TT causes a frameshift mutation resulting in additional amino acids and a premature stop codon. This change to the amino acid sequence can alter protein shape ultimately effecting its function. Understanding its protein molecular processes and its function in vesicle trafficking to and between lysosomes can be beneficial for HPS gene therapy experiments.

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Tables and Figures:

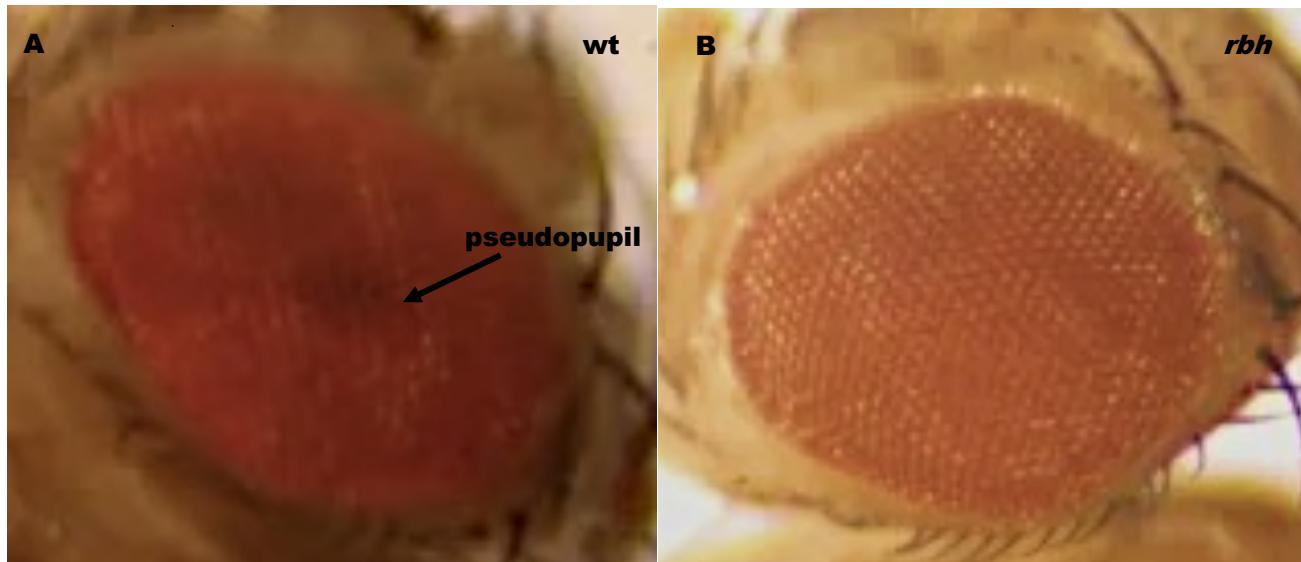


Figure 1: Wildtype flies compared to *red blush(rbh)* mutant flies. (A) Wildtype flies have brighter true red eye pigmentation with pseudopupil. (B) *rbh* mutant flies appear to have a dull lighter red eye color pigmentation with no pseudopupil.

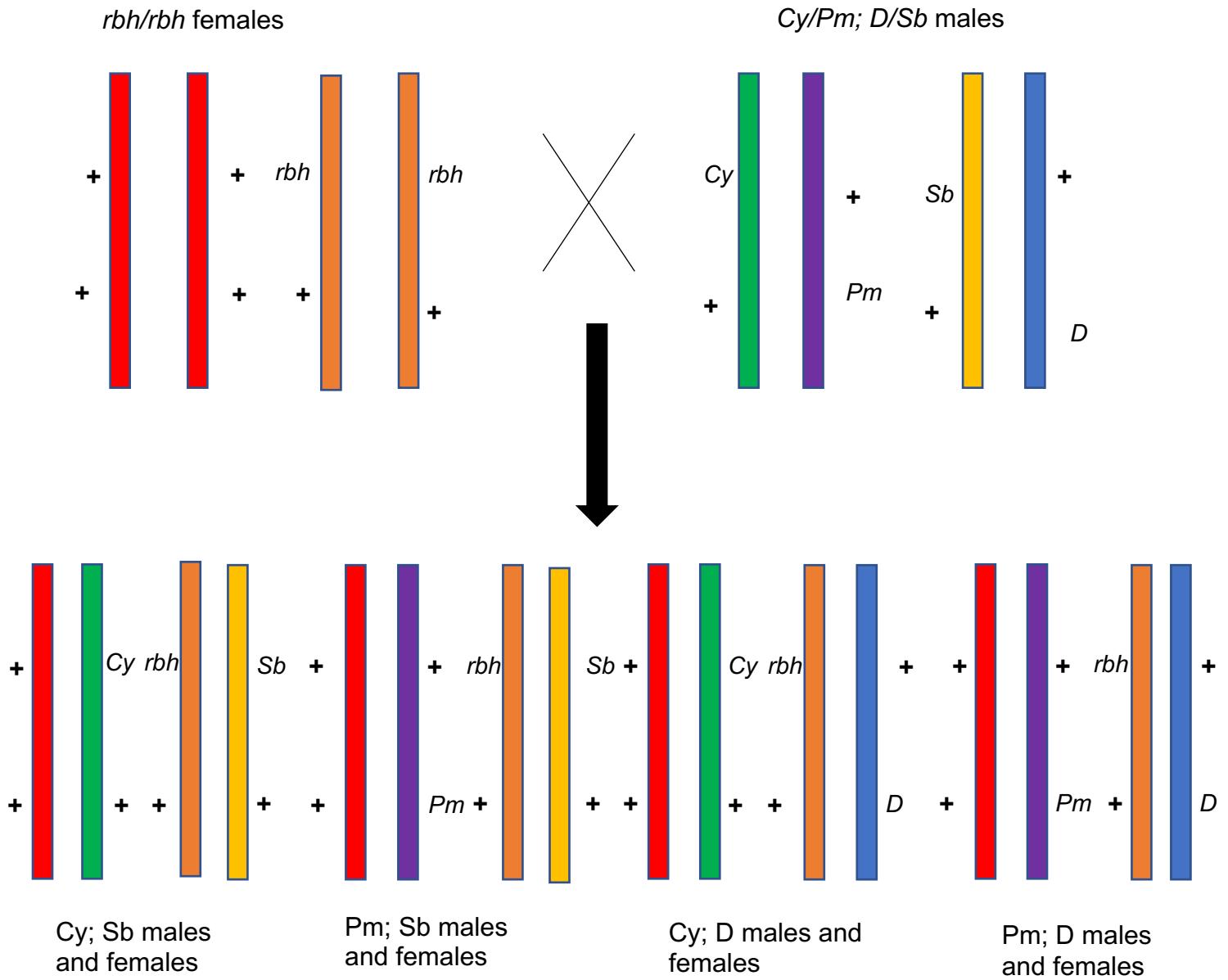


Figure 2: Marker DC1 chromosomal outcome for *rbh*, an autosomal recessive gene.
 Parental cross of marker DC1, homozygous *rbh/rbh* virgin females and *Cy/Pm; D/Sb* marker males, is indicated in the top row and F1 progeny is indicated below. Because *rbh* is autosomal recessive males and females are present in each phenotypic class. There are four expected phenotypic class: *Cy;Sb*, *Pm;Sb*, *Cy;D* and *Pm;D*. Dominant markers will independently assort and will appear in progenies since only one copy is needed. Since *rbh* is autosomal recessive, no progeny displayed phenotype for *rbh* gene because only one copy of the gene is present.

Class	Phenotypes	Males	Females
1	Cy;Sb	27	23
2	Cy; D	12	16
3	Pm;Sb	31	24
4	Pm; D	16	19
Total		86	82

Table 1: *rbh* is an autosomal recessive gene. Homozygous *rbh/rbh* virgin females were crossed with *Cy/Pm;D/Sb* dominant marker males to give four phenotypic class of F1 progeny as expected. Note that *rbh* is masked in all progeny showing characteristics of a recessive allele.

Class	Phenotype	Observed	Expected	O-E	(O-E)²	(O-E)²/e
1	Cy; Sb	50	42	8	64	1.52
2	Cy; D	28	42	-14	196	4.67
3	Pm; Sb	55	42	13	169	4.02
4	Pm; D	35	42	-7	49	1.17
						$\chi^2=11.38$

Table 2: Chi Square Analysis of Marker DC1 cross. Chi square value at 3 degrees of freedom(df) is 7.82 at p=0.05. Because *rbh* is an autosomal recessive allele, males and females of the same phenotypic class are grouped together. The chi square value for this data is 11.38 which is greater than 7.82 suggesting that recessive mode of inheritance is not statistically significant. However, this deviation from expected may be due to limited amount of progeny scored.

