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# Structure and Phenotypic Analysis of Notch Mutations

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# Structure and Phenotypic Analysis of *Notch* Mutations

By

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# ABSTRACT

The *Notch* gene encodes a transmembrane protein that functions as a receptor for intracellular signals in *Drosophila melanogaster*. The *Notch* gene is required for cell proliferation as well as differentiation. A *Notch* mutation, $N^{nd\text{-}p}$, is caused by the insertion of a P element into the first exon upstream of the start of translation of the *Notch* gene. The insertion causes a weak *Notch* mutation. This *Notch* mutation is lethal in the presence of an *enhancer of rudimentary* mutation. This lethality of the X chromosome that contains $N^{nd\text{-}p}$ and $e(r)^{27\text{-}1}$ was hypothesized to be caused by an interaction between these two mutations. In order to support this hypothesis, two aims were done. The first aim was to revert $N^{nd\text{-}p}$ and then examine the viability in the presence of $e(r)^{27\text{-}1}$. The second aim was to revert the lethality of the $N^{nd\text{-}p}$ $e(r)^{27\text{-}1}$ chromosome and then examine the $N^{nd\text{-}p}$ mutation. It was determined that the $N^{nd\text{-}p}$ mutation is the cause of the lethality. In the first case where $N^{nd\text{-}p}$ was reverted, the lethality was also reverted. In the second case where the lethality was reverted, $N^{nd\text{-}p}$ was concurrently reverted. The two $N^{nd\text{-}p}$ revertants, $R(N^{nd\text{-}p})^4$ and $R(N^{nd\text{-}p})^9$, were determined to be imprecise excisions of the P element where the P element left some of its DNA behind. New *Notch* mutations caused by the mobilization of the P element $N^{nd\text{-}p}$ were also isolated. Each of them ($N^2$, $N^3$, *and* $N^5$) resulted in a further decrease in *Notch* activity. Each of the mutations were analyzed by PCR and DNA sequencing in order to determine the precise structure of the mutation. The mutant phenotypes were studied by examining viability of both the larvae and the adults. By examining the viability, the point in the *Drosophila* life cycle where the lethal interaction occurs can be determined. Crosses were set up between males with the X

chromosome tagged with GFP and female containing a *Notch* mutation on one of the X chromosomes and GFP on the other. The males and females were scored based on their visible phenotypes. The larvae were observed through the 1st, 2nd, and 3rd Instar stages. The $N^2$ and $N^3$ mutants were found to be embryonic lethal. Observing the viability of the larvae, shows that the *e(r)* gene can affect *N* expression in the embryo stage. The *Notch* mutant larvae did not give rise to adult flies, except for one $N^3$ male that survived to adulthood. This revertant of $N^3$ was found to have a wild type *Notch* gene probably due to recombination with the GFP-containing X chromosome. The lethal interaction between $N^{nd\text{-}p}e(r)^{27\text{-}1}$ was previously shown to be during the pupal stage, however, $N^5e(r)^{27\text{-}1}$ was found to be lethal during embryogenesis. These results indicate that *e(r)* is necessary for *Notch* activity both during embryogenesis and metamorphosis.

# BACKGROUND AND SIGNIFICANCE

***Drosophila melanogaster* as a model organism.**

The fruit fly, *Drosophila melanogaster*, serves as a well-known model organism, since its entire genome has been sequenced and homologues of many of its genes are also found in mammalian genomes. There are more than 13,500 genes present. Approximately sixty percent genes are conserved between *Drosophila melanogaster* and homosapiens. Therefore, approximately 75% of known human disease genes have a recognizable match in the genetic code of fruit flies. For example, both humans and *Drosophila* have a tendency to develop cancer as a result of mutations in the genes that control the cell cycle. It is believed that sixty seven known human cancer genes have related genes in *Drosophila* (1). There are four pairs of chromosomes (three pairs of autosomes and two sex chromosomes). A few of those genes will be discussed and examined in this thesis. All of the genes that will discussed here are located on the X chromosome.

Neurological diseases such as Alzheimer's and Parkinson's continue to be thoroughly studied and there are a great deal of similarities between humans and *Drosophila.* Flies bred with the human gene "alpha-synuclein," show symptoms of brain damage that not only mimic those in humans but also appear to be closer in the fly than those used in mouse models of Parkinson's. The symptoms observed in the flies are loss of motor coordination and tremors. The flies could become a model for the testing of new anti-Parkinson's therapy quickly and effectively, since the lifespan of *Drosophila* is short and multiple generations form quickly. Other genes have been discovered in *Drosophila* that suppress the onset of the disease for about 10 days. And yet other genes have been discovered that prevent the formation of "Lewy

bodies", abnormal lumps inside nerve cells, in Parkinson patients and in *Drosophila* (2)

**Life Cycle of *Drosophila*.**

The males and females are easily identifiable and there is little cost and care needed for their survival. Flies that are wildtype have characteristic red eyes, a yellow-body, and black rings across their abdomen; the base of the male's abdomen is black. Females are approximately 2.5 millimeters in length and males are slightly smaller than the females. When certain mutations occur in the *Drosophila melanogaster*, phenotypic changes are visible in comparison to a wildtype fly.

There are four stages in the life cycle of *Drosophila*; embryonic, larval, pupal, and adult stage (Figure 1). The life cycle takes approximately 12 days from start to finish at 25°C; 8 days in the embryo and three larval stages and 6 days in the pupal stage. The females lay eggs upon the food source, and this is when the *Drosophila* begins its life as an embryo. The 1$^{st}$ instar larvae hatch from the eggs, crawl into the food source and eat. After approximately 1 day, the 1$^{st}$ instar larva molts, crawling out of its external cuticle, and becomes a 2$^{nd}$ instar larva. The larvae have molting periods, because in order to shed the cuticle, mouth, hooks, and spiracles are all shed. The larvae are called instars during the period of growth before and after molting. After two days, the larvae molt again and become 3$^{rd}$ instar larvae which, crawl out of the food source and the cuticle becomes the pupal case after two days.

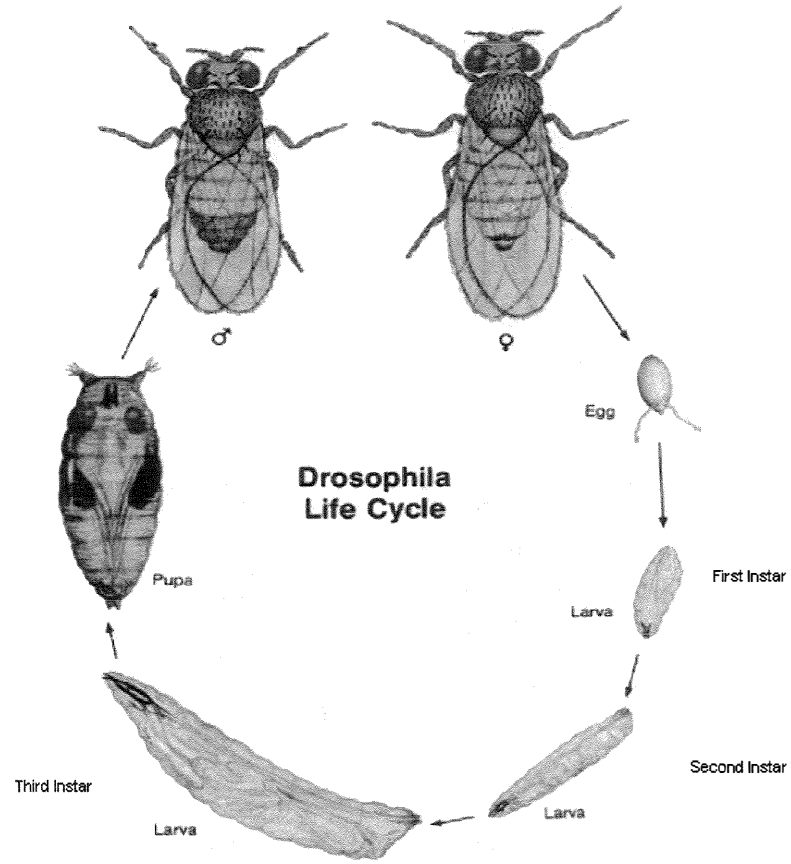



**Figure 1:** The following figure represents the *Drosophila melanogaster* life cycle in pictorial form beginning with the embryonic stage and ending with the adult stage. There are 3 larval stages noted on the chart as 1st Instar, 2nd Instar and 3rd Instar. (3)

After the third instar phase, the larva stop moving and form a puparium, this becomes hard in texture and dark in color. They remain in the puparium for approximately 6 days, and it is here that metamorphosis occurs. During metamorphosis, adult structures such as wings, legs, and eyes develop. Approximately, 24 hours before the adult emerges, the wings and the pigment of the eyes can be seen through the puparium (Figure 1). After metamorphosis is completed, the adult fly forces its way through the operculum, the anterior end, of the

puparium. The newly emerged fruit fly appears light in color with a long abdomen and wings that are unexpanded. Within a few hours, the fly becomes darker in color, rounder in the abdomen, and the wings become extended. After forty eight hours, from emerging from the puparium, females start laying eggs. For the first twelve hours a female can be considered a virgin after emerging from the puparium. After reaching maturity, the flies are fertile for the remainder of their lives.

**The *Notch* gene.**

The gene *Notch*, *N*, discovered in *Drosophila melanogaster*, is involved in signaling processes, such as lateral inhibition, and cellular differentiation. A mutation in the *Notch* gene produced notches in the wings of *Drosophila*. The notches appeared as a result of a partial loss of function of the *Notch* gene.

The *Notch* gene encodes a 300 kDa receptor which is a large single pass type I transmembrane proteins. Whereas mammals have four Notch paralogs that display redundant and unique functions, and *Caenorhabditis elegans* have two redundant Notch receptors, *Drosophila melanogaster* possesses only one Notch receptor (4). The Notch receptor belongs to a family of transmembrane proteins that are conserved from *Drosophila* to vertebrates and are thought to be involved in the fates of cells. *Notch* is required for nervous system development of the *Drosophila*, but also for the proper formation of the mesoderm, germ line ovarian follicle cells, larval Malphigian tubules, adult sensory bristles and eye structures (5, 6). The Notch receptors are composed of functional extracellular (NECD), transmembrane (TM) and intracellular (NICD) domains. The extracellular domain of the receptor contains 36 tandem

epidermal growth factor (EGF)-like repeats along with three cysteine rich Notch/LIN-12 repeats. Within the extracellular domain, the EGF repeats are followed by a unique negative regulatory region (NRR), which is composed of three cyteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain. The negative regulatory region plays a critical role in preventing receptor activation in the absence of ligands (4).

The activation of the *Notch* receptor in the wing disc induces expression of the wing margin patterning genes, vestigial and wingless as well as strong mitotic activity. By modulating *Notch* signaling in specific precursor cells during *Drosophila* imaginal disc development, Masahiro *et al* demonstrated that *Notch* activity can influence cell proliferation (7).

**_Notch_ Signaling Pathway.**

*Notch* signaling is a conserved mechanism used by organisms in order to control cell fates and embryonic development through cellular interactions. *Notch* activity effects differentiation, proliferation, and apoptotic processes, which in turn influence organ formation and morphogenesis. *Notch* signaling demonstrates the ability of sensing a small change in cell fate and amplifying it, acting as a sort of contrast enhancement mechanism in cell fate determination (8).

Signaling exchanges that occur between neighboring cells, through use of the *Notch* receptor, amplify and consolidate molecular differences. Signaling exchanges control how cells respond to intrinsic and extrinsic developmental cues that are necessary for specific developmental processes. The effect of *Notch* signaling is the

lateral inhibition of one cell, from a cell cluster, for a given fate and the inhibition of those cells not elected to differentiate. Lateral inhibition occurs repeatedly in *Drosophila* development (9).

The Notch receptor functions in the three germ layers during embryonic development, in the germ cell line, and in imaginal discs during the larval and pupal stages. For example, the *Notch* pathway is involved in germ cell line maintenance and the development of the sensory bristle, retinal development in the eye antennal disc and wing margin morphogenesis. The *Notch* pathway is involved in the maintenance of epithelial structures in the ectoderm such as the optic lobe, Malphigian tubule, salivary glands, foregut and trachea. *Notch* is involved in mesodermally derived tissues such as pericardial and peritracheal cells and in endodermally derived tissues such as the maintenance of epithelial cells, and the transition of midgut precursors that become midgut rudiments (10).

The *Notch* receptors are activated by the *Delta* and *Serrate* families of membrane bound ligands. The interaction of *Notch* receptor with these ligands is essential for the initiation of intracellular events. These two ligands interact with the Ankyrin repeats of the intracellular domain of the *Notch* receptor. Some of these ligands float free around the outside of the cell and others are bound to the surface of the cells. The interaction of *Notch* with its ligand triggers a chain of intracellular events resulting in lateral inhibition, the prevention of a specified cell fate. Through the study of genetic and molecular interactions, a number of proteins were identified that may be involved in the transmission or regulation of *Notch* signals. The extracellular domain of the ligands for the *Notch* receptor interacts with the

extracellular domain of the *Notch* receptor present on an adjacent cell. Posttranslational events seem to regulate the activities of the *Notch* receptor and the ligands. The interaction of the receptor with its specified ligand causes the cleavage of the intracellular region of the *Notch* protein, which travels into the cytoplasm where it triggers the activation of transcription factors within the cell's nucleus. The quantities of the receptor or the ligands present on the surface of the cells are important for successful *Notch* signaling (8).

ER and Golgi processing of *Notch* receptors in the signal-receiving cell results in cleavage of the receptor and produces a glycosylated, $Ca^{2+}$ stabilized heterodimer composed of NECD non-covalently attached to the TM-NICD inserted into the membrane. The receptor is then translocated to the plasma membrane for the ligand to bind to the receptor. Once the ligand binds to the *Notch* receptor, the NECD is cleaved from the TM-NICD domain. The NECD remains bound to the ligand and the complex undergoes endocytosis and recycling/degradation within the signal sending cell. In the signal receiving cell, a third cleavage event releases the NICD from the transmembrane, which translocates to the nucleus and associates with the transcription factor complex. This association results in subsequent activation of the *Notch* target genes (11).