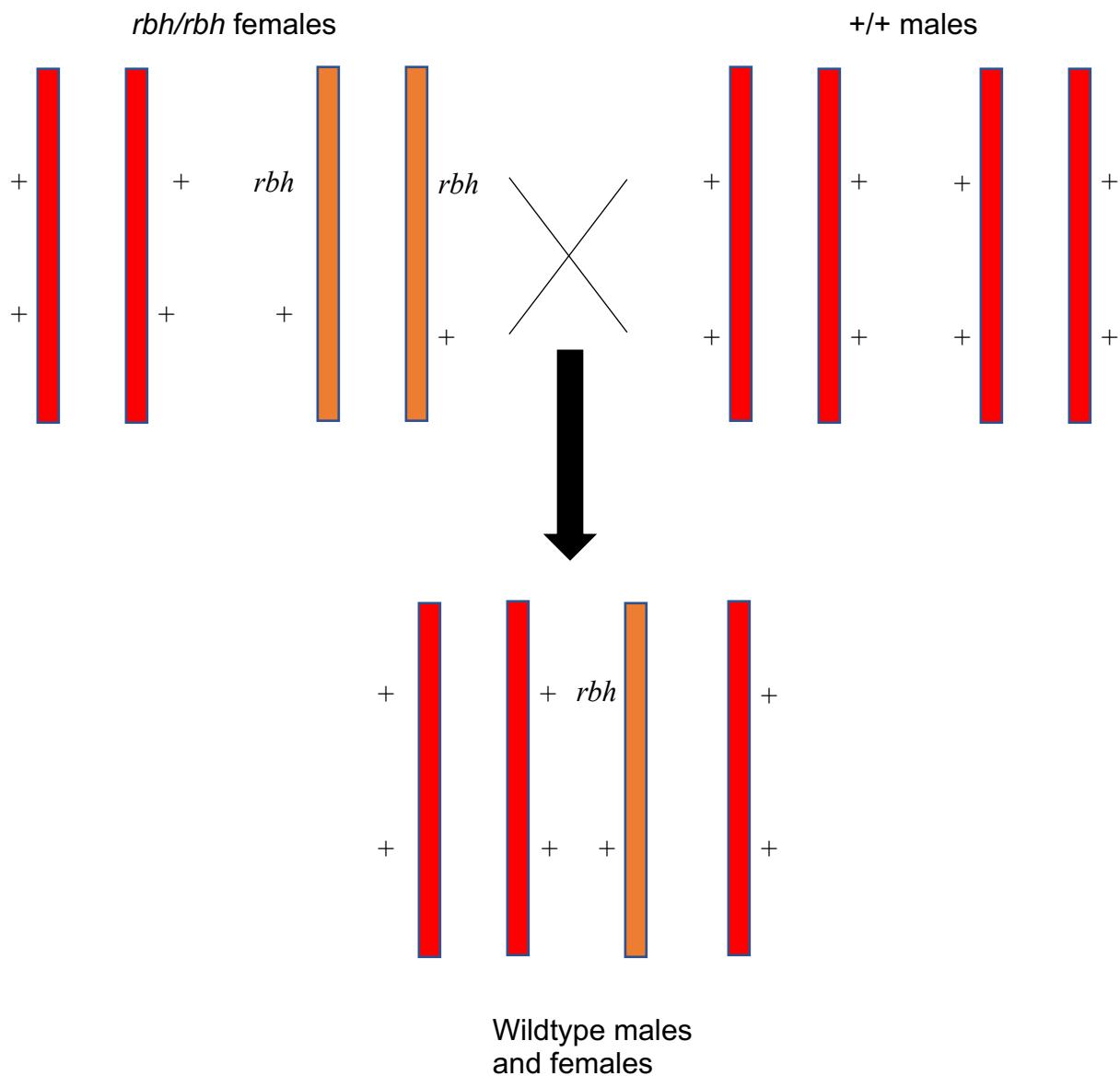


Figure 3: WT DC1 chromosomal outcomes. *rbh* virgin females are crossed with wildtype males. Expected F1 progeny will be wildtype because recessive allele is masked.

Class	Phenotype	Males	Females
1	wildtype	109	117

Table 3: WT DC1 cross to determine if dominant marker have recessive alleles that interfere with *rbh* gene. Homozygous *rbh/rbh* virgin females are crossed with *+/+* males to give all wildtype F1 progeny. As expected, one copy of *rbh* gene does not result in *rbh* mutants due to its recessive properties.

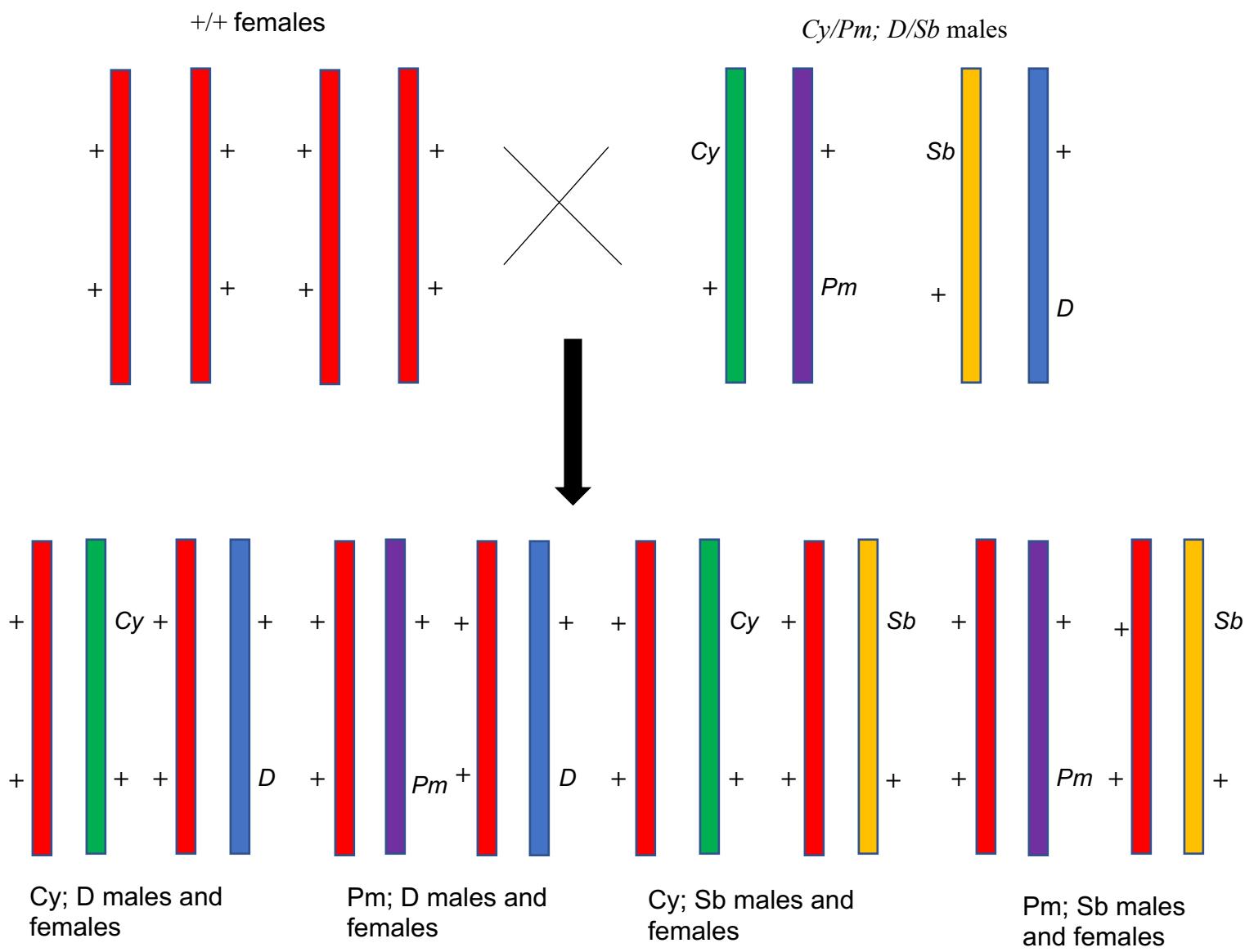


Figure 4: Marker DC1 control chromosomal outcome if Mendelian independent assortment of dominant markers occurs. Parental cross of marker DC1 control, $+/+$ virgin females and *Cy/Pm; D/Sb* marker males, is indicated in the top row and F1 progeny is indicated below. There is a 1:1:1:1 ratio of four expected phenotypic class: *Cy;Sb*, *Pm;Sb*, *Cy;D* and *Pm;D*. Dominant markers will independently assort and will appear in progenies since only one copy is needed.

Class	Phenotype	Males	Females
1	Cy; Sb	47	34
2	Cy; D	33	21
3	Pm; Sb	37	46
4	Pm; D	29	38
Total		146	139

Table 4: Marker DC1 control proves Mendelian's independent assortment. Homozygous +/+ females are crossed with Cy/Pm;D/Sb males to give four phenotypic F1 progeny. Cy and Pm, alleles on chromosome 2, are never seen together in a progeny. The same is true for D and Sb on the 3rd chromosome. Because of independent assortment, the expected ratio for the phenotypic classes is 1:1:1:1. The expected amount of progeny for each phenotypic class is 71 but little deviation is observed.

Class	Phenotype	Observed	Expected	O-E	(O-E)²	(O-E)²/E
1	Cy; Sb	81	71	10	100	1.41
2	Cy; D	54	71	-17	289	4.07
3	Pm; Sb	83	71	12	144	2.03
4	Pm; D	67	71	-4	16	0.23
						$\chi^2=7.74$

Table 5: Chi square analysis of marker DC1 control of independent assortment. Chi Square at degree of freedom (df) of 3 is 7.82 at p=0.05. The data chi square value of 7.74<7.82 indicating that independent assortment of dominant marker is statistically significant.

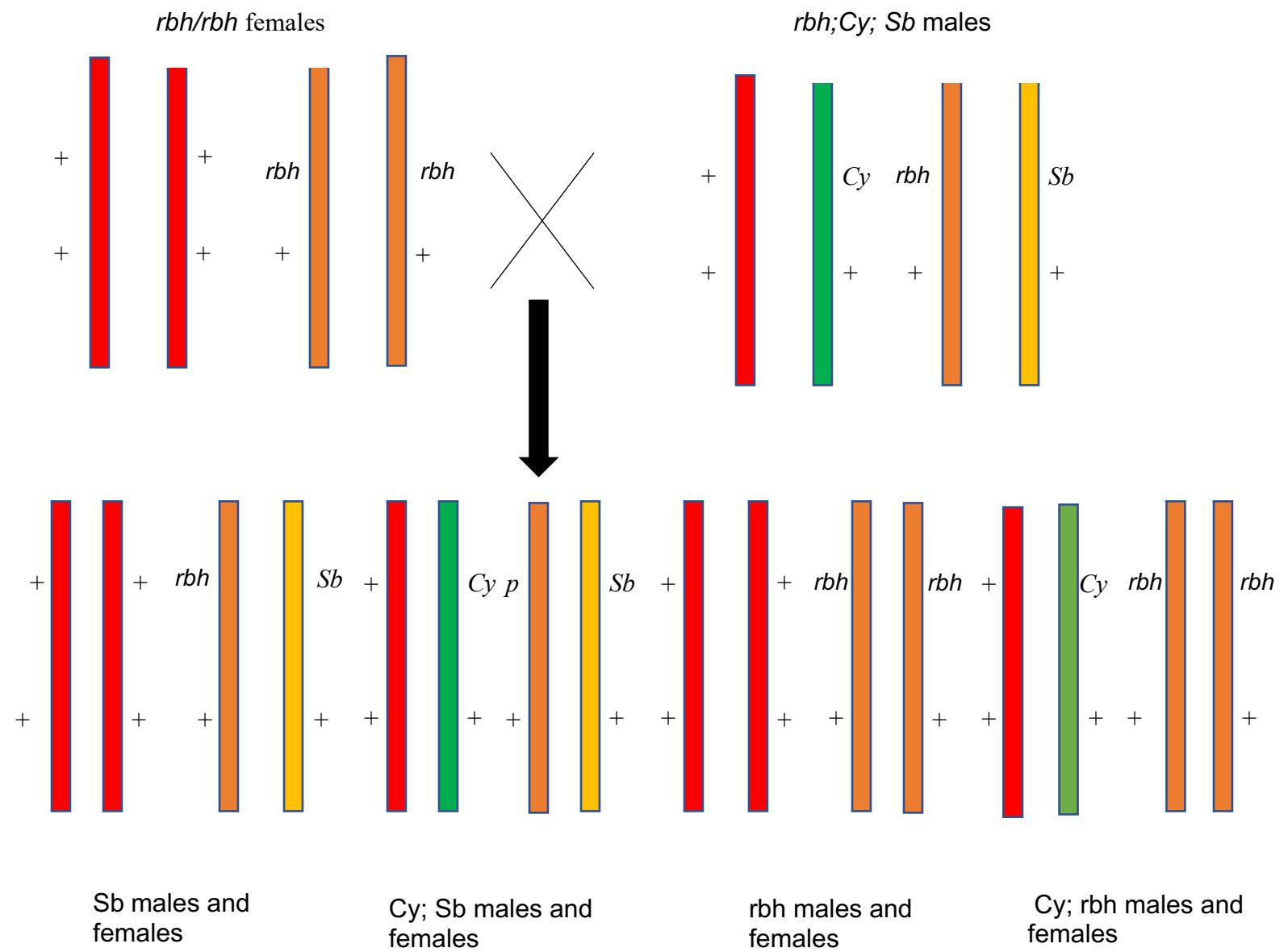


Figure 5: Marker DC2 chromosomal outcome if *rbh* is on the third chromosome.
 Homozygous *rbh/rbh* virgin females were crossed with *rbh;Cy;Sb* marker DC1 F1 males to produce four phenotypic classes: Sb; Cy;Sb, rbh, Cy; rbh. Because *rbh* phenotype showed up with chromosome 2 dominant marker, Cy, but not chromosome 3 dominant marker, D, indicates that *rbh* is on the third chromosome. Dominant markers assort independently to generate a 1:1:1:1 ratio.

Class	Phenotype	Males	Females
1	Sb	49	35
2	Cy; Sb	41	50
3	rbh	44	56
4	rbh; Cy	28	35
Total		162	176

Table 6: Data of Marker DC2 cross indicates that *rbh* is on the third autosomal chromosome. Homozygous *rbh/rbh* virgin females are crossed with *rbh; Cy; Sb* F1 males to produce four phenotypic classes: Sb, Cy;Sb, rbh, and rbh;Cy. Due to independent assortment, these phenotypic classes should have an expected 1:1:1:1 ratio.

Class	Phenotype	Observed	Expected	O-E	(O-E)²	(O-E)²/E
1	Sb	84	85	-1	1	0.01
2	Cy; Sb	91	85	6	36	0.42
3	rbh	100	85	15	225	2.65
4	rbh; Cy	63	85	-22	484	5.69
						X ² =8.77

Table 7: Chi Square Analysis of Marker DC2 indicates that *rbh* is on the third chromosome. Chi square value is 7.82 at p=0.05 for 3 degrees of freedom (df). The data has a chi square value of 8.77>7.82 indicating that *rbh* on chromosome 3 is not statistically significant. Deviation from expected chi square value may be due to the amount of progeny scored.