Mutations in *deltex* have been shown to suppress lethality of certain *Notch* mutations. However, over expression of *deltex* and activated *Notch* both generate similar adult phenotypes. These results suggest that *deltex* is a positive regulator of the *Notch* pathway. If the function of *deltex* is lost, the phenotypes are rescued by activated *Notch*, suggesting that *Notch* activation bypasses the requirement for *deltex*

and therefore, *deltex* is most likely upstream of *Notch.* Suppressor of Hairless, a transcription factor, appears to be a downstream effector of *Notch* signaling (12).

**Mutations in the *Notch* gene.**

Mutations in the *Notch* gene are known to affect many developmental processes in the segregation of different cell types in both embryonic and imaginal development. In the epidermis of the wing, *Notch* mutations affect the veins; *Notch* loss-of-function alleles have thicker veins and notches in the wing margin. The *Notch* gain-of-function alleles result in larger wings with a lack of veins (13).

Embryos homozygous for a null mutation in *Notch* are lethal. In the absence of *Notch*, cells in the neurogenic region all differentiate as neural cells because they are unable to acquire an epidermal fate. In the peripheral nervous system, loss of *Notch* results in hyperplasia of smooth muscle cells at the expense of epidermal cells. *Notch* is known for its pleiotropy because of its affects on numerous tissues and organs such as the ovary and its requirement for the differentiation of sensory organs such as the eye. Over expression of activated *Notch* results in an overproduction of epidermis at the expense of neural structures. Therefore, *Notch* mutants lack an epidermis that participates in the specification of muscle progenitor cells and muscle differentiation. *Notch* is believed to regulate three processes in embryonic myogenesis; control of the initial segregation of muscle progenitors from among competent myoblasts, control of a phase when founder cells identity remains sensitive

to Notch activity until myoblast fusion. Finally, *Notch* can suppress muscle development by regulating a nonautonomous signal from the ectoderm (5).

**The *rudimentary* gene.**

The *rudimentary* gene, *r*, on the X chromosome, is approximately 15kb in size and is involved in RNA and DNA synthesis. It encodes the first 3 enzymes in the pyrimidine biosynthetic pathway: Carbamoyl phosphate synthetase (CPSase), Aspartate transcarbamoylase (ATCase) and Dihydroorotase (DHOase). All three of the enzymes are translated on a single polypeptide and have a characteristic developmental pattern of expression. The pathway is activated in proliferating cells due to an increase in demand for nucleotides needed for DNA synthesis. Pyrimidine nucleotides play a critical role in cellular metabolism serving as activated precursors of RNA and DNA. The Pyrimidine biosynthesis pathway occurs universally in all organisms and is subject to diverse regulatory mechanisms. The highest levels of activity are seen in the embryos and first instar larvae of *Drosophila.* The level of activity decreases during development and is lowest in pupae and adults. There are differences observed in the levels of activities between tissues. The 3$^{rd}$ instar larvae, which have low levels of *r* gene product, have high levels of the product in the imaginal discs. High levels are observed in ovaries and developing eggs of adult females (14, 15)

Mutations of the *rudimentary* gene result in pyrimidine auxotrophy, where the mutants are unable to synthesize pyrimidines needed for proper growth. Null and

hypomorphic mutants at the *r* locus result in rescuable maternal effect lethality and an oblique truncation of the wings. The lethality is an indication of sterility of females. This lethality can be rescued by introducing the wild-type gene via the sperm (15). The wing truncation is caused by a lack of the *r gene* product in the wing during metamorphosis. The female sterility directly correlates with the severity of the wing truncation (16).

Since most *Drosophila* media contains pyrimidines, *rudimentary* mutants are able to survive to adulthood. However, the truncation phenotype, apparent in both males and females, suggests that the *rudimentary* gene is involved in ensuring normal development of wings. This reasoning is supported by the fact that *rudimentary* expression is highest in embryos, adult females and imaginal discs (14). The *rudimentary* mutant wing phenotype is extremely sensitive to changes in the *rudimentary* expression. Therefore, this phenotype is a powerful tool in isolation of cis-acting regulatory mechanisms at the *rudimentary* gene and trans-acting mutations which affect the wing phenotype (15, 16).

***Enhancer of rudimentary* gene.**

The *enhancer of rudimentary* gene, *e(r)*, encodes a 104 amino acid protein, ER, involved in pyrimidine biosynthesis and the cell cycle. The *e(r)* gene acts as a recessive enhancer of a weak *r* mutant wing phenotype. *Enhancer of rudimentary* is a regulator of the *rudimentary* gene and was isolated as a recessive enhancer of the mutant wing phenotype of *rudimentary* mutants. The *e(r)* gene encodes 2 transcripts, a 1.0kb and 1.2kb transcript, which have the same 5' ends and differ only in their 3'

untranslated regions. This difference is a result of alternative polyadenlyation sites spaced 228bp apart (17). The 1.0kb transcript is present in both males and females, where as the 1.2kb transcript is found only in adult females and is believed to be involved in oogenesis. The longer transcript is found in the nurse cells of developing ovaries and in preblastoderm embryos. Expression of *e(r)* is found in the nurse cells of the ovary and the transcripts are maternally deposited into the egg. Since the *e(r)* gene is involved in the biosynthesis of pyrimidines, mutations in *e(r)* are believed to be null mutations and are thought to be recessive lethal (18).

The ER protein is nuclearly localized, which is consistent with its proposed role as a transcription factor (18). The *enhancer of rudimentary* protein is highly conserved (19). The proteins encoded by the human and murine *e(r)* both contain three α-helices and four β-sheets, which constitutes a novel fold (20-22). The β-sheets mediate dimerization of two monomeric ER proteins. The ER proteins also contain hydrophobic pockets, which suggest that the protein-protein interactions play a role in the function of the protein (20).

**Mutations in *Enhancer of rudimentary* gene.**

Hypomorphic mutations of *e(r)* show an enhancement of the hypomorphic *rudimentary* mutant wing phenotype. These mutants in the wild type background are viable, fertile and morphologically wild type (18). The high conservation of *e(r)* suggests that it encodes a vital function. Mutants with deficiencies of the *e(r)* gene are viable and morphologically wild type; however null males and females have decreased viability and for females decreased fertility. The pleiotropic effects of *e(r)*

null mutations and their interaction with different genes point to the involvement of *e(r)* in a number of different pathways such as pyrimidine biosynthesis, nerve cell specification and ovarian development. The expression of *e(r)* in the nurse cells of the ovary is consistent with low fertility and suggests an ovarian function (18).

**P elements.**

The four *Notch* mutants that were analyzed were created by the excision of the P element upstream of the start of translation of the 1st exon of the *Notch* gene. The *Drosophila* has many transposable elements that have been extensively studied. The P family of transposable elements has been studied quite thoroughly. It is widely believed the P elements have existed in the *Drosophila melanogaster* genome for less than 100 years. Based on this theory, the elements were introduced through a rare horizontal transmission event. The P element copies were acquired by *Drosophila melanogaster* from another *Drosophila* species followed by rapid spread through the global population (23).

An autonomous P element encodes a functional transposase, which binds to the ends of the transposon and catalyzes the movement of the transposon to another part of the genome. The structure of an autonomous P element is 2,097 bp sequence. The sequence features a 31-bp terminal inverted repeat and an 11-bp subterminal inverted repeat (24). The repeats are needed in cis for efficient transposition, however, they are not sufficient for it (25). Internally, there is a transposase gene composed of four exons. The transposase gene is required in trans for transposition

and a part of the gene is necessary for regulation of P mobility (26). The P element transposase is an 87kD protein encoded by autonomous P elements (23). Transposase is a double-stranded endonuclease needed for the transformation event. Transposase binds to subterminal regions at both ends of the element and represses transcription (27). GTP is also bound by transposase and is needed for transcription occurring in vitro (28).

When P elements are mobilized they produce traits known as hybrid dysgenesis in *Drosophila*. Some of these traits include temperature independent sterility, elevated rates of mutation, chromosome rearrangement and recombination. Sterility results as a loss of germ cells early in development (23,29). In females, there are fewer germ cells so this trait is seen more readily. Mutations arise, like the ones studied here, because the P element inserts into the genes or by an imprecise excision of the element near the genes. Chromosome rearrangements result from a breakage at the sites of 2 or more P element insertions. Following the breakage, the chromosome segments rejoin in a different order. Recombination induced by a P element occurs in genetic intervals containing mobile P elements. Recombination occurs usually within 2kb of the insertion site (30-31).

When the excision of the P element occurs, there are 3 possible excision outcomes as seen in Figure 2. During an imprecise excision, a portion of the P element is left behind. The remaining base pairs may or may not cause a mutation. When the P element jumps out cleanly, a precise excision occurs, leaving the DNA in the same manner as before the P element was inserted, having no effect. The final excision outcome is a deletion. This occurs when the P element jumps out, taking

some of the DNA with it. These 3 possible excisions may or may not cause a visible mutant phenotype. When the excision of the P element occurs, the P element can imbed in another portion of the genome or it can be lost altogether.

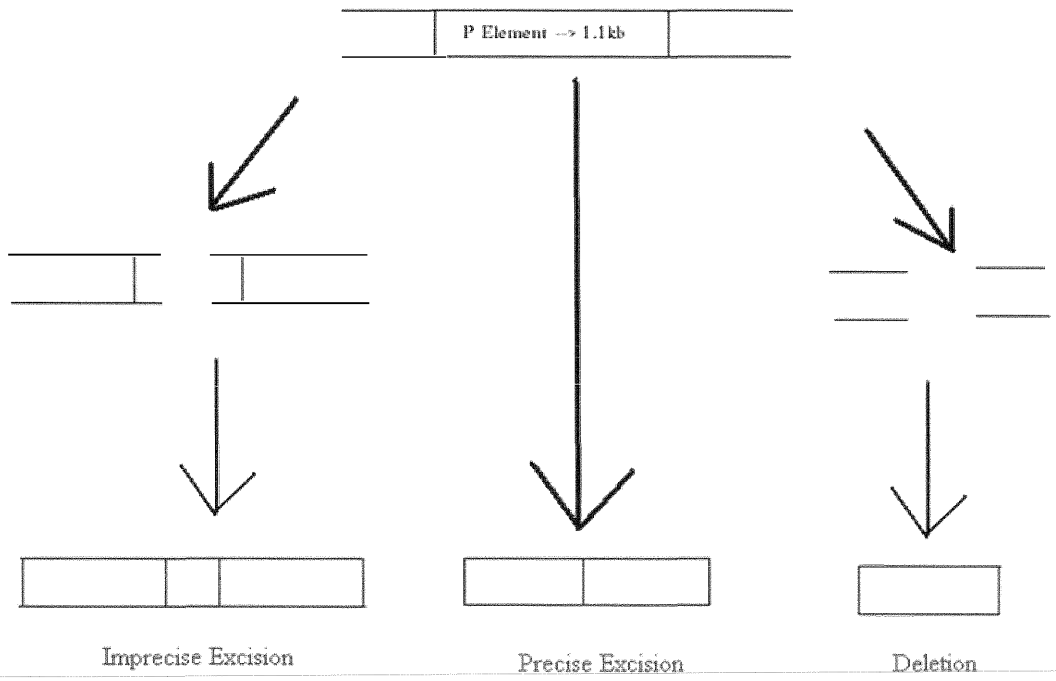



**Figure 2:** A diagram representing the three possible outcomes when the excision of the P element occurs. The three possible outcomes are imprecise excision, precise excision and deletion. The imprecise excision occurs when DNA from the P element is left behind, and the precise excision occurs when no DNA from the P element is left behind. A deletion occurs when a portion of the flanking DNA is taken when the P element is excised.

The P element used in this research is non-autonomous. Some of the P elements occur naturally through internal deletions of the autonomous element. These P elements lack the transposase gene yet still have parts of the sequence required in cis for transposition. Mobilization of these P elements occurs only if there is at least one autonomous P element present to supply transposase (26).

**Creation of $N^{nd\text{-}p}$ Mutants.**

The mutation $N^{nd\text{-}p}$, caused by the excision of the non-autonomous P element, was selected (Dr. Stuart Tsubota personal communication). A recessive *Notch* allele, $N^{nd\text{-}p}$, was isolated when lethality was observed in combination with an *e(r)* allele, $e(r)^{p2}$. As single mutants both mutants are viable. The lethality is rescued by an $e(r)^{+}$ transgene, which demonstrates the lethality observed was created by a combination of the two mutations. In an $e(r)^{+}$ background, $N^{nd\text{-}p}$, is homozygous, viable, and fertile. Mutant stocks were generated. New *Notch* mutations were caused by the mobilization of the P element were isolated. Three of them $N^{2}$, $N^{3}$, and $N^{5}$ resulted in a further decrease in *Notch* activity. The fourth mutant and the ninth mutant examined is a revertant of the original $N^{nd\text{-}p}$ mutation. Each of the mutations were analyzed by PCR and DNA sequencing in order to determine the precise structure of the mutation. The mutant phenotypes were studied by examining both the viability of the mutant larvae and adults.

**Viability.**

The Viability of $N^{nd\text{-}p}$ mutation has been documented to be approximately 40% viable in the presence of the wild type *e(r)* gene. However, if *e(r)* is deleted and a mutation occurs in *Notch* to give $N^{nd\text{-}p}$, lethality will occur. Lethality occurs because $N^{nd\text{-}p}$ reduces *Notch* expression and since e(r) is an activator of *Notch*, the mutation in *e(r)* causes a reduction in expression of the *e(r)* gene. This reduction of *e(r)* causes the activation of *Notch* to be significantly decreased and therefore the lethality occurs (32).

Figure 3, shows which *Notch* mutants are viable and lethal. When there is a wild type $N^{+}$ and $e(r)^{+}$ there is 100% viability. When there is a mutation in both *N* and *e(r)* a lethality will occur. $N^{4}$ and $N^{5}$ mutants in the presence of a wild type *e(r)* are viable. The *Notch* mutants $N^{2}$, $N^{3}$and $N^{5}$ in the presence of mutant *e(r)* is lethal, however $N^{4}$ mutants are viable.