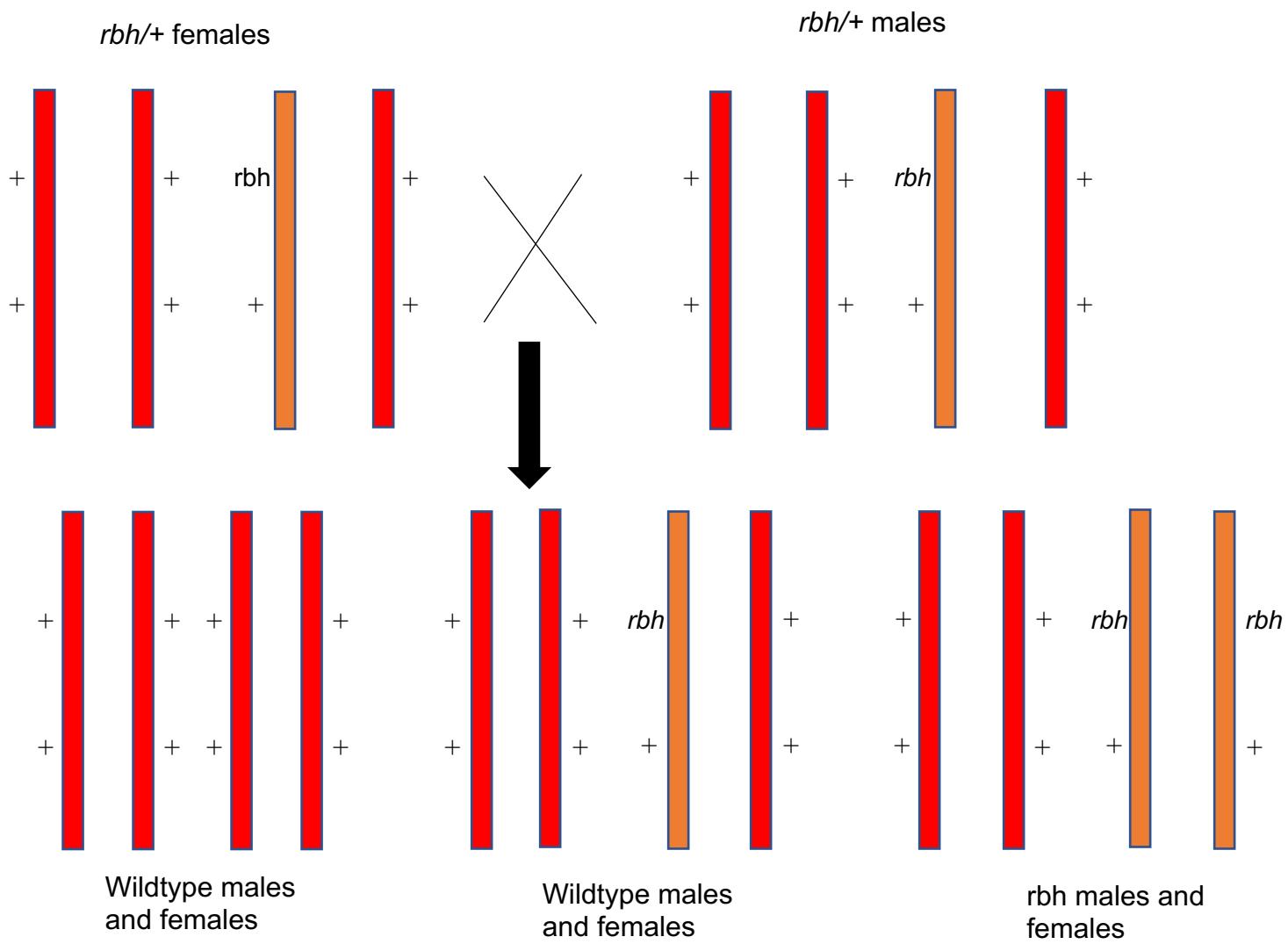


Figure 6: WT DC1 F1 cross chromosomal outcome with Mendelian genetics. On the top row, *rbh/+* males and females from WT DC1 are crossed. F2 progeny chromosomal outcome are shown below. Since *rbh* is autosomal recessive, F2 generation display Mendelian ratio of 3:1 wildtype to *rbh* phenotypic ratio.

Class	Phenotype	Males	Females
1	rbh	15	15
2	+/+	49	58
Total		30	107

Table 8: WT DC1 F1 cross follows expected classic phenotypic ratios. Males and females from F1 progeny of WT DC1 are crossed together to generate an F2 generation. Since *rbh* is autosomal recessive an expected 25% of *rbh* progeny should be observed in F2. This cross generates two phenotypic classes in a 3:1 wildtype to *rbh* ratio.

Class	Phenotype	Observed	Expected	O-E	(O-E) ²	(O-E) ² /E
1	rbh	30	34	-4	16	0.47
2	+/+	107	103	4	16	0.16
						X ² =0.63

Table 9: Chi square analysis for expected Mendelian ratios for WT DC1 F1 progeny. At one degree of freedom, the chi square value of p=0.05 is 3.84. Data collected scored a chi square value of 0.63 < 3.84 indicating that result is statistically significant.

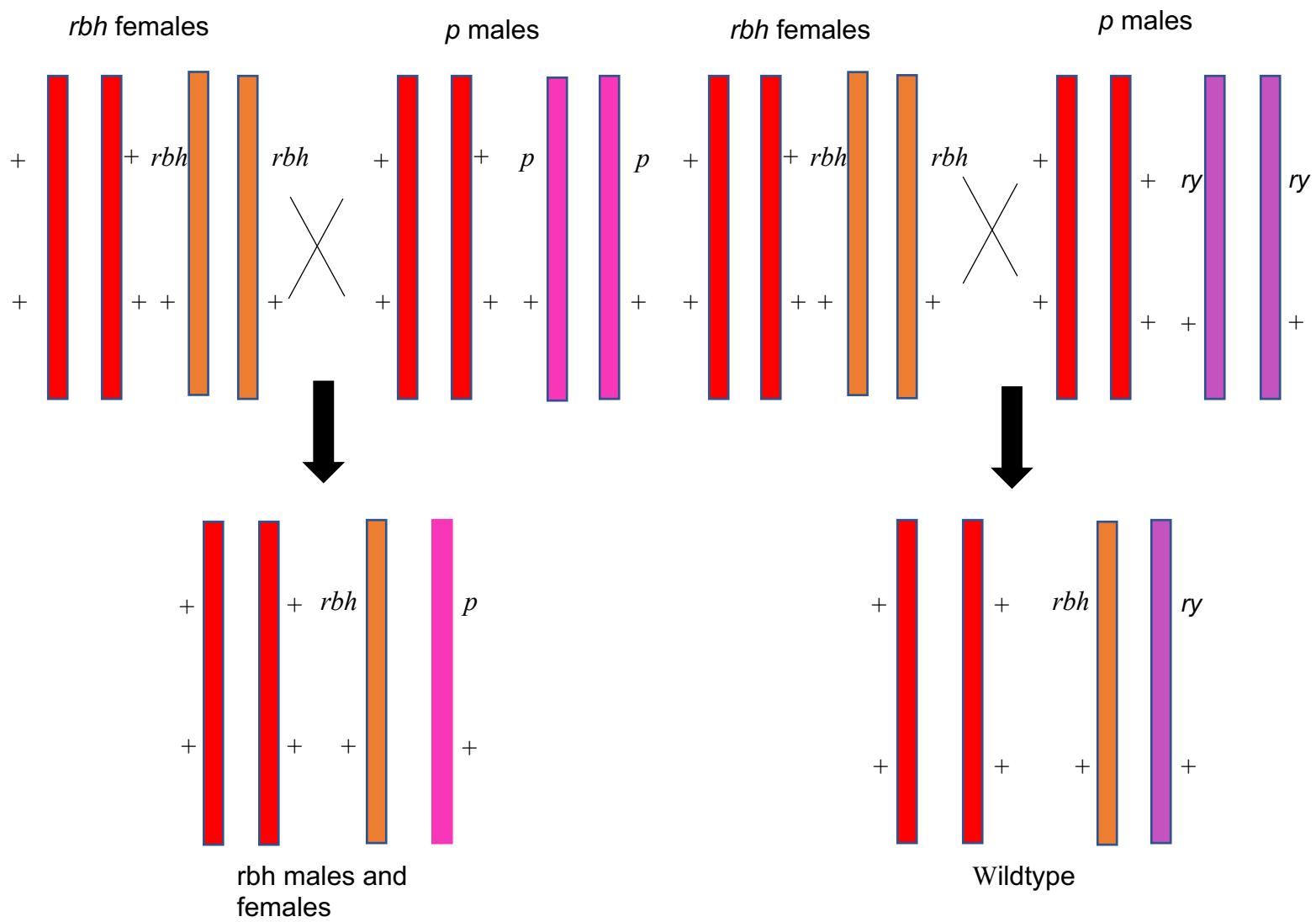


Figure 7: Complementation crosses of *rbh* with *pink(p)* and *rosy(ry)*. On the left, complementation cross of *rbh* and *p* is shown. All progeny of this cross displayed pink phenotype and suggests that they are on the same chromosome and *rbh* is *pink*. On the right, complementation cross of *rbh* and *ry* is shown. All progeny of this cross resulted in flies that appeared wildtype indicating *rbh* and *ry* are on different chromosomes.