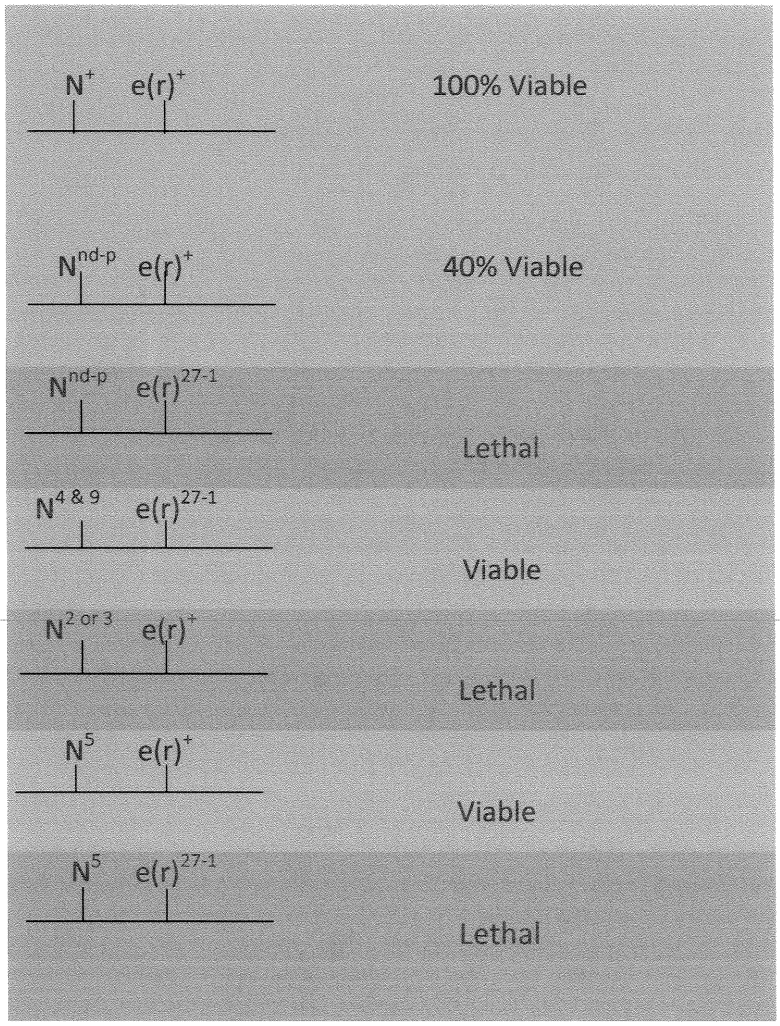



**Figure 3:** A chart that showing how viability is affected by a mutation in *Notch*, and/or a mutation in *e(r)*. A $N^{nd-p}$ mutation in the presence of wild type *e(r)*, has a viability of 40%. A *Notch* mutation and an *e(r)* mutation is lethal, as is the $N^{5}$ mutation, however the $N^{4}$ mutation is viable. Mutants $N^{2}$ and $N^{3}$ are lethal in the presence of wild type *e(r)*, where as $N^{5}$ mutants are viable.

The lethality that is observed in the fruit flies contains $N^{nd\text{-}p}$ and $e(r)^{27\text{-}1}$. The *e(r)* gene is believed to be a positive regulator of *Notch*. It has been shown that a wild-type transgene of *e(r)* rescues the lethality, therefore, $e(r)^{27\text{-}1}$ is believed to be necessary for the lethality. There is a possibility that another mutation that maps close to *Notch* is causing the synthetic lethality with $e(r)^{27\text{-}1}$. Therefore, there are two possible hypotheses: 1) the lethality is caused by $N^{nd\text{-}p}$, and 2) the lethality is caused by an independent mutation. In order to test the hypotheses, the first approach was to revert $N^{nd\text{-}p}$. If $N^{nd\text{-}p}$ is the cause of the lethality observed, then the revertant will be lethal when combined with $e(r)^{27\text{-}1}$. If the lethality is the cause of an independent mutation, the reverting of $N^{nd\text{-}p}$ will not revert the lethality. Determine the DNA sequence of the revertant of $N^3$ to obtain clues as to its origin.

The new *Notch* mutants, $N^2$, $N^3$, and $N^5$ appear at a phenotypic level to be hypomorphic mutations. To support this hypothesis, the mutations were examined at the DNA level. The mutations were all believed to be deletions or imprecise excisions that leave the coding region intact.

The lethal interaction between $N^{nd\text{-}p}$ and $e(r)^{27\text{-}1}$ was examined, in order to indicate when *e(r)* activity is necessary to activate *Notch* activity. During the life cycle of the *Drosophila*, the larvae were collected and examined under a microscope with a GFP filter at the 3 larval stages. The $N^2$, $N^3$, and $N^5$ mutants and a double mutant $N^5e(r)^{27\text{-}1}$ were examined at the larval stages. $N^{nd\text{-}p}e(r)^{27\text{-}1}$ is lethal during the pupal stage, indicating that *e(r)* is necessary for *Notch* activity during this stage and not earlier. The DNA sequence of revertant $N^3$ was generated in order to obtain clues as to the origin of the $N^3$ mutant.

# Materials and Methods

## Analysis of *Notch* mutants

### Genetic Crosses.

Genetic markers were used to differentiate the mutants from those carrying GFP. The *yellow* gene, *y*, controls the melanotic pigment patterns for the cuticle of the adult fly. However, this locus also controls the pigmented mouth parts and denticle belts of the larval cuticle, the protective layer of the epidermis in Drosophila. The *white* gene, w, is involved in production and distribution of the ommochrome (brown pigments) and pteridine (red pigments). These pigments are found in the eyes and ocelli of adult flies and in the sheath of the testis, as well as in larval Malphigian tubules. The function of the protein encoded by this gene is not known, but it is suggested that it is a membrane-associated ATP-binding transport protein for pigment precursors both in the ommochrome and pteridine pathways. The *crossveinless gene,* cv, is a trace or absence of the crossveins present. The wings are affected due to an excessive contraction in the pupal stage, obliterating the cavity which should normally remain between the epithelia to form the vein (33, 34).

The *Notch* mutants were generated by Dr. Stuart Tsubota of The College at Brockport. In the initial screen X linked lethals that were rescued by an $e(r)^+$ transgene were separated. The starting X chromosome contained an *e(r)* allele, $e(r)^{p2}$, which had the only P element in stock. In the first cross, the P element Δ2-3 was introduced to the stock in order to mobilize the P element present in $e(r)^{p2}$. In the second cross, Tr[$e(r)^+$] was introduced into the stock to cover any X linked lethal that could be rescued by $e(r)^+$. Males containing the transgene and a mutant X

chromosome were crossed to C(1)DX, yf females. Any males that contained an X linked lethal that was rescued by Tr[$e(r)^+$] gave rise to male progeny which all carried Tr[$e(r)^+$]. These stocks were chosen by looking for males lacking white eyes.

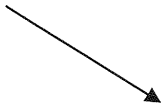
Revertants of $N^{nd\text{-}p}$, R($N^{nd\text{-}p}$), should be white-eye, notched wing females. A female fly with $N^8/N^{nd\text{-}p}$ is lethal. A set of $N^8$/ y w R($N^{nd\text{-}p}$) cv females were isolated from vial #4. From the females that were isolated a stock of y w R($N^{nd\text{-}p}$)cv / FM7c was isolated. The stocks were presumed to contain the same revertant and were labeled with 4.1, 4.2, 4.3 and 4.4, etc.

Another cross generated new *N* alleles generated by the excision of the P element in $N^{nd\text{-}p}$. These new alleles would be isolated as notched wing females, y w $N^{new}$ cv/FM7c. Vials numbered 2 and 3 produced a set of females. The females from vial 2 had more severely notched wings than the females that were isolated from vial 3. Successive crosses showed females from vial 3 often had wild-type wings. The stocks were labeled 2.1, 2.2, etc. and 3.1, 3.2, etc.

## Generation of Revertants of $N^{nd\text{-}p}$ and $N^2$, $N^3$, and $N^5$ mutants:

## Cross #1:

*y w* $N^{nd\text{-}p}$*cv*/ FM7c females X pn;ry$^{506}$Dr P{ry[+t7.2]=Delta2-3}99B/TM6 males



*w* $N^8$/FM7c females X *y w* $N^{nd\text{-}p}$*cv*; ry$^{506}$Dr P{ry[+t7.2]=Delta2-3}99B/+ males

Revertants of $N^{nd\text{-}p}$ can also be isolated by the reversion of the lethality of the double mutant, $N^{nd\text{-}p}e(r)^{27\text{-}1}$. In experiment 2, a female, *y w* $N^{nd\text{-}p}e(r)^{27\text{-}1}$/ FM7c, was crossed with a male, FM7C;pn;ry$^{506}$Dr P{ry[+t7.2]=Delta2-3}99B/+. Males that were *y w* Rev($N^{nd\text{-}p}$) *cv* $e(r)^{27\text{-}1}$, would be seen as *y w cv* males. Two of these males were isolated from vial 9 and were designated R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$. Each male was crossed with *w* $N^8$/FM7c to establish stocks. This cross revealed that the revertant X chromosome is lethal over $N^8$. The R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$ both carried *cv* which should not be present on the chromosome, suggesting that a contamination may have occurred.

From vial 2 two *y w cv* males with scalloped wings were isolated. They were crossed to *w* $N^8$/FM7c females to set up stocks, but both males were sickly and the crosses failed. Since it was possible that the same mutant alleles were present as R($N^{nd\text{-}p}$)*cv* $e(r)^{27\text{-}1}$/FM7c from the same vial, these females were isolated and crossed to FM7c males. From vial 2, scalloped wing females were isolated and it was possible the mutation represents the same mutation as the two scalloped wing males. The female was crossed with an FM7c male to establish a stock; the vial was

designated #5. The chromosome contained *cv+*, which was seen when *y w* $N^{nd-p}$ *cv* males were crossed to heterozygous females. The new $N^5$ was viable over $N^{nd-p}$ and the females had normal crossveins.

## Cross #2:

*y w* $N^{nd-p}$ $e(r)^{27-1}$/FM7c females X *FM7c*;pn; $ry^{506}$Dr P{ry[+t7.2]=Delta2-3}99B/+

*y w* $N^{nd-p}$ $e(r)^{27-1}$/*FM7c*;
$y^{506}$Dr P{ry[+t7.2]=Delta2-3}99B X FM7c males

## Primers.

| Samples/Revertants | 5' primer | 3' primer | Expected Product Size (bp) |
|---|---|---|---|
| $N^4$ | Notch 5 prime D | Notch 3 prime D | 306 |
| $N^5$ | Notch 5 prime C | Notch 3 prime C2 | 1,146 |
| | Notch 5 prime B | Notch 3 prime C2 | 1,724 |
| | Notch 5 prime 2 | Notch 3 prime C2 | 1,473 |
| $N^2$ & $N^3$ | Notch 5 prime C | Notch 3 prime B3 | 2,069 |
| | Notch 5 prime D | Notch 3 prime B3 | 1,735 |

**Table 1:** Summary of primer sets used for each specific samples in order to determine the precise structure of the mutation caused by the mobilization of the P element.

In order to create a 100μM primer stock to be used for PCR , the tube containing the primer salt was centrifuged. The primer salt was dissolved with a specified of 1X TE buffer. The specified volume of TE buffer needed was determined by multiplying the amount of primer in nanograms times 10 and then the tube was centrifuged at 21,000 x g for 2 minutes. The tube was incubated at 37°C for 30 minutes. The primer stock was mixed and then centrifuged again at 21,000 x g for 2 minutes. To create the working solution from the primer stock, a 1:10 dilution was prepared.

**DNA Extractions for PCR.**

DNA preps were completed and used for amplification in PCR. A single fly was placed in a 1.5 ml tube and mashed with a pipette tip containing 50 μl of buffer (10 mM Tris-Cl pH 8.0, 1mM EDTA, 25 mM NaCl and 200 μg/ml of Proteinase K). The preps were incubated in a 37°C water bath for 30 minutes, in order to allow the proteinase K to digest the tissues and release the DNA. After the 30-minute incubation period, the preps were heated at a temperature of 95°C for 2 minutes in order to inactivate the Proteinase K. The inactivation of proteinase K is very important due to the fact that Taq polymerase is an important protein used in PCR and it can be digested and inactivated by proteinase K. Samples were centrifuged for 3 minutes to pellet the debris. These samples were stored in the -20°C freezer for future use.

## Recipes for TAE Buffers.

### 25X TAE Buffer (pH 8.0).

To make the buffer 121g of Tris Base, 28.55mL of glacial acetic acid with a pH of 2.4 and 50mL of 0.5M EDTA with a pH of 8.0 was added to a 1000mL beaker containing a stir bar. Then 500mL of distilled water was added to the beaker. The solution was brought up to the 1000mL line by adding distilled water.

### 1X TAE Buffer.

In order to make 2L, 80mL of 25X TAE (pH 8.0) was added to a 2L graduated cylinder and filled to the 2000mL line with distilled water.

### Mutation Analysis Using Polymerase Chain Reaction and Gel Electrophoresis.

Polymerase chain reaction was performed to estimate the size of the deletion of *Notch*. The total volume of the PCR reaction was 25 µl, consisting of 12 µl of sterile $H_2O$, 10 µl of Master Mix from BioRad, 1 µl of the chosen 5' primer, 1µl of the chosen 3' primer and 1 µl of the template DNA. The Master Mix contains the Taq Polymerase, dNTPs, and $Mg^{2+}$.

A 1.2% agarose gel was made, to run the PCR samples on, by adding 0.54g of agarose to 45mL of 1X TAE (Tris Base, Glacial Acetic Acid and 0.5M EDTA) and heating the solution for 3 minutes. At the conclusion of 3 minutes, the agarose solution was removed and swirled to determine the agarose is fully dissolved. The solution was heated for an additional minute and then removed. The solution was allowed to cool by swirling the beaker in a 35°C water bath and then once the

solution was cool to the touch 4.5uL of ethidium bromide was added to the solution. The agarose solution was added to the gel box and left to harden.

Once the gel hardened, Hyperladder I, from Bioline, and the PCR samples were added to the wells of the gel. Hyperladder I was added to the gel, so that the size of the DNA bands could be determined as well as the amount of DNA present in each of the bands. The amount of DNA present in each band is important when the samples were to be sent out for sequencing. The Hyperladder was prepared by adding 1μL of Hyperladder I to 10uL of distilled water along with 1uL of the loading dye from Bio-Rad. The entire 12μL of solution was then added to the gel. Hyperladder I contains 14 spaced bands, ranging from 200bp – 10,000bp. Approximately 20μL of the PCR product were added to the gel along with 2μL of loading dye. The gel was run at 80 volts for two hours.