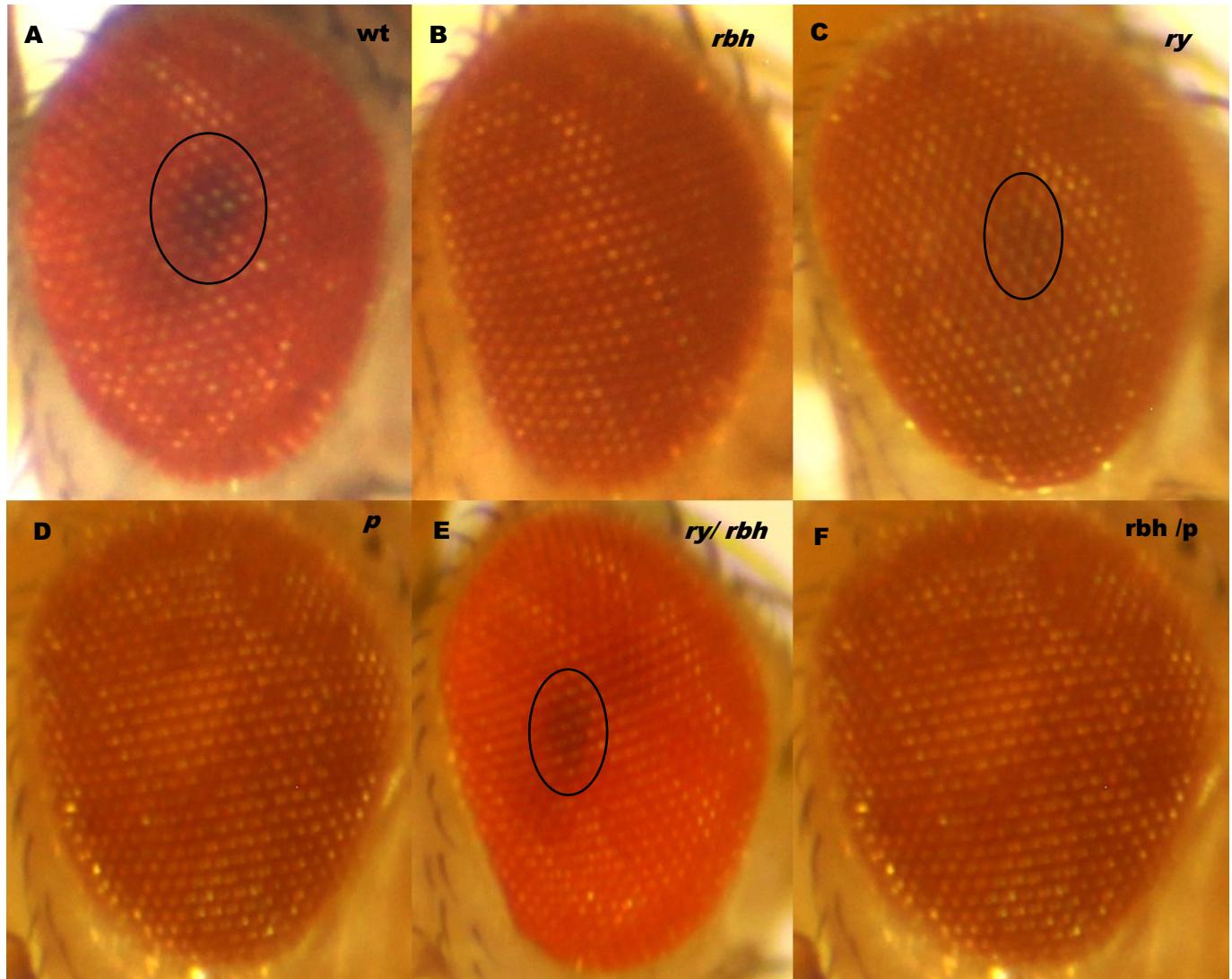


Figure 8: Complementation test between *rbh* and two other eye color pigmentation mutants. (A) Bright red wildtype eyes with pseudopupil present. (B) Homozygous *rbh* mutant eye appears darker red with lack of pseudopupil. (C) Homozygous *rosy* (*ry*) with dark red eyes with lighter pigmented pseudopupil. (D) Homozygous *pink*(*p*) show darker colored eyes with lack of pseudopupil similarly to *rbh*. (E) Heterozygous *rbh* and *ry* adult appears wildtype with presence of pseudopupil and bright red eyes. (F) Heterozygous *rbh/p* adult flies with dark red eyes and no presence of a pseudopupil.

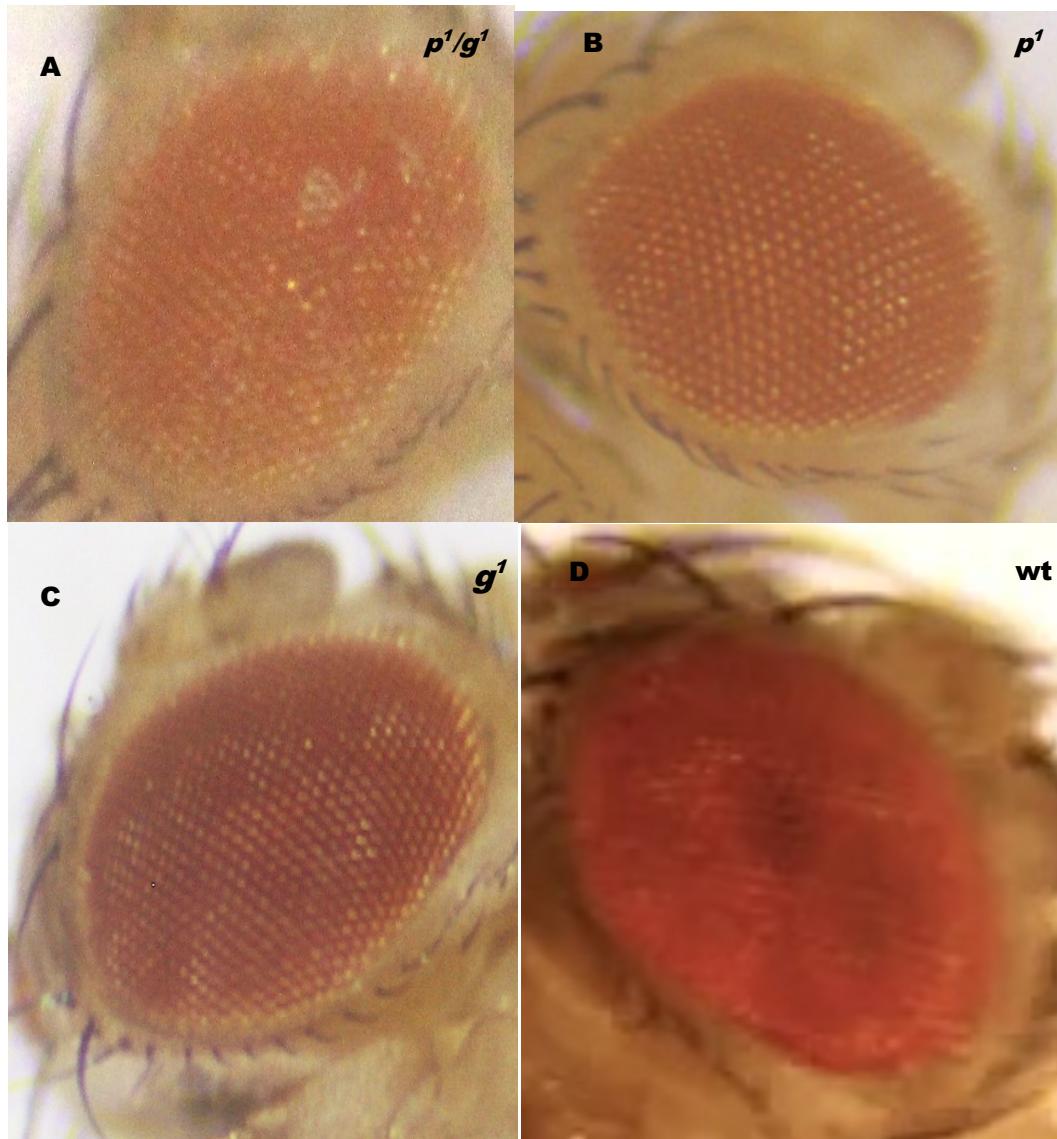


Figure 9: Genetic interaction cross with p^1 and g^1 mutants. (A) p^1/g^1 double mutants display eyes with less pigmentation than both single mutants. Lack of pseudopupil with light bright eyes indicates a more severe mutant phenotype. (B) Flies with p^1 show a similar phenotype to garnish with a lighter red and brown pigmentation. (C) g^1 mutants have more brownish eye pigmentation with no pseudopupil. (D) Wildtype flies show a bright red eye pigmentation with a pseudopupil.