A reamplification was performed by one of two methods. The first way was to take 1μL of the previously run PCR reaction. That 1μL represents the DNA source in a typical 20μL reaction. The reaction is run in the same manner as the first PCR reaction. The second method is to take 1μl of the previously run PCR reaction and dilute it in 99μL of distilled water. Then 1μL of the dilution was used in a reaction and run in the same manner as the first PCR reaction. Once the reamplification was completed, only 1uL of the PCR product was run on the gel. The PCR product to be run on the gel was prepared by adding 1μL of the PCR product to 10μL of Tris EDTA Buffer (Tris Base, Glacial Acetic Acid, 0.5M EDTA). The Hyperladder I solution added to the gel when performing PCR reamplifications, was the same as when performing normal PCRs. The gel was run at 80 volts for two hours.

**Gel Extraction.**

In order to analyze the $N^2$ and $N^3$ mutants, the smaller of the two bands seen in Figure 10 at approximately 1400bp was cut out of the gel using a hand held UV light which has longer wavelengths to keep DNA damage to a minimum. The gel was placed in a microcentrifuge tube with approximately 100μL of distilled $H_2O$. The microcentrifuge tube was then placed in a 37°C water bath in order to melt the gel.

Once the gel had melted completely, 1μL of the melted gel solution was used in a normal PCR reaction as the DNA. The PCR and gel was run as normal and once again the band at approximately 1400bp was cut out and the whole process of melting the gel was repeated. For a second time, a PCR and gel were ran as normal. The sample was purified and sent out for sequencing.

**DNA Purification.**

The DNA purification was performed using the QIAquick PCR purification kit from Qiagen The column was hen placed in a 1.5 ml microcentrifuge tube. In order to elute the DNA, 10 μl of Buffer EB (10Mm Tris-Cl pH 8.5) and 10 μL of distilled water or 0.1x TE (1mM Tris; 0.01mM EDTA) pH 8.0 was added to the center of the membrane in the column. The column was allowed to sit for a minute and then centrifuged for one minute at 16,000 x g. The purified DNA was kept in the 1.5 ml centrifuge and placed in the -20°C freezer.

**DNA Sequencing.**

To prepare samples for DNA sequencing, a specified amount of DNA was needed. The amount of DNA needed for sequencing was determined by dividing the number of basepairs for the bands on the gel by 5. The number obtained tells you the amount of DNA, in ng, needed for sequencing. The amount of DNA present in each band needed to be determined. The bands from the sample were compared to the bands from the molecular ladder. If 1μL of the Hyperladder and 1μL of the reamplification product was added to the gel, and both of these bands were the same intensity and size, then the bands have equal amounts of DNA present. The specified amount of DNA was added to a screw cap vial along with 0.8 μL of the 5 prime primer (10μM) used in the reaction in a final volume of 20μL. If there is enough DNA for a second vial to be sent for sequencing, then 0.8μL of the 3 prime primer used in the reaction can be sent out as well. The samples were sent to Cornell University's DNA Sequencing Facility.

## Analysis of Viability

**Scoring Adults.**

The flies were examined using the Zeiss dissecting microscope and the male and female flies were separated. All of the males were then separated into two separate groups. Males with a GFP X chromosome had red, and kidney bean shaped eyes with a yellow body. The males with a mutation in the *Notch* gene had large white eyes, a yellow body and were missing the crossvein. The females were then separated into two separate groups. Females that were homozygous for GFP have

small red, and kidney bean shaped eyes with a yellow body. The females with a mutation in the *Notch* gene have larger, slight kidney bean shaped eyes that are orange in color and have a yellow body.

**GFP Tagging.**

GFP stands for green fluorescent protein and was originally isolated from the bioluminescent jellyfish *Aequorea Victoria.* In *Aequorea victoria* a protein called aequorin releases blue light once it has bound to calcium. The blue light given off is absorbed by the GFP, which causes a green light to be emitted. GFP is important in the monitoring of gene expression both in vivo and in invitro. GFP is used widely as a fluorescent marker in molecular and cell biology. GFP's fluorophore is derived from post-translational cyclization of the serine-tyrosine-glycine tripeptide of GFP, followed by dehydrogenation of the tyrosine (35). Since GFP requires no exogenous moiety for fluorescence, it makes it a useful marker for studies in vivo.

The wild type GFP contains 238 amino acids and residues 65-67 in the sequence form the fluorescent chromophore p-hydroxylbenzylideimidazolinone (36). The amino acids are folded into a series of 6 alpha helices and 11 beta strands connected by loops. The beta strands form a classical beta barrel, a cylindrical beta sheet with anti-parallel strands. The fluorophore is buried deep within the beta barrel. The fluorophore interrupts the alpha helix that runs through the center of the barrel; therefore it is protected from interactions with solvents by the beta strands. This accounts for the stability of GFP fluorescence (35-36).

When the gene for GFP is fused to the gene of a protein or in this case the X chromosome of the *Drosophila,* the X chromosome retains its normal activity and the GFP protein retains its fluorescence. This way the location and activities of the studied chromosome can be studied by microscopic monitoring of GFP fluorescence. Having GFP tagged to different proteins or chromosomes allows for the effect of chemical inhibited mutations and gene knock outs to be studied (35-36).

The $N^2$, $N^3$, $N^5$ mutants are lethal and the stage in the life cycle at which they are dying can be determined through the use of GFP. One of the X chromosomes for the female flies had all of the wild-type genes as well as the GFP tag. The second X chromosome has the Notch mutation and the e(r) null mutation. The males have the wild-type genes and a GFP tag on their X chromosome and the Y chromosome remains unchanged. The progeny are observed at the 1st instar, 2nd instar, and 3rd instar and adult stages. For the larva stages the fly is observed under a dissecting microscope with a GFP filter, which allows for the larva to be separated into expressing and non-expressing groups. When the flies become adults, they are examined under a dissecting scope and the different progeny are separated as described in the counting crosses method.

When a cross is done between a wild type male and a female with a *Notch* mutation on one of the X chromosomes, four possible progeny are expected as seen in Figure 4; a female and male with a chromosome containing GFP, will have small red kidney bean shaped eyes and a yellow body, a female with one of the X chromosomes containing the *Notch* mutation, will have large kidney bean shaped eyes that are orange in color and yellow body, a male with a *Notch* mutation in the X chromosome

will have large white eyes and a yellow body. When scoring the adult flies, it is easy to separate the females and males that are wild type from the females and males that have a *Notch* mutation.

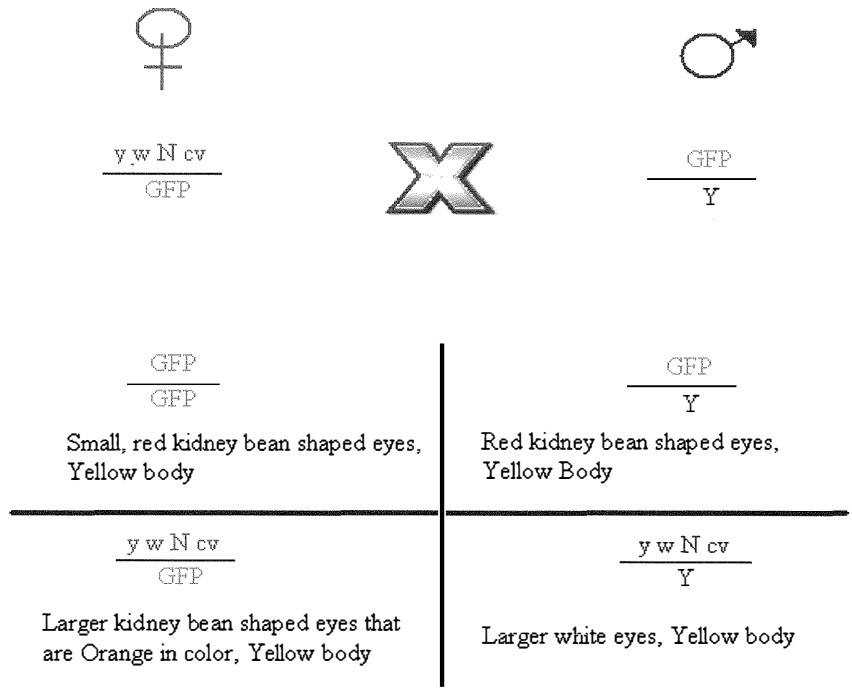



**Figure 4:** A cross between a wild type male and female with a *Notch* mutation on of the X chromosome. Four progeny are possible, a wild type male and female and a mutant male and female.

When the *Drosophila* are in the larval stage, the mutant larvae are easily separated from the wild type larvae under a dissecting scope using a GFP filter. The larvae that contain a chromosome tagged with GFP will fluoresce whereas; the male mutants will not glow. Therefore, theoretically if there are no viability differences, 75% of the possible progeny in Figure 4 will contain a GFP gene and will fluoresce under the GFP filter, but 25% of the progeny which are the male mutants will not.

# RESULTS

*Notch* loss of function alleles in Drosophila melanogaster have thicker veins and notches in the wing margin. In Figure 5, four wings are shown, each taken from a different fly with a different *Notch* mutation.

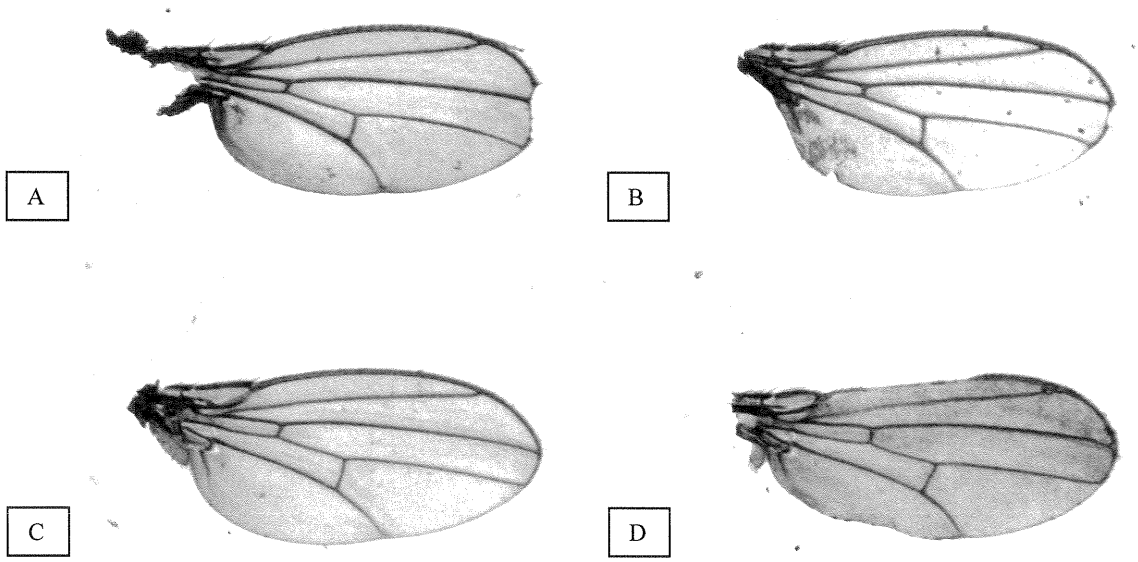



**Figure 5:** These wings from *Drosophila melanogaster* show the phenotypic results of a *Notch* mutation. Wings A-C were taken from heterozygous females. The wing seen in 5a represents a $N^8/N^+$ female, where as 5b represents a $N^3$ mutant that is $N^3/N^+$. Figure 5c, represents a wing from a $N^2$ mutant that is $N^2/N^+$. The wing seen in D was taken from a hemizygous male. The wings are ordered from the wing with the most *Notch* activity (A) to the wing with the least *Notch* activity (D). The wing seen in Figure 5d has the least *Notch* activity because it is $N^5$/Y. The $N^5$/Y wing represents a $N^5$mutation, and since the only *Notch* allele present is mutated, this male has no *Notch* activity (32)

## R($N^{nd\text{-}p}$)$^4$ Mutants.

The first aim was to determine the cause of the mutation in the $N^4$ gene. For example, did the P element have a precise excision and not leave any of its DNA behind or take any of the sample's DNA with it? Did the P element cause a deletion when it jumped out; removing some of the sample's DNA? Or did the P element have an imprecise excision, leaving behind some of its own DNA?

Revertants of $N^{nd\text{-}p}$ should be white-eye, notched wing females as heterozygotes ($N^8/N^4$). A female fly with $N^8/N^{nd\text{-}p}$ mutation is lethal. A set of $N^8$/ y w R($N^{nd\text{-}p}$) cv females were isolated from vial #4. From the females that were isolated a stock of y w R($N^{nd\text{-}p}$)cv / *FM7c* were isolated. The stocks were presumed to contain the same revertant and were labeled with 4.1, 4.2, 4.3 and 4.4, etc. In order to determine the cause of the mutation the DNA was extracted from four of the $N^4$ mutants 4.2, 4.3, 4.5 and 4.6. A primer set of *N* 5 prime D and *N* 3 with a wild type band of 587 bp was chosen. The primer set was chosen because the two primers flanked the region in which the P element was inserted.

As seen in Figure 6, a band of approximately 650 bp was visible for each of the four $N^4$ mutants on the agarose gel. The bands visible around approximately 200 bp represent the remaining primers. Since the band at approximately 650 bp differed from the expected wild type band of 587 bp for each of the four samples and there was one single clear, concise band, the PCR products for the R($N^{nd\text{-}p}$)$^4$ mutants were purified for sequencing. Since all 4 mutants gave the same band at approximately 650 bp, samples 4.2 and 4.3 were chosen to be sent out for sequencing. The bands, seen at

approximately 650 bp, are larger than expected so one possibility is that there are additional base pairs present in those bands.

The Cornell University DNA Sequencing Facility was able to sequence the DNA for both of the R($N^{md\text{-}p}$)$^4$ mutants and the sequences are found in Figure 7. The sequence was compared to the wild type sequence and analyzed by eye. After analyzing the sequences for samples 4.2 and 4.3, there are two things that are apparent. First, samples 4.2 and 4.3 are identical sequences. Secondly, there is an additional 48 bp present around the P element's insertion site when compared to the wild type sequence.

## R($N^{nd-p}$)$^4$ Mutants

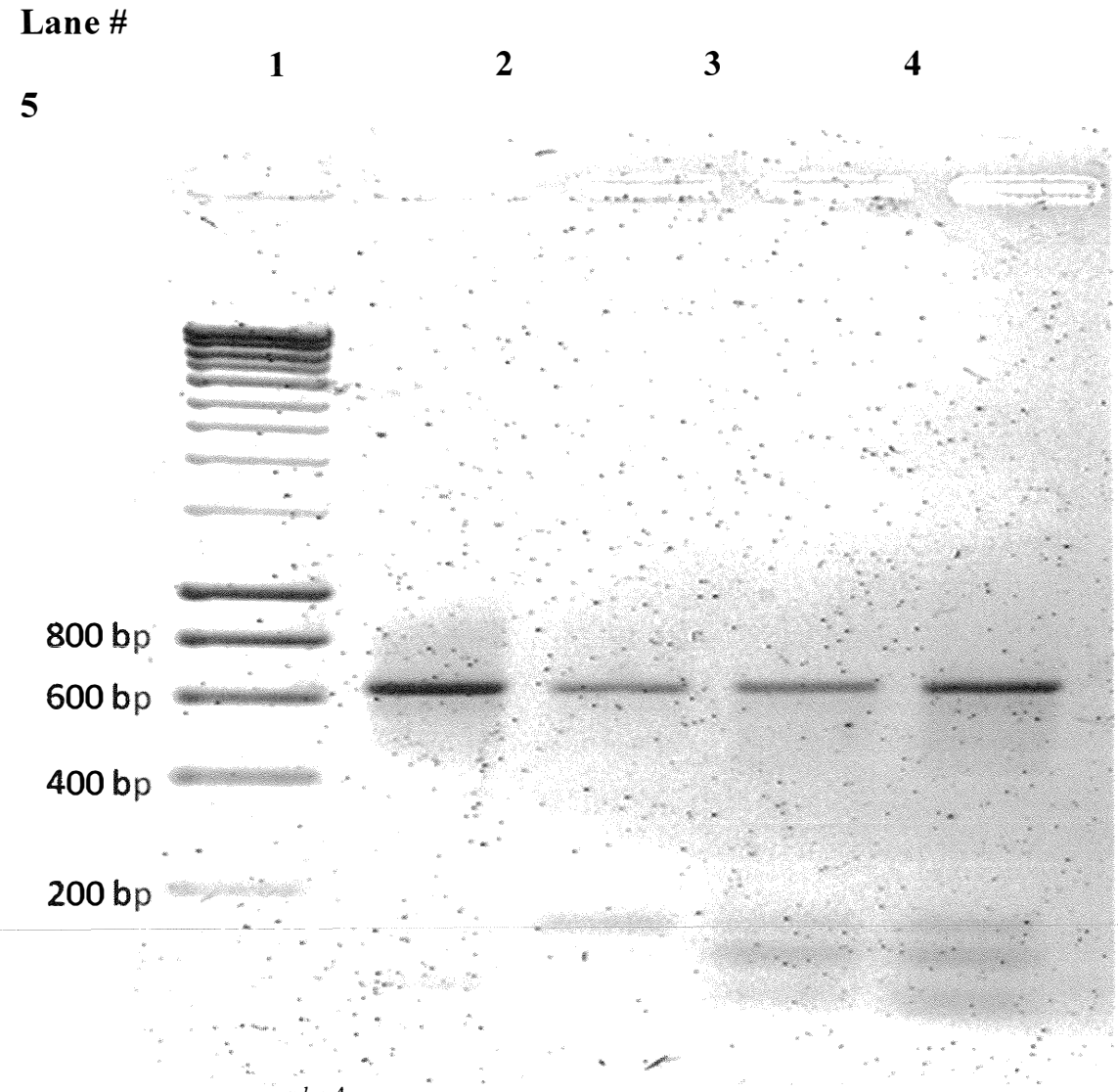



**Figure 6:** PCR of R($N^{nd-p}$)$^4$ mutant DNA shown on a 1% Agarose Gel. Lane 1 contains 1μL of a 2 kb Hyperladder. A primer set of *N* 5 prime D and *N* 3 with a wild type band of 587 bp was chosen Samples 4.2, 4.3, 4.5 and 4.6 are shown in sequential order starting in lane #2. Lanes 2-5 contain 20μL of the specific sample.

## Wild type Sequence

...AGAGAGCAAGAGTGCGG***CGTCGGTT***TGAATTTGAATTT
GTGGCTCGATCCTCGCGAAGAGAAAAGCAAGCAAAAGATACAC
GAAAAGCGTTCTTTTTTTGCCACTTTTTTTTTTATGTTTCAAAA
CGGAAAATGTCGCC...

## R($N^{nd-p}$)$^4$ Sequence

...AGAGAGCAAGAGTGCGG***CGTCGGTT***CATGATGAAATAACAT
CATCACATAACATAATATGTTATTTCATCATG***CGTCGGTT***TGA
ATTTGAATTTGTGGCTCGATCCTCGCGAAGAGAAAAGCAAGCA
AAAGATACACGAAAAGCGTTCTTTTTTTGCCACTTTTTTTTTTA
TGTTTCAAAACGGAAAATGTCGCC...

**Figure 7:** A comparison of the R($N^{nd-p}$)$^4$ sequence to the wild-type sequence (40). The target duplication sites are italicized and bolded, and the additional 48bp are underlined. The base pairs that are in normal script are the same in the $N^4$ sequence and the wild-type sequence.

## R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$ Mutants.

In order to test whether the observed lethality is caused by $N^{nd\text{-}p}$ or whether the lethality is caused by an independent mutation, we isolated R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$ by the reversion of the lethality of the double mutant, $N^{nd\text{-}p}e(r)^{27\text{-}1}$. Two males with *y w* Rev($N^{nd\text{-}p}$) *cv* $e(r)^{27\text{-}1}$ were isolated from vial 9 and were designated R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$. The males were crossed with *w* $N^{8}$/FM7c to establish stocks.

The primer set chosen was Notch 5 prime D and Notch 3 prime D, which has a wild-type band size of 306 bp. As seen in Figure 8, a band of approximately 370 bp was visible for each of the R($N^{nd\text{-}p}$)$^{9}$ mutants in lanes 2 and 3 on the agarose gel. The bands at approximately 370 bp differed from the expected band at 306 bp which was anticipated and there was one single clear, concise band. The PCR products for the R($N^{nd\text{-}p}$)$^{9A}$ was purified and sent out for sequencing since both mutants gave the same band at approximately 370 bp. The bands, seen at approximately 370 bp, suggest that there are additional base pairs present in those bands. The additional band seen in lane 3 could be due to the non-specific binding of the primers.

The Cornell University DNA Sequencing Facility was able to sequence the DNA for R($N^{nd\text{-}p}$)$^{9A}$ and the sequences are found in Figure 9. The sequence was compared to the wild type sequence and analyzed by eye. After analyzing the sequence for sample R($N^{nd\text{-}p}$)$^{9A}$, there are two things that are apparent. First, sample R($N^{nd\text{-}p}$)$^{9A}$ is identical to samples 4.2 and 4.3. Secondly, there are an additional 48 bp present around the P element's insertion site when compared to the wild type sequence.

## R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$

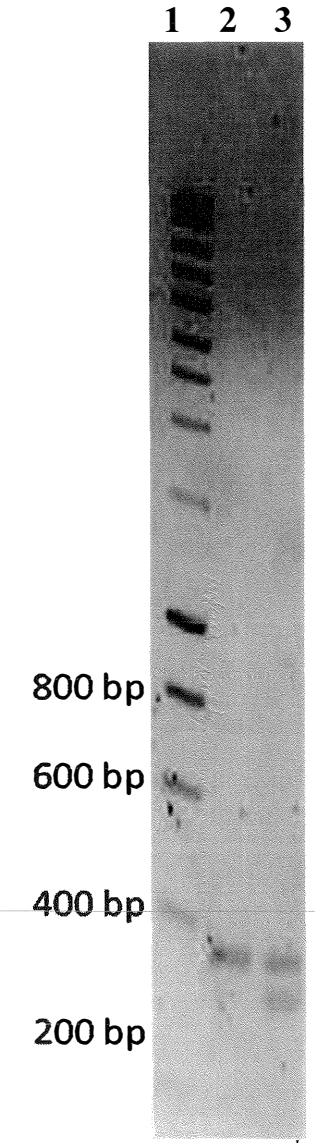



**Figure 8:** PCR of R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$ mutants DNA shown on a 1% Agarose Gel. Lane 1 contains 1uL of a 2 kb Hyperladder. Samples R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$ are shown in sequential order starting in lane #2. Lanes 2-3 contain 20μL of the specific sample.

## Wild type Sequence

...AGAGAGCAAGAGTGCGG***CGTCGGTT***TGAATTTGAATTT
GTGGCTCGATCCTCGCGAAGAGAAAAGCAAGCAAAAGATACAC
GAAAAGCGTTCTTTTTTTGCCACTTTTTTTTTATGTTTCAAAA
CGGAAAATGTCGCC...

## R($N^{nd\text{-}p}$)$^{9A}$ Sequence

AGAGAGCAAGAGTGCGG***CGTCGGTT***CATGATGAAATAACNTCA
TCACATAACATAATATGTTATTTCATCATG***CGTCGGTT***TGAAT
TTGAATTTGTGGCTCGATCCTCGCGAAGAGAAAAGCAAGCAAA
AGATACACGAAAAGCGANGNNNNTTNTGTGTGCTTTCGCGCNC
CTNGNAAATTTNNGNNNNGGANATTTNNACTNNGGACAN

**Figure 9:** PCR was performed and sequencing carried out at Cornell University. A comparison of the R($N^{nd\text{-}p}$)$^{9A}$ sequence to the wild-type sequence (40). The duplication sites are italicized and bolded and the additional 48 bp are underlined.

## *Notch*$^2$ *and Notch*$^3$ *Mutants.*

A cross between w $N^8$/ *FM7c* females X y w $N^{nd\text{-}p}$*cv*; $ry^{506}$ Dr P {ry[+t7.2] =Delta2-3}99B /+ males mobilized the P element in $N^{nd\text{-}p}$, creating new *N* alleles. This cross generated notched-wing females, y w *N cv* / *FM7c.* Two vials, 2 and 3 generated a set of females. The females from vial 2 had more severely notched wings than the females from vial 3. Crosses conducted later showed that females from vial 3 often had wild-type wings. The mutants are hemizygous lethal and the stocks were referred to as 2.1, 2.2, etc., and 3.1, 3.2, etc.

Since the DNA was obtained from heterozygous female flies two different, single and concise bands were expected. The two expected bands are a wild type band and a band different in size than the expected wild type band from a primer set, representing the mutation. In order to determine the cause of both mutations, two different primer sets were used in PCR reactions in the hopes that one of the primer sets would flank the deletion.

The first primer set chosen was Notch 5 prime D and Notch 3 prime B3 which amplifies a wild type band of 1,735 bp. The second primer set was Notch 5 prime C and Notch 3 prime B3 which amplifies a wild type band of 2,069 bp. The samples chosen are *Notch* 2.5, 2.7, and *Notch* 3.2, and 3.4. The PCR reactions were analyzed by gel electrophoresis as seen in Figure 10.

As seen in Figure 10, in lanes 1-4, the first primer set was used and three bands are visible on the gel. The largest and brightest band represents the wild type band of 1.735bp, the second band represents a hybrid and the smallest and final band represents the possible deletion. In lanes 6-9, the second primer set is used and two

distinct bands are visible for each of the four samples. The wild type band of 2,069 bp is the larger of the two bands and the second band represents the possible deletion. For both primer sets an approximate 800 bp deletion was seen based on the size of the smaller band in comparison to the wild-type band.

The lower band at approximately 1400 bp in lane 6 was cut from the gel, and placed in a tube with water and melted in a water bath. A reamplification was done using 1μL of the melted gel solution and then run on a gel. This procedure was repeated and the PCR sample was run on the gel seen in Figure 11. The band seen at approximately 1400 bp represents the band that is believed to represent the deletion. Since this band was able to be amplified, sample 2-5 was sent out for sequencing.

The Cornell University DNA Sequencing Facility was able to sequence the DNA for the $N^2$ mutant; however the DNA sequence came back with a lot of Ns present. Since there were so many Ns present the sequence was not analyzed further.

## *Notch*$^2$ and *Notch*$^3$

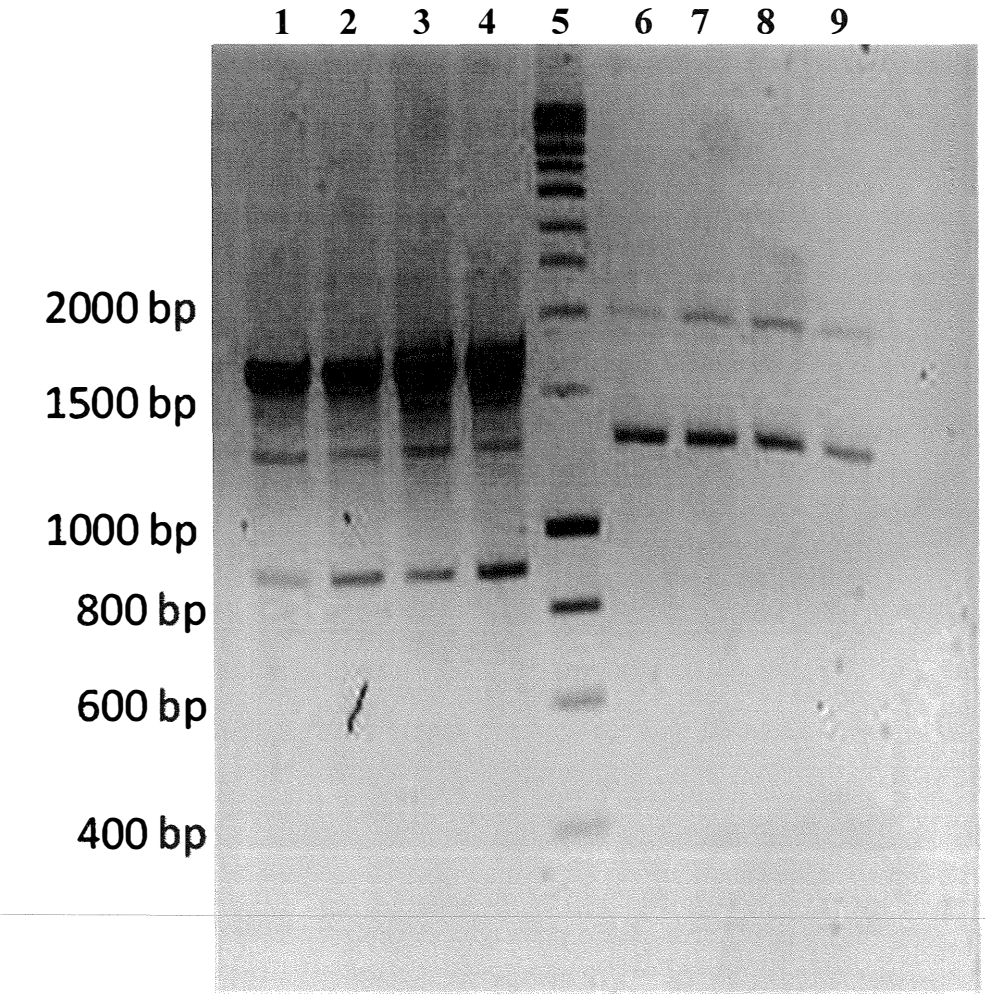



**Figure 10:** PCR of *Notch* 2 and *Notch* 3 Mutant DNA shown on a 1% Agarose Gel. Lane 5 contains 1uL of a 2 kb Hyperladder. Samples 2-5, 2-7, 3-2 and 3-4 are shown in sequential order starting in lane #1 and lane #5. Lanes 1-4 and 5-9 contain 20µL of the specific sample. Primer Set Notch 5 prime D and Notch 3 prime B3 is used in lanes1-4, whereas Notch 5 prime C and Notch 3 prime B3 is used in lanes 5-9.