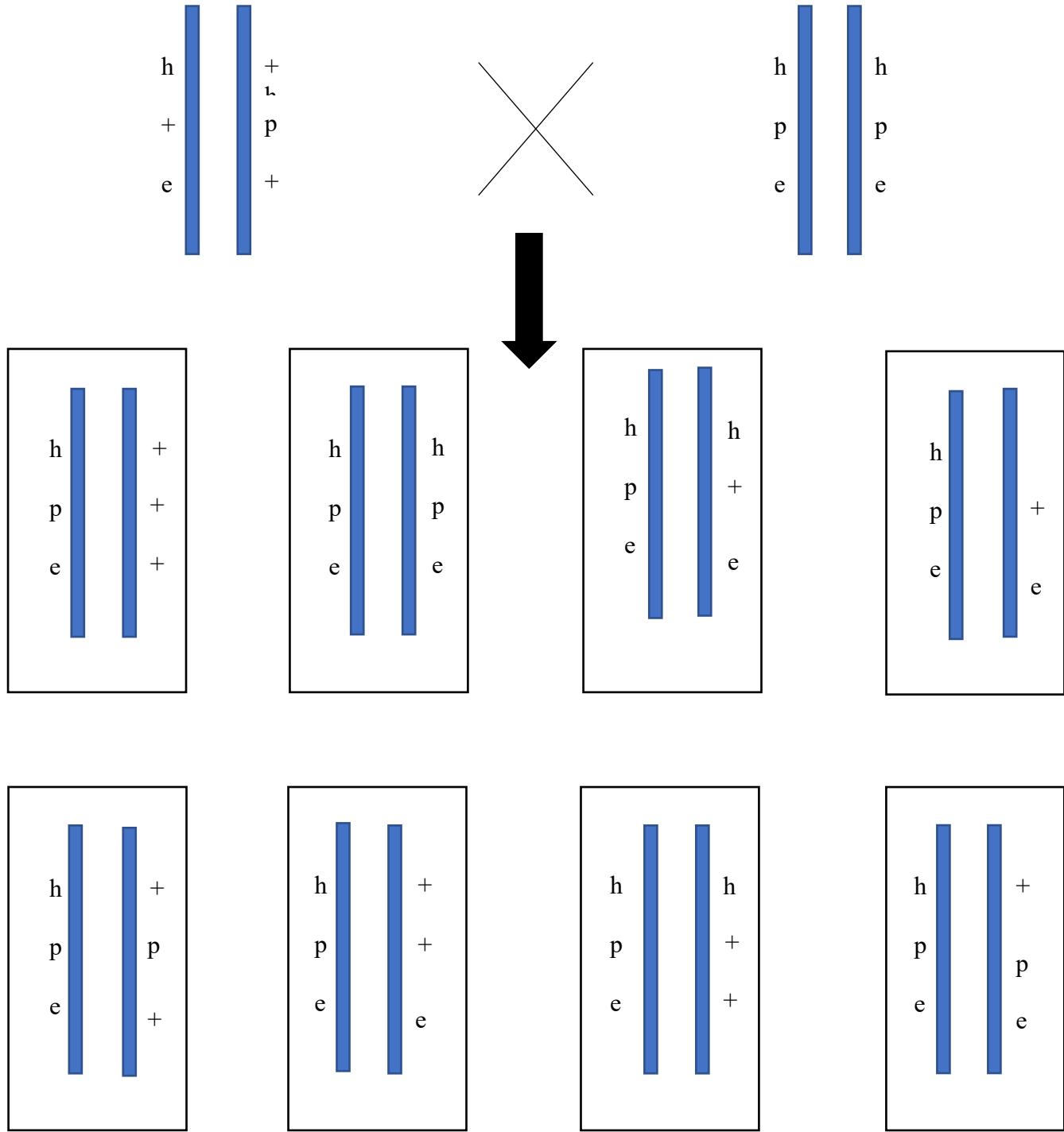


Figure 10: Recombination outcome for mapping cross 2(MC2) displays eight phenotypic classes. For the parental cross, *h*/*+*, *+/p*, *e*/*+* are crossed with *h*, *p*, *e* marker males. Since only females can undergo recombination in *Drosophila melanogaster*, WT, *hpe*, *hp*, *he*, *pe*, *p*, *e* and *h* are 8 different phenotypic outcomes for the F1 progeny. *he*, and *p* represents the parental progeny class. *hp*, *pe*, *e* and *h* represent the single recombination progeny classes. WT and *hpe* represent the double recombinant progeny classes. Expected progeny is 22%, 23% and 5.1% is expected for the first single recombinant, second single recombinant, and double recombinant phenotype respectively.

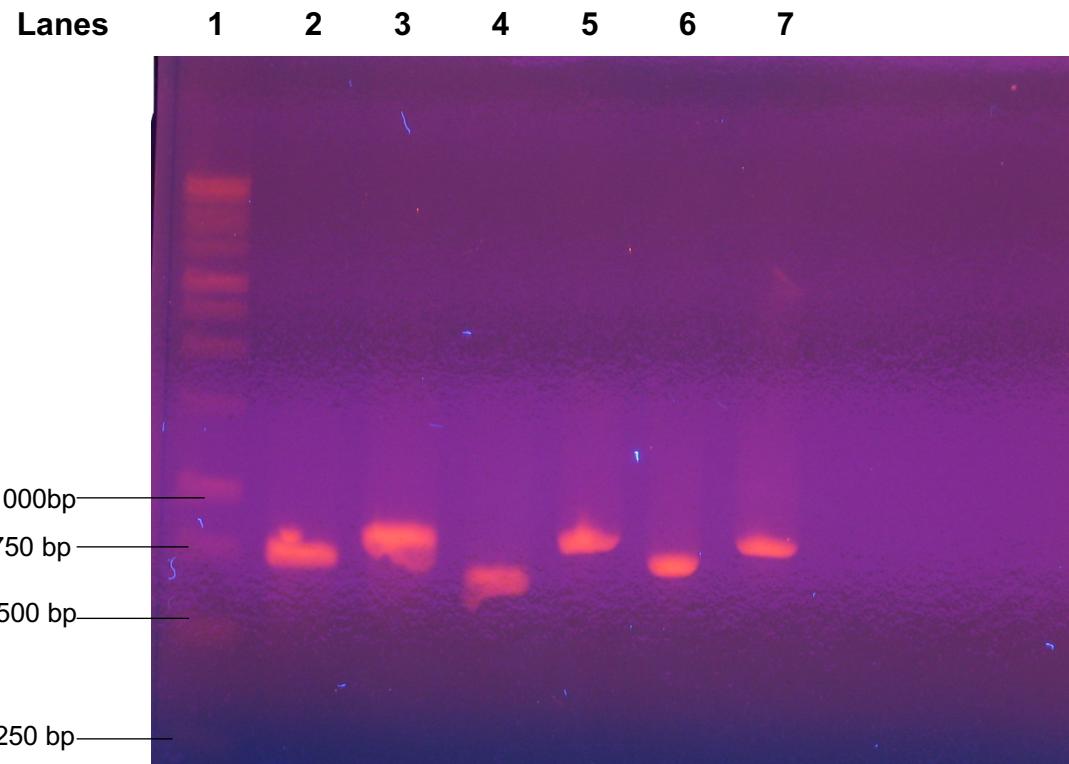


Figure 11: Agarose gel electrophoresis analysis suggests *p* is a small-scale mutation. Lane number is indicated above each well. Lane 1 is the 1kb ladder used to estimate the band length of each PCR DNA product and ladder is labeled with band sizes to the left. Lane 2, 4 and 6 is wildtype, *ry* and *p* DNA is amplified with *ry* forward and reverse primer, respectively. For lanes 3, 5, and 7, forward and reverse *p* primers are used to amplify wildtype, *ry* and *p* DNA, respectively. For Lane 3 and 5, wildtype and *ry* DNA amplified with *p* primers will have the same wildtype *p* DNA fragment. Lane 6 will result in *p* DNA fragment when *p* primers are used to amplify *p* DNA. However, all three lanes appear to be the same size of ~ 800bp. This suggests that *p* is a small point mutation such as insertion, deletion and substitutions. Lanes 2 and 6, wildtype and *ry* DNA is amplified with *ry* primer to result in wildtype *ry* DNA sequences of ~750bp. For lane 4, *ry* DNA amplified with *ry* primers shows a band length of ~650bp which suggests a large deletion.