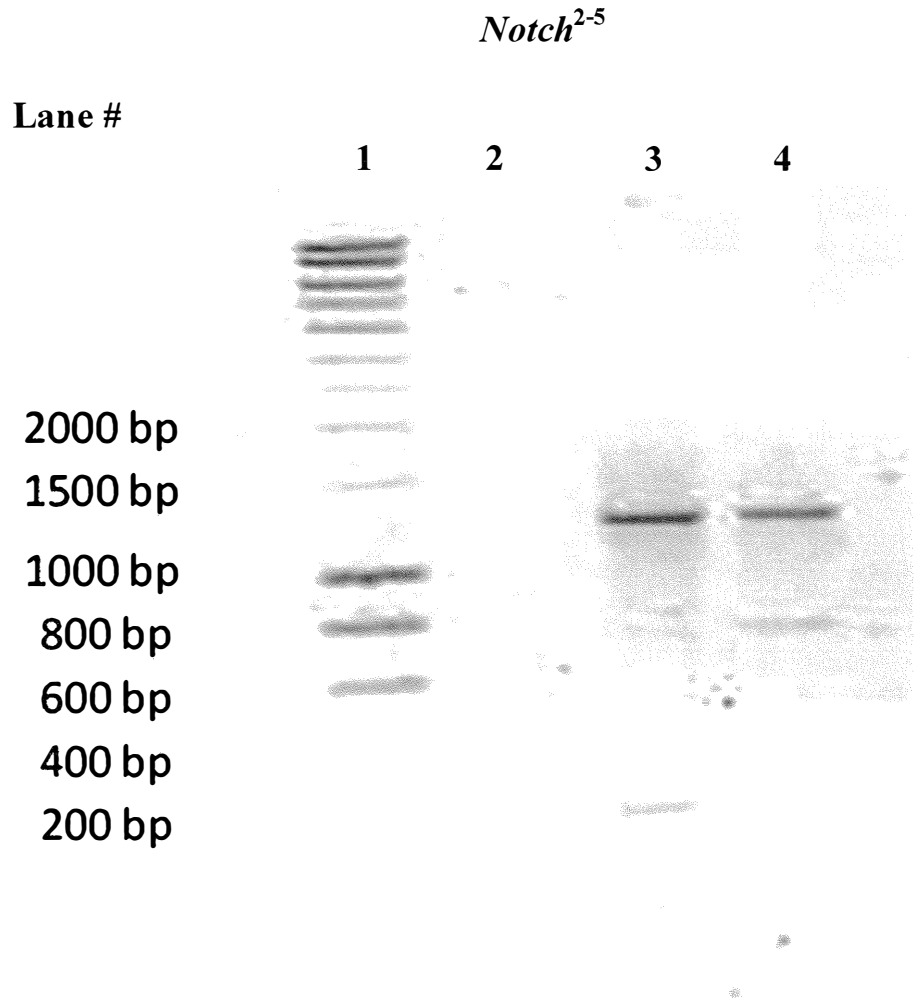



**Figure 11:** Reamplification of sample *Notch* 2-5 once the band at approximately y1400 bp was isolated. Lane 2 contains 1uL of Hyperladder I and Lane 4 contains 5uL of sample 2-5. The band at approximately 1400 bp represents the suspected deletion and the light bands seen at approximately 200 bp represent the remaining primers.

## *Notch$^5$ Mutants.*

From vial 2, a scalloped wing female was isolated and crossed to FM7c males to establish a stock. The flies collected from this screen, were designated vial 5. The chromosome was *cv+*. This was shown when *y w* $N^{nd\text{-}p}$*cv* males were crossed to heterozygous females. This new mutation $N^5$ was found to be viable over $N^{nd\text{-}p}$ and the females had normal crossveins. A cross was set up with wild-type to replace $e(r)^{27\text{-}1}$ with $e(r)^+$. Males that were *y w* were isolated which had scalloped wings. The males were tested for $e(r)^{27\text{-}1}$ and $e(r)^+$.

Many primer sets were chosen to try and amplify the $N^5$ mutation. However, no bands that were larger or smaller than the wild-type band were seen. The presence of these bands suggests that a deletion occurred or an imprecise excision of the P element. One primer set chosen was $N^{nd\text{-}p}$ 5 prime and $N^{nd\text{-}p}$ 3 prime, with a wild-type band of 97 bp as seen in Figure 12. The two bands seen at approximately 1200 bp on the gel represent the 97 bp from the primer set and the 1100 bp from $N^{nd\text{-}p}$. The $N^5$ mutant male was in lane 4 and the $N^{nd\text{-}p}$ male was in lane 5. Both of the males had $e(r)^+$ present. For both samples, there should have been only one band visible on the gel since both samples were male DNA; however, multiple bands were visible. Also, both samples had the same bands, and similar insertions were not expected.

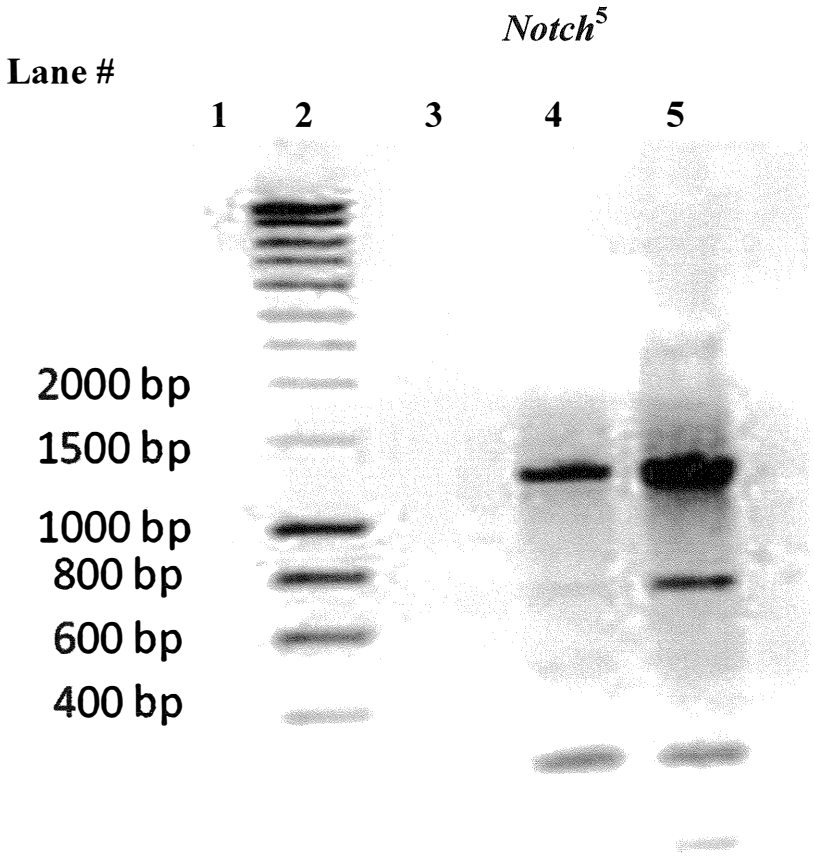



**Figure 12:** PCR of *Notch 5* Mutant DNA shown on a 1% Agarose Gel. Lane 2 contains 1uL of a 2 kb Hyperladder. The *Notch 5* male sample is seen in lane 4 and the $N^{nd\text{-}p}$ male was seen in lane 5. The lane contains 20µL of each sample.

## Viability Results.

In order to determine at which stage of the life cycle the three *Notch* mutants are dying, the larvae were examined under a dissecting scope with a GFP filter. The GFP filter was used to detect the X chromosomes that are tagged with GFP. In each case the mutant chromosomes were GFP$^-$. For all of the larvae that were examined in the third instar stage, there were no GFP – larvae present. In the third instar stage, all of the larvae were found to be glowing, showing that the larvae contained GFP on at least one of their chromosomes.

| SAMPLE | FIRST INSTAR LARVA | | SECOND INSTAR LARVA | | THIRD INSTAR LARVA | | ADULT | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | GFP + | GFP - | GFP + | GFP - | GFP + | GFP - | Males GFP + | Females Hetero | Females Homo | Males GFP - |
| $N^2$ e(r)$^+$ | 182 | 1 | 463 | 2 | 2000 | 0 | 331 | 402 | 216 | 0 |
| $N^3$ e(r)$^+$ | 160 | 4 | 350 | ***1*** | 1814 | 0 | 457 | 462 | 229 | 0 |
| $N^5$ e(r)$^{27-1}$ | 272 | 8 | 423 | 1 | 1377 | 0 | 12 | 181 | 56 | 0 |

**Table 2:** A summary of the viability data for first, second and third instar larvae and adults. The larvae were examined under a dissecting scope with a GFP filter, the larvae were examined for the presence or absence of GFP. There were no third instar larvae that were non-GFP expressing, and very few first and second instar larvae that were non-GFP expressing. There was one second instar larva, which was non-GFP expressing, that survived to adulthood and was used to set up crosses. This second instar larva is $N^3e(r)^+$ and is represented in bold and italics in the table. As for the adults, most of the females examined were heterozygous meaning one chromosomes contained GFP, however, there were no males scored that were non-GFP expressing.

In the second instar stage, there were less non-GFP expressing larvae seen in comparison to the first instar larvae. There were two larvae for the $N^2$ mutants that did

not glow, however when placed in a separate tube the larvae never grew and therefore did not survive. Also, for $N^5$ there was one larva that did not glow, however, like $N^2$ the larva never grew into the third instar stage and therefore did not survive. However, for $N^3$ the one larva that did not glow in the second instar stage, did survive and produced a male with white eyes, which was later sequenced and will be discussed later.

In the first instar stage, there was one larva for $N^2$ that did not glow, however when separated, the larva did not grow and eventually died. For $N^3$, there were four larvae that did not glow but like $N^2$ these larva did not produce adult flies. For $N^5$, there were eight larvae that did not glow but just like $N^2$ and $N^3$ the larvae did not produce adult flies.

Adult flies were also examined in order to determine the ratio of adults that contain a chromosome with GFP to those males that did not contain a chromosome with GFP. For all of the males examined, the males had small red eyes which suggest the presence of a chromosome with GFP. There were no males present with white eyes, which suggest that there were no males present that did not contain GFP on a chromosome. As for the females, the majority examined were females that were heterozygous, meaning that the adult females only have one chromosome containing GFP. Females that have GFP on both chromosomes is less than half of the heterozygous females.

Since no adult males were present with white eyes, it is apparent that at some point in life cycle these males are dying. Observing the viability of the larvae, shows that the *e(r)* gene can affect *N* expression in the embryo stage. The *Notch* mutant

larvae did not give rise to adult flies, indicating that *Notch* activity is necessary both during embryogenesis and metamorphosis.

## The Revertant of $N^3$ Male.

Examination of the larvae in the viability study produced one male from the $N^3$ that survived to adulthood. This male had white eyes, which suggested that this was a male that contained the $N^3$ mutation. In order to determine if the male contained the $N^3$ mutation, the male was used to generate stocks as detailed in materials and methods. The DNA from a male progeny with white eyes was sequenced.

The primer set chosen to try and amplify the deletion or imprecise excision was Notch 5 prime D and Notch 3 prime C2. The primer set has a wild-type band of 812 bp. In Figure 13, a band close to the expected wild-type band was seen. The sample was amplified and purified before being sent out for sequencing.

The Cornell University DNA Sequencing Facility was able to sequence the DNA for the $N^3$ mutant and the sequences are found in Figure 14. After analyzing the sequence for sample $N^3$, it was apparent that sample $N^3$ is identical to the published wild-type *Notch* sequence, not the progenitor of $N^{nd\text{-}p}$ sequence seen in Figure 14. These data suggest that a double recombination or gene conversion had occurred. This recombination event also explains why the band seen on the gel in Figure 13 resembles the wild-type band for that primer set.

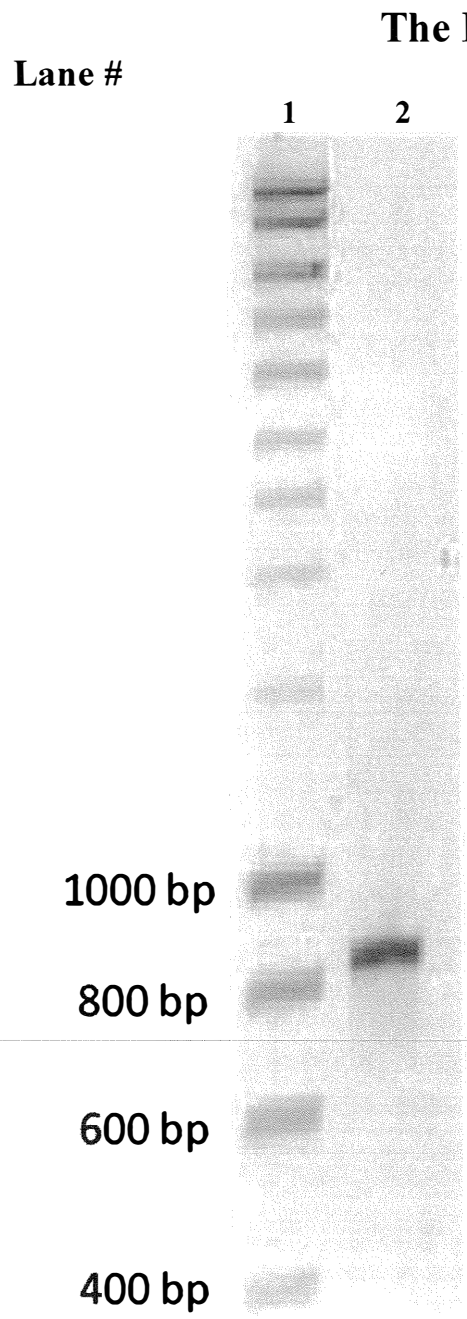



**Figure 13:** PCR of $N^3$ mutant DNA shown on a 1% agarose Gel.
Lane 1 contains 1uL of Hyperladder I and lane 2 contains 5uL of sample 2-5.
The band at approximately 812 bp represents a wild-type band.

## Wild-type Sequence from Progenitor $N^{nd\text{-}p}$

CGCAAGAGGAAGAGA. . . . . .GCAAGAGTGCGGCGTCGGT
TTGAATTTGAATTTGTGGCTCGATCCTCGCGAAGAGAAAAGCA
AGCAAAAGATACACGAAAAGCGTTCTTT

## The Revertant of $N^{3}$ sequence using Notch 5 prime D

CGCAAGAGGAAGAGA***GCAATAC***GCAAGCGTGCGGCGTCGG
TTTGAATTTGAATTTGTGGCTCGATCCTCGCGAAGAGAAAAGC
AAGCAAAAGATACACGAAAAGCGTTCTTT

## *Notch* sequence from published wild-type allele

CGCAAGAGGAAGAGA***GCAATAC***GCAAGCGTGCGGCGTCGGT
TTGAATTTGAATTTGTGGCTCGATCCTCGCGAAGAGAAAAGCA
AGCAAAAGATACACGAAAAGCGTTCTTT

**Figure 14:** The $N^{3\text{-}4}$ mutant was found to have the same sequence as the above published wild-type *Notch* (41). The duplication sites are underlined. The $N^{3\text{-}4}$ mutant does not have the same sequence as $N^{nd\text{-}p}$(40) . The difference between the revertant's sequence and the $N^{nd\text{-}p}$ sequence is one base pair and the deletion of 7 base pairs represented by the dots in the $N^{nd\text{-}p}$ sequence. The $N^{3\text{-}4}$ mutant has a C present where as the $N^{nd\text{-}p}$ progenitor sequence has an A.

# DISCUSSION

The *Notch* gene encodes a transmembrane protein that functions as a receptor for intracellular signals in Drosophila (4). The *Notch* gene is required for cell proliferation as well as differentiation (8). Mutations in the *Notch* gene are known to affect many developmental processes in the segregation of different cell types in both embryonic and imaginal development (13). A *Notch* mutation, $N^{nd\text{-}p}$, is caused by the insertion of a P element into the first exon upstream of the start of translation of the *Notch* gene. This insertion causes a weak *Notch* mutation, which is lethal in the presence of the *enhancer of rudimentary* mutation, $e(r)^{27\text{-}1}$ (32).

The first question to be examined was whether the lethality of the X chromosome that contains $N^{nd\text{-}p}$ and $e(r)^{27\text{-}1}$ was caused by an interaction between these two mutations. The gene *e(r)* is believed to be a positive regulator of *Notch* (32). Mutations in *e(r)* are believed to be null mutations and are thought to be recessive lethal (18). Previously it was shown $e(r)^{27\text{-}1}$ is required for the lethality, because a wild-type transgene of *e(r)* rescues the lethality. Since there was no data for *Notch's* involvement in the lethality like there was for $e(r)^{27\text{-}1}$, it was possible that there was a mutation that mapped close to *Notch* that was causing the lethal interaction with $e(r)^{27\text{-}1}$. There were two hypotheses suggested at the beginning of this thesis: either the observed lethality is caused by $N^{nd\text{-}p}$ or the lethality is caused by an independent mutation. In order to determine which hypothesis was correct, two approaches were done. The first approach was to revert the $N^{nd\text{-}p}$ mutation. If the $N^{nd\text{-}p}$ mutation was the cause of the lethality, then the revertant would have been found to

be viable when combined with $e(r)^{27\text{-}1}$. If the lethality was caused by an independent mutation then reverting the $N^{nd\text{-}p}$ mutation would not revert the lethality.

$N^{8}/N^{nd\text{-}p}$ heterozygotes are lethal. The lethality is a result of $N^{nd\text{-}p}$, since $N^{8}/N^{+}$ heterozygotes are viable and $N^{+}$ rescues the lethality. This property was used to isolate a revertant of $N^{nd\text{-}p}$, R($N^{nd\text{-}p}$)$^{4}$. Females with a genotype of *y w* Rev($N^{nd\text{-}p}$) *cv* / FM7c were isolated. The stocks were presumed to contain the same revertant and were labeled 4.1, 4.2, 4.3, 4.4, etc. PCR revealed that each sample gave a slightly larger than wild-type band, indicating an imprecise excision of the P element. Therefore, approximately 1,050 bp of the P element was excised properly and these base pairs are believed to be the cause of the $N^{nd\text{-}p}$ mutation and subsequently the lethality. When R($N^{nd\text{-}p}$)$^{4}$ was combined with $e(r)^{27\text{-}1}$, it was found to be viable. Therefore, reverting $N^{nd\text{-}p}$ reverted the lethality observed when $N^{nd\text{-}p}$ is in the presence of $e(r)^{27\text{-}1}$. The observation that R($N^{nd\text{-}p}$)$^{4}$ was viable in the presence of $e(r)^{27\text{-}1}$ disproves that the lethality is caused by an independent mutation and supports that the observed lethality is caused by $N^{nd\text{-}p}$.

The second approach to test the cause of the lethality observed when the X chromosome contains $N^{nd\text{-}p}e(r)^{27\text{-}1}$ was to revert the lethality of the $N^{nd\text{-}p}e(r)^{27\text{-}1}$ chromosome and then examine $N^{nd\text{-}p}$. If the lethality has nothing to do with $N^{nd\text{-}p}$ then reverting the lethality will not affect $N^{nd\text{-}p}$. Males that were *y w* $e(r)^{27\text{-}1}$, would be seen as *y w cv* males. Two of these males were isolated from vial 9 and were designated R($N^{nd\text{-}p}$)$^{9A}$ and R($N^{nd\text{-}p}$)$^{9B}$. Each male was crossed with *w* $N^{8}$/FM7c to establish stocks. The *Notch* DNA was examined by PCR and DNA sequencing and found to contain additional base pairs. These extra 56 bp consisted of a duplication

site, which consists of 8 bp and 48 bp of the P elements DNA that was left behind when the excision occurred. The 48 bp are identical to the 48 bp left behind by the P element seen in R($N^{nd-p}$)[4]. While uncommon, identical but independent mutations caused by P element excisions are not unheard of (18, 23, 24). The data for R($N^{nd-p}$)$^{9A}$ and R($N^{nd-p}$)$^{9B}$ further support hypothesis 1 and further disproves hypothesis 2, since reverting the lethality reverted $N^{nd-p}$.

New *Notch* mutations caused by the mobilization of the P element were isolated. Three of them ($N^2$, $N^3$, *and* $N^5$) resulted in a further decrease in *Notch* activity. The second question to be examined focused on the new *Notch* mutations; $N^2$, $N^3$, and $N^5$ and their appearance to be hypomorphic mutants. At the DNA level, one would predict that these mutants are a result of a deletion or an imprecise excision which leaves the coding region intact. The new $N^2$ and $N^3$ mutants produced the same sized bands, which could suggest that both mutations have the same deletion. However, $N^2$ and $N^3$ have different wing phenotypes and were collected at different time points, which support the idea that these mutations are in fact different. The $N^2$ mutant had slight notches in the wing; whereas the $N^3$ mutants had wild type wings. When the $N^{2.5}$ sample was sent out for sequencing, the resulting sequence was not usable since there were so many Ns in the sequence. For both of the $N^2$ and $N^3$ mutations new primer sets should be designed that flank the suspected deletion. Another possible approach is to clone the DNA into a vector and sequence or another possible approach is the use of a Western Blot.

In the presence of an *e(r)* mutation, $e(r)^{27-1}$, a lethality will occur for $N^5$ mutants. However, over the GFP transgene, a heterozygous fly with wild type wings

should be present. The $N^5$ mutant fly used in this experiment was isolated as a result of visible notches in the wings, suggesting the possible mutation $N^{nd\text{-}p}$ may have changed. Also, similar insertion bands were seen between the $N^5$ male and the $N^{nd\text{-}p}$ male suggesting that the $N^5$ mutant may in fact be the same as the $N^{nd\text{-}p}$ mutation. For the males, only one band should be present on the gel when primers are chosen to flank the deletion. Since there have been multiple bands visible for a multitude of primer sets that were used on the $N^5$ males, sequencing has been very difficult. The presence of multiple bands could be due to non-specific binding of the primers.

All of the primers chosen from Table 1 for *Notch* 5 were unable to bind to the DNA, due to a possible larger deletion. If this theory is true, than the deletion may extend farther out than what was expected. If this deletion does in fact extend farther out, then a possible approach is to design new primers that surround the known insertion site of the P element. Primers could be designed to amplify a region close to the P element to see if any changes have occurred in this region. Also, primers could be designed upstream and downstream of the P element's insertion sight in order to determine how far out the possible deletion may have gone. Through the use of these new primers, the length of the deletion can hopefully be determined through PCR, gel electrophoresis, and sequencing.

Another possible approach is to sequence the DNA from the band seen at 1200 bp in lane 4 of Figure 5 to see if the DNA sequence of $N^5$ is the same as the $N^{nd\text{-}p}$ sequence. If the sequences are not the same or the sequence is not usable than a possible approach is to clone the DNA into a vector and sequence; or another possible approach is the use of a Western Blot.

Lastly, the reason we may not be getting good results is that there could possibly be an inversion or a point mutation in the region that we are trying to amplify. When P elements are mobilized they produce traits known as hybrid dysgenesis in *Drosophila.* Some of these traits include temperature independent sterility, elevated rates of mutation, chromosome rearrangement and recombination (23, 29). If there is an inversion present, the primer sets that we have designed will not be able to bind properly.

The $N^{nd\text{-}p}$ mutation has been shown to be lethal in the presence of an *enhancer of rudimentary* mutation, $e(r)^{27\text{-}1}$. The data presented in this thesis suggests that the observed lethality is caused by the interaction between $N^{nd\text{-}p}$ and $e(r)^{27\text{-}1}$. By reverting $N^{nd\text{-}p}$, the lethality was also reverted. Also, by reverting the lethality of the $N^{nd\text{-}p}e(r)^{27\text{-}1}$ chromosome, $N^{nd\text{-}p}$ was concurrently reverted.

## Timing of lethal interaction between *Notch* and *e(r).*

For the observed lethality caused by the interaction of $N^{nd\text{-}p}$ and $e(r)^{27\text{-}1}$, it is important to determine when this lethal interaction occurs in the life cycle of *Drosophila.* The point at which the interaction occurs is important since it indicates when *e(r)* activity is necessary to activate *Notch* activity or the *Notch* signaling pathway. A $N^{nd\text{-}p}e(r)^{27\text{-}1}$ mutant has been shown to be lethal during the pupal stage, which indicates that *e(r)* is necessary for *Notch* activity during this stage and not earlier, since the larvae are able to survive to the pupal stage (32). However, the viability results suggested in this thesis indicate otherwise. The $N^{5}e(r)^{27\text{-}1}$ double mutant was found to be lethal during embryogenesis. The lethality occurring during

embryogenesis is apparent since most of the embryos do not make it into the first instar larval stage. When the larvae were examined under the dissecting scope, there were a large number of embryos present that were not glowing. These embryos never formed into larvae. Since the $N^5e(r)^{27\text{-}1}$ was found to be lethal during embryogenesis, $N^2e(r)^+$ and $N^3e(r)^+$ mutants should be analyzed to determine at what stage in the life cycle they are dying. This analysis can be done in the same manner as the other viability studies in this thesis.

Therefore *e(r)* is believed to be necessary for *Notch* activity during embryogenesis as well as during the pupal stages.

The revertant of $N^3$ fly that came from the second instar larva that did not express GFP was a male with white eyes. This $N^{3\text{-}4}$ mutation was derived from $N^{nd\text{-}p}$ and the revertant of $N^3$ was derived from $N^{3\text{-}4}$. The revertant of $N^3$ was isolated as a male, whose mother was $N^{3\text{-}4}$/GFP. The male revertant of $N^3$ was crossed with a wild type female and these adults produced some males with white eyes. The DNA from one of the males with white eyes, which was second generation progeny, was extracted as usual and sent out for sequencing. The revertant of $N^3$ sequence data came back as being identical to published wild type allele for *Notch.*

Males that are *y w* $N^3e(r)^{27\text{-}1}$ are known to be lethal. The revertant of $N^3$ was a male; therefore the *Notch* allele in this revertant must come from GFP since the revertant of $N^3$ sequence and the wild type *Notch* sequence from GenBank are identical. Therefore, the chromosome is still *y w* $e(r)^{27\text{-}1}$, but has the wild type *Notch,* this was believed to have occurred by the natural process of gene conversion. Gene conversion is defined as a process by which DNA sequence information is transferred

from one DNA helix (which remains unchanged) to another DNA helix, whose sequence is altered (38). In the case of the $N^{3-4}$ male with white eyes, the wild-type *N* gene on the GFP chromosome was transferred to the chromosome containing the $N^3$ mutation. The wild-type *N* gene replaced the $N^3$ mutation. When the excision of the P element occurs a gap is left behind. The exonuclease chews away the remaining DNA and uses the wild-type chromatid, containing the wild-type *Notch* gene, as a template. The replacement of the $N^3$ mutation with the wild-type *N* gene is from the X chromosome tagged with GFP and this is what allowed the male to be viable.

A $N^{nd\text{-}p}e(r)^{27\text{-}1}$ mutant shown to be lethal during the pupal stage indicates that *e(r)* is necessary for *Notch* activity during the pupal stage and not earlier (32). However, the viability results suggested in this thesis suggest the importance of *e(r)* earlier in the life cycle. The $N^5e(r)^{27\text{-}1}$ double mutant was found to be lethal during embryogenesis. Therefore *e(r)* is believed to be necessary for *Notch* activity during embryogenesis as well as during the pupal stages.

# Appendix 1: Sequence Surrounding $N^{nd\text{-}p}$

aaaga*tcttgggttgtaaatgcaactg*acttggatttatctgatatggctgaaattcgagc
actaagaatgtgactgctttcgtttgtaaatttgtaacgtacagctcccaaaaacaaaaat
aaaagagaaaagagttcaacacacccttgagtaaaaaaaagaaatgtgtataaaaacgaaa
aacaaaacacaaaaaaaaaaaaacagaagaaaacaaaaaccgtattgtgatttatagcgtt
aattattttatggctgagctcgatgagtttcgcagcggcaaattatatcaactagtgcaaa
tcttaagctacttatcgagtaaaaaatcgtatatcaattaaacacgaaacgcgtatcgaac
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tacaattttaaaacaaaaccgaaaatcaattcaattgtatacattatgacaaaacaaaaag
cctgtgtagttagttggaactaaaaaaaaacaaaaataatataaaagcaaacgaaaatgaa
acgtaaatgaaaagtaaagcagcagcagcagaaagcaaaaacaattttctaagccaaaatt
tatcaaatttttagacttaattacgtatacatcaaacgaaaccaactgaatgcagtgaga
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acatatataactattattttgcacacaaacaaacaaaattgaaattgaattgaaaactata
ttttacgggatgctacagtattattgcaatttaatcgataatccccaagccgcaaaaaac
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accaccaacatatatatatatatatatatatatagttcccactttccaacttttgatc
gggttttcagtggcacagcatttcagcatccatgtgaaaagttgaatatttttttgtctag
ccctaagcgcgtaaatgatatttgaacttaaatcaattatgtaaagaagcaaatgaaatat
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aacttaaaaagcttagaaacaatttgtaaaacatttgagcgatctaccaaaaaaacacaaa
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gggttcttaattcactaagccaatactgtaattaattaactaatttattgtttcatatttt
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aaatgc*ttaaaaataatgcccgttcgtt*taaactgatttatacactcgaatctaattctat
tctttccgtgggccaattgcgccgagcttttaactgggatagcgttccgaaaatcggaga
taagtatggtgttacatatttttttttgttttggataattgtcaaacgattcaattaagcg
ttcaattatatagacgttacacacgaaacgaaatgaaattcaaatgaaatcctattaagcc
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ctaagaacgtattgcgggaaaacctaacttaagtcgagaacaactcaaattagttgaaata
aagatacgagaacaaaaggaaaataaaatacttaaaaaatgctacaagtgcgtttttcaat
caaatttatgcacatttttttccagtgtctacgaattttacattgtcgaattttacattat
acatagcgtaatttctacgaatcttacattgtcgaattttacattttt*acatagcgaaattt*
*ctacataacgc*tatgacagcactaaagcgccattc*ggcgaaatggggaaactact*catgca
agcggctcggagcccggctaacgttatttgttcaccaaccgatctcgcaacgctgcgaaag
agcgcgctgccaaaatggctccccgccatacggtatctttttcttgcaccgacgcggtcac
actgccgATTTGAAACAGATCGCTTTTTTCCAGTGGACGAAACGGTTGTGAAAGCGGACGA
GCGTTAGGCAGACGAACCTGGAAAGCGCAGAGCACAGTTCTCAACATTTATTTTTTTTTTG
AATGTGTGTGCAACAACGCACGTAAAATCGCGCTGCCAACAGGATATACAAACAAATCAAT
TAC*ACAGCAAGCAAATGCAATGA*AATGAAAAGGATGGCCCCAGCGGGAAAGCCGTTCAGCA
AGAGCAAGGAGTGCCTGTCGCAGGGATAGCAACGAGAGAGCGACACAGAGAGCGAGAGAGA
GAGAGGGAGAGAAACAAGGATTTTCGAAAAGTGTATCTACCTCGAGTCGCGCGTGTGTGAG
AGTGAGACGAAAGCCGAGTGCAAGAAGCGCAAGAGGAAGAGAGCAAGAGTGCGG*CGTCGGT*
*T*TGAATTTGAATTTGTGGCTCGATCCTCGCGAAGAGAAAAGCAA*GCAAAAGATACACGAAA*
***AGCG***TTCTTTTTTTTGCCACTTTTTTTTTTATGTTTCAAAACGGAAAATGTCGCCGTCGTCGG
GAGAGTGCCTCCTCTTAGTTTATCAAATAAAGCTTTAAAGTCTGAAGTTCAAAACTATAAA
AAACAAAAAAAAAAAACAAAAAAACAACAAACGCGCTAAAACAAAAAATTGCTTAAACTACT
GAGACTAGCATACTAAACCTAAACTCGCAGTTAAACATATCCTCAAAGAGAGACCAAAGAG
GAGCGCTAAAG*AAACCGAGAAGCGAGTAGAGG*TGAAAAACTGGAAAAGTGGTTAACTGGAA
AACTGGATTACAATGCAATCGCAGCGCAGCCGACGTCGCAGTCGCGCACCAAACACTTGGA
TTTGTTTTTGGATTAACAAAATGCACGCCGTTGCGTCGCTGCCGGCGTCGCTGCCTCTGCT
GCTGTTGACGCTGGCGTTTGCGAATTTGCCAAACACCGTTCGCGGAACTGGTGAGTAAAGC
GG*AGACGCCGATCAGAACAACT*AGGCGGGGGGGAGGGCAGGGTGACAGGCGGGGTCGCCCGA
AAATAGGGTGGGTGCACAAACTGGAATTCCAGGAGGCTCAAAACGTTCCAAAACGCTTGCC
AAAGAACGGTTTTTTACAGAACCAAAACTATGAGATAAGATGATCTTGTATAGCTGAAAGA
TGTGGATCTCAACTAATAGTACTGAATCGCATAATTGTTTGGTCTTAGATGTCAGTTTTAA

Notch 5 prime B

Notch 5 prime 2

Notch 5 prime C

Notch 5 prime D

$N^{md\text{-}p}$ insertion site

Notch 3 prime D

Notch 3 prime

Notch 3 prime C2

Notch 3 prime B2

ATGGAGCATACTTAGTATGAAACAACTAAAATTGCAATTACAAAATATACATACATTTGCA
TTGGACTAGTTCCGCTTAAACCCGTTGGATAGATCGCTACATTGCATAGCTAGGAAATGTT
GGTCTATAAAATCGGATGCATTCGCAACACATATAAGTGACTGAGCAAACCCATGCAACTG
TAATCCAAAAAGGAAGTGATGCTACTTAATCTGCTTAATAAGCCTATAGGATTGTTGCAAA
TCCTACCTTAAAGAATTTC*ACTTTAAACGCAATCGTCGG*AATCCGCTCCCAAATGCGTTCT
CTGTGTTTCTCCATTCCCTTTTCGATCCTCTTCGTTTGTCTCCCTTTTGGGCAGCTCCTCT
TCGCTCTTCGCCTTCCTTTTCATGGCTTGTATTCACCGCGCTTCCTTTTGCGCTGTCTTCC
TTGGCTTCGCTTTGTTGTCGCTTTTCGGACAGGTGGTCGTTTTTTTTTCCTCTCTCTTTCT
CTCTCTCTCTCTCCTTGTTGTCTCGCTGATCTCGTTCTCGCTCTCGCTCGATCGCTGTCGC
TGTCTCGCTCTCCGCCGACTGCGCATGCTCTCTCAGATTTGGAAACCCACCTTTTCGAAAC
AAGAAAGACCGTTTTTACCGTCGGCAAAAGGCGAGTTTTTCCATTCCCCTAGCACTCTAGG
CACTCTTTCC*TTTGTGTTTTCTCACTGCGC*TATTTGCTCGCTAGTAGTGAGTGGCCAACTT
CCTTTCCAGCTTTGGAAGCGAACTCTCCTCCTCCACACGCTGTGTTGTGCCGCATGCACAT
GGGGCTTACTTGGGTATTTGCATATGGAGCGGTCGTTGTCTAATCACTGAACAAACACCGA
ATCGTATCGGATCGGATCGGATCGGTTGTAGGTGTACTTAGGGTCGATTGCCCCAGTAAAA
TGTCAACTTTCCATGGAACTCGAAATCTCTTCGACGGAATTCTCGGCTCAACGATGAGAAA
GTATGGGGAATGCAGCTCGATATCATAGTATTTTGAAACAAATTTGATAATGTCAGAGAAT
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GCAATTAAATGATCATTACTAGAAATTTGATCCCTGAGCACTGGGCAACTTTATTATTACG
CACTCCATGATCTCTGCTATTTTTTTTATATTTTTTAACAGCTGCAACCGAAGAGCGTTGA
ACTATTATGTTTATAACTTTTACCTGGGCATTTCCTGAGCTCGAGTTTCCGCCTCCTAAAC
TCTTTTTTTTTCTGGCTTTATTAGCCATGGGTAATCGTGTGATCTTTGCAGTTTTAGCCAA
AGGAAGTTGTAGTAAAAGTTAAATAAACGGTATAAAAAAAGTATACAAATGCATATGCATAT
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AAAAAGTGTTCGAAGATGGAAAAAAAAAAACCAAAAAAAAAAAACAAAAATCTCTCGAATCTT
CATGTACATTTTTGTACCTATTAAGTTCTCACAAATTTACGCCCCATTAGCTCCAATCCAA
GTTTGTTCAACTTTGTGCGATCGAACGCCTGCATTTAGCCACAAATTTAGGGTATTATTTT
TTTGCAGGTTTAATGTGGCACGTAACGGTTTCGAGAAACGAGAACCAGAACCAACCACCGC
ATTCGATTTCATCATATATGTACACAACCAGGCGCATATAGAAAGAATGTAGTTGGAATAA
ACGACAGCTGGGCTCCTGGAGCCAAAAGTGAAACAGCAGCAGGAGAAGCAGGAGCAGCAGG
AGCAACCCCTGAGTCCTGGCATATTGCACATGCGCGTCCTTTTTGCTGTTTGTTCAAAGAT
CAAATTACGATATGCTATGGCATGGCATCGCACGGCATGGTGTTGTTGTTTGTATCGTGAG
CGAGAAGGGAAATGAGCGGGCATAGAGTAGAGATACTTTTGTATCTGCGCGCTCTCTCGGC
TATTTCCTGTATATCCTGTGCGCACCCCAAAAACCAAAGAAGTAAAAACCGAAAACACATT
TCTTGACTTTCGTAAGTCACGCACACGGCGGATCCCTAATTAGCCCTCGCCTAGACTTCAA
GTCAATATAAATATACTACATAAAGTACTATATATGCATGTACATATATACGTAAGTATGT
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ATGGATCGTAAAGTGTGCACTTTATGGTCGGAATGGATCATCCACATAAAGTTGCAACCAA
ATTTAATGGAAATAAAAATTTAAGAGGGGAAAATGGAGGGAGGGCACTTAA

Notch 3 prime B3

# Appendix 2: Viability and Phenotype of $N^{nd\text{-}p}$ Mutants

| Fly | Viability | Phenotype |
|---|---|---|
| $N^{+}e(r)^{+}$ | Viable | Wild type wings |
| $N^{nd\text{-}p}e(r)^{+}$ | Viable | Notches in the wings |
| $N^{nd\text{-}p}e(r)^{27\text{-}1}$ | Lethal | Severe notching in the wings |
| $N^{4}e(r)^{+}$ | Viable | Notches in the wings |
| $N^{9}e(r)^{+}$ | Viable | Notches in the wings |
| $N^{2}e(r)^{+}$ | Lethal | Scalloped wings |
| $N^{3}e(r)^{+}$ | Lethal | Wild type wings |
| $N^{5}e(r)^{+}$ | Viable | Notches in the wings |
| $N^{5}e(r)^{27\text{-}1}$ | Lethal | Severe notching in the wings |

# Bibliography


1. Rubin G.M., Yandell M.D., Wortman J.R., Miklos G.L.G., Nelson C.R., Hariharan I.K., Fortini M.E., Li P.W., Apweiler R., Fleischmann W. Comparative genomics of the eukaryotes. Science. 2000;287:2204–2215.
2. Feany M.B., Bender W.W. A *Drosophila* model of Parkinson's disease. Nature. 2000;404:394–398
3. Dr. Paula Kavathas (SEOP Program Director and Professor Laboratory Medicine, Genetics and Immunobiology
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4. Kopan R., Ilagan M. Xenia G. The Canonical *Notch* Siganling Pathway: Unfolding the Activation Mechanism. Cell. *2009*;137:216–233.
5. Tsaknonas Spyros Artavanis, Matsuno Kenji, Fortini M.E. *Notch* Signaling. Science.1995;268:225-232.
6. Wharton K.A., Johansen K.M., Xu T., Artavanis-Tsakonas S. Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. Cell; 1995;43:567-581.
7. Go Masahiro J., Eastman D. S., Artavanis-Tsakonas S. Cell proliferation control by *Notch* signaling in *Drosophila* development. Devlopment.1998;125:2031–2040.
8. Artavanis-Tsakonas S., Rand M.D., Lake R.J. Notch Signaling: Cell Fate Control and Signal Integration in Development. Science; 1999; 284:770-776.
9. Brody Thomas B. Interactive Fly. 1996 http://www.sdbonline.org/fly/aimain/cites.htm
10. Hartenstein A., Rugendorff A., Tepass U., Hartenstein V. The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. Development; 1992; 116:1203-1220.
11. Cell Signaling Technology 1999-2009. http://www.cellsignal.com
12. Matsuno K., Eastman D., Mitsiades T., Quinn A.M., Carcanciu M.L., Ordentlich P., Kadesch T., Tsaknonas Spyros A. Human *deltex