pink	314	TGGGAAGTGGACGACGCTACGC _{GGG} GATCACATAGCTGCCGGATTGTGCTGCTAAATACT	373
ry/p1	313	TGGGAAGTGGACGACGCTACGC _{GGG} GATCACATAGCTGCCGGATTGTGCTGCTAAATACT	372
pink	374	TCGC _A AATT _T CGGAGATTGTCAAGTGTGAGCATTGCTCGTTCCCCCTTCGCTTCGATACC	432
ry/p1	373	TCGC _A AAAT _T CGGAGATTGTCAAGTGTGAGCATTGCTCGTTCCCCCTTCGCTTCGATACC	432
pink	433	TCCTGCCAGTATCATGAGCTTGGAGCAGTGCTCCTGCGATACTTCTGGTCCC _T GGCGAG	492
ry/p1	433	TCCTGCCAGTATCATGAGCTTGGAGCAGTGCTCCTGCGATACTTCTGGTCCC _T GGCGAG	492

Figure 12: BLAST sequence shows ATG substitution with TT resulting in a frameshift mutation. *pink* DNA amplified with *pink* primers are aligned with *rosy* DNA sequenced with *pink* primers(*ry/p1*). ATG sequence in wildtype *p* gene is substituted with TT in *p*¹ allele which results in a frameshift mutation. E Value and score of 0 and 767 respectively suggests that substitution is statistically significant.

ry/p1:	303	ATGTATTCGATTACCTGGATCCGGAGATGATCTGGGAAGTGGACGACGCTACGC _{GGG} GAT	362
3R:	1721	ATGTATTCGATTACCTGGATCCGGAGATGATCTGGGAAGTGGACGACGCTACGC _{GGG} GAT	1662

ry/p1:	363	CACATAGCTGCCGGATTGTGCTGCTAAATAACTTCGCAAAT _G CGGAGATTGTCAAGTGT	422
3R:	1661	CACATAGCTGCCGGATTGTGCTGCTAAATAACTTCGCAAAT _G CGGAGATTGTCAAGTGT	1602

ry/p1:	423	GAGCATTGCTCGTCCCCCTCGCTTCGATAACCTCCTGCCAGTATCATGAGCTTGGAGCA	482
3R:	1601	GAGCATTGCTCGTCCCCCTCGCTTCGATAACCTCCTGCCAGTATCATGAGCTTGGAGCA	1542

Figure 13: Sequence alignment of *ry* DNA using *p* primers matches with wildtype *p* gene. *ry* DNA is amplified using *p* primer(*ry/p1*) is aligned with wildtype genomic *p* gene shows a 100% sequence match identity. This suggests that the two strands are identical and that *ry* mutants are wildtype at *p* locus. This serves as a molecular analysis of complementation of *p* and *ry*. E value and score of 0 and 760 suggests that comparison of the two DNA strands are statistically significant.

T C G C A A A T T C G G A G A T T
410 420

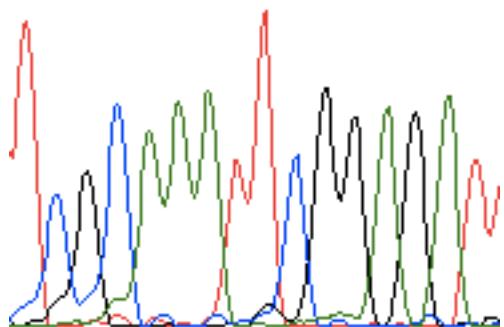


Figure 14: Region of mutation in *p* DNA chromatogram. *pink* primers were used to amplify *p* DNA in PCR. Highlighted region shows TT substitution.

T C G C A A A A T G C G G A G A T T
400 410

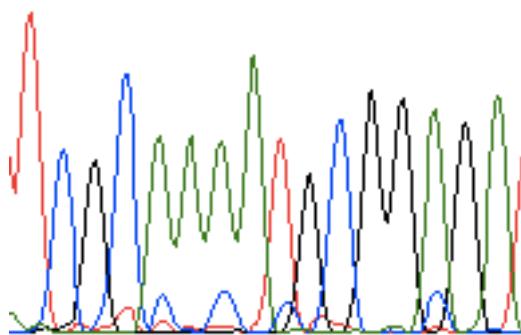


Figure 15: WT DNA of *p* gene chromatogram. Wildtype DNA obtained by PCR amplification of *r*y DNA with *pink* primers. Highlighted region shows wildtype ATG nucleotides.

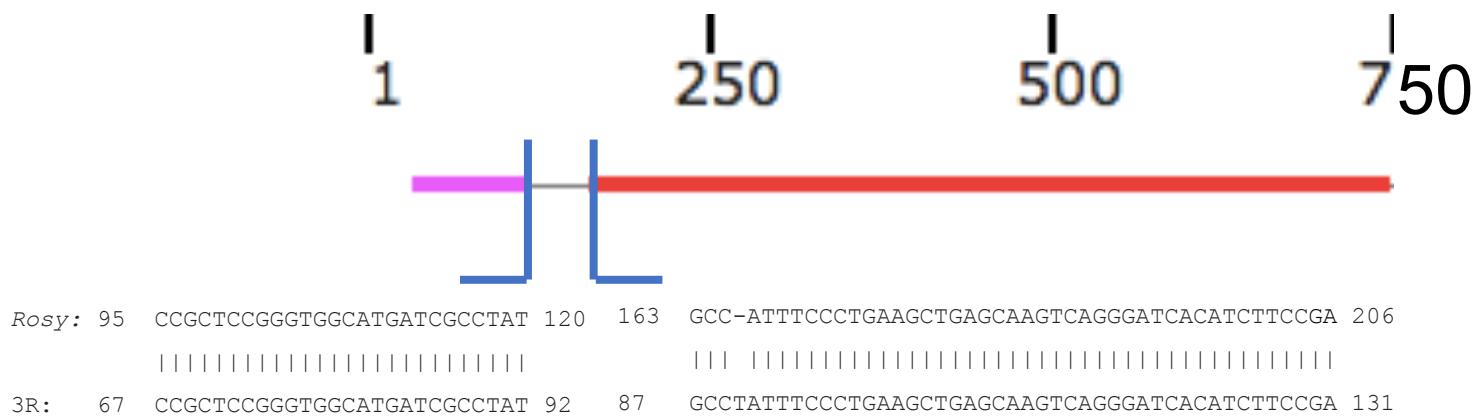


Figure 16: *ry* DNA and WT DNA sequence alignment suggests large deletion. *ry* DNA amplified with *ry* primer is aligned with WT DNA sequences at *ry* locus. Alignment suggests a 42bp deletion noted by missing DNA sequences between 120-163 nucleotides. E value of 8E10⁻⁴⁰ and score of 647 indicates that sequence identity is statically significant between *ry* and wildtype DNA.

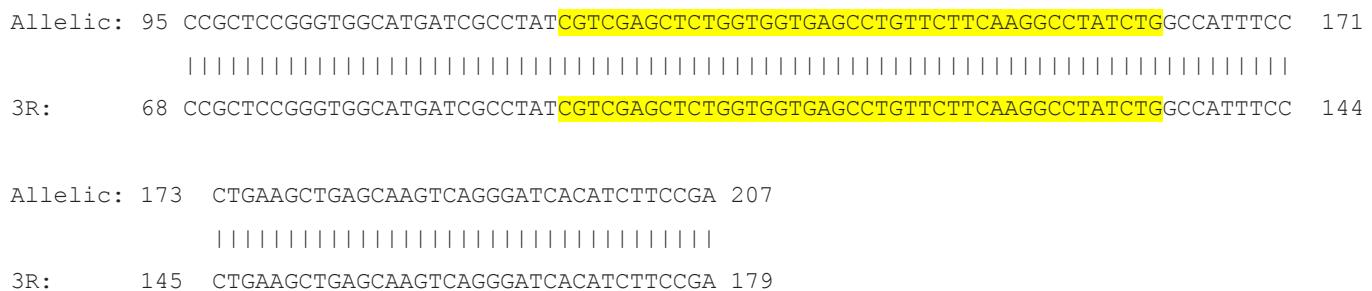


Figure 17: *p* DNA with *ry* primer and wildtype *ry* DNA sequence alignment. *p* DNA amplified with *ry* primer(query) and WT DNA sequence(subject) at *ry* locus. Note that sequence identity for both strand is 100% indicating that the DNA sequences are the same. This supports the claim that *p* mutant is wildtype at *ry* locus therefore complementation occurs. Because *p* is wildtype at *ry* locus, 42 bp deletion from nucleotide 120-163 missing in *ry* DNA (indicated in the highlighted) is present. E-value and scores of 8E10⁻⁴⁰ and 647 respectively, suggests that identical matches of both DNA sequences is statistically significant.

Drosophila	25	NRIKYTCFDISDSYIIFGASSGSLYLFNRNG-KFLLLIPNKHGAITSLSISA-NSKYVAF	82
		+R+K T +S ++ G+S G L+L + G K L + ++ GAI+ ++ + YVA	
Humans	33	SRLKCTSIAVSRKWALGSSGGHLHIQKEWKHRLFLSHREGAISQVACCLHDDDYVAV	92
Drosophila	83	ATQRSLICVYAVNLSAQATPQVIFTHLD-QSVQVTCIHWTDQDEKQFYGGDSRGQVSLVLL	141
		AT + L+ V+ +N + P+ ++ + + +VT + W + + GD G+VS + L	
Humans	93	ATSQGLVVVWELNQERRGKPEQMYVSSEHKRRVTALCWDTAILRVFVGDHAGKVSAIKL	152
Drosophila	142	SS---FIGHSLLFNMTVHPLLYLDSPIVQIDDFEYLLLVSNCTKCILCNTEYEDYKQIGN	198
		++ + V + +DS +VQ+D + LL+S+ T+ LC+TE E + +IGN	
Humans	153	NTSKQAKAAAFAVMFPVQTITTVDSCVVQLDYLDGRLLISSLTRSFLCDTEREKFWKIGN	212
Drosophila	199	RPRDGAFGACFFVSPQESLQPSR IYCARPGSRVWEVDFEGEVIQTHQFKTALATAPARIQ	258
		+ RDG +GACFF Q IYCARPGSR+WEV+F+GEVI THQFK L+ P +	
Humans	213	KERDGEYGACFFPGRCGGQQPL IYCARPGSRMWEVNFDGEVISTHOFKKLLSLPPLPVI	272
Drosophila	259	RPGSGTDELDANAELLDYQPQNLQFAKVQRLNDDFLLAFTTELGLYIFDIRRSAVVLWS--	316
		S + D A Q+L F K+ L++ +L +TE G+YIF + V+LWS	
Human	273	TLRS-EPOYDHTAG----SSQSLSFPKLLHLSEHCVLTWTERGIYIFIPQNQVLLWSEV	327
Drosophila	317	NQFERIADCRSSGSEIFVFTQSGALYSVQLQTLQ	350
		+ +A CR +E+F +G + + L +++	
Humans	328	KDIQDVAVCR---NELFCLHNGKVSHLSLISVE	358

Figure 18: Alignment of pink protein using BLAST shows sequence homology between pink and human Hermansky- Pudlak syndrome 5(hps5) gene. Sequence identity between pink protein(query) and hps5 protein(subject) is 33% indicated by the 110 out of 334 identical amino acid shared between both proteins. Additionally, there are an addition 72 similar amino acid sequence shared between both organism resulting in a positive value of 54%. Highly conserved regions between both proteins are highlighted. Of the sequences, 16/334 or 4% are gaps between both sequences indicative of insertion or deletion of amino acids. Blanks between each strand indicates regions of proteins where amino acid sequences between the two organisms are not conserved. E value and score of $3e10^{-44}$ and 176 indicates that homology between highly conserved regions is statistically significant.

	Phenotype	Observed	Expected	O-E	(O-E)²	(O-E)²/ E
Parental	h, e	376	402	-26	676	1.68
Parental	p	394	402	-8	64	0.16
Single c/o	h, p	126	113	13	169	1.50
Single c/o	e	118	113	5	25	0.22
Single c/o	p,e	137	120	17	289	2.41
Single c/o	h	130	120	10	100	0.83
Double c/o	wt	35	34	1	1	0.03
Double c/o	h,p,e	22	34	-12	144	4.24
	Totals	1338	1338		X ² =	11.06

Table 10: Three-point mapping cross between h, p, and e chi-square analysis. Heterozygous h/+, +/p, e/+ virgin females from MC1 were crossed with triple mutants h, p, e males. Calculations for expected single recombination is (total # of progeny x distance between gene) – (# of double recombinant progeny). For single recombination between h and p, ((1338 x (0.48-0.26) - (1338 X 0.22 X 0.23)) / 2 is 113. For single recombination between p and e, ((1338 x (0.71-0.48) - (1338 X 0.22 X 0.23)) / 2 is 120. Expected double recombinant progeny calculation is (1338 X 0.22 X 0.23) / 2 is 34. Possible deviation in the number of double recombinants is because of an interference of 0.16. Expected parental progeny is calculated by subtracting all the other classes such as 1338-227-240-67= 804/2= 402. We observed slightly less parental cross than expected and more double recombinant than expected. However, for the other crosses, small deviations from expected are seen. At 7 degrees of freedoms (dfs), expected chi square value is 14.07 at p=0.05. Our chi square value of 11.06<14.07, indicating that our data is statistically significant.

Gene pair	Observed	Expected	(O-E)	(O-E) ²	(O-E) ² /E
<i>p-e</i>	24.22	23.02	1.20	1.44	0.06
<i>h-e</i>	38.19	34.83	3.36	11.29	0.32
<i>p-h</i>	22.50	21.97	0.53	0.28	0.01
				X ² =	0.40

Table 11: Chi- Square analysis for recombination frequencies between *h*,*p*, and *e* suggests that *p* is located at 3-48. Observed and expected recombination frequency between gene pairs are listed above. At 2 dfs, $0.40 < 5.99$ at $p=0.05$ for the calculated data. This indicates that recombination frequency between *p*,*h*, and *e* are statistically significant.

A

Gene Pairs	Map Distance
<i>p-h</i>	3.50
<i>p-e</i>	46.78
Total	50.28

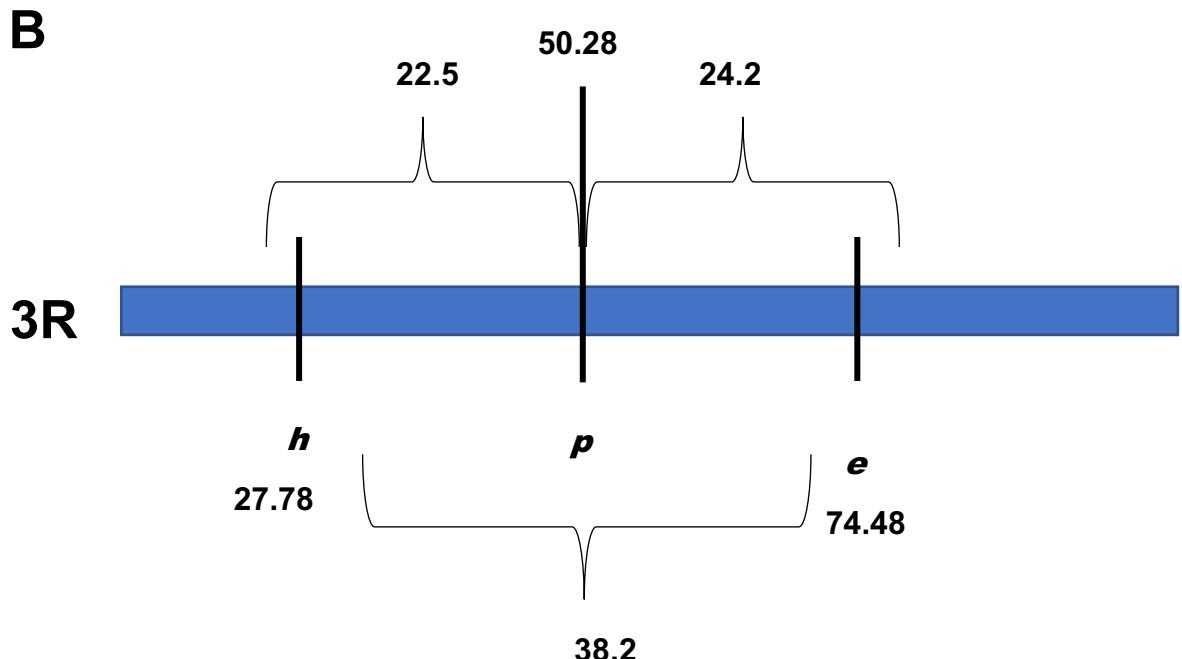


Figure 19: Predicted Genetic Map for *p* and marker genes *h* and *e* on chromosome 3.(A) Calculated mapping distance for *p* with respect to markers *h* and *e*. Studies show that distance for *h* and *e* are 26.0mu and 71.0mu respectively. Based on our data, *p* has a recombination frequency of 22.5% and 24.22% with *h* and *e* respectively. To get the experimental location of *p*, experimental recombination frequencies are subtracted from known marker distances and averages ie $((26-22.5)+(71-24.22))= 50.28$. To find experimental marker location, experimental location of *p* is subtracted or added to observed frequency of *h* and *e* respectively. (B) Genetic map shows *p* location with respect with *h* and *e* using experimental data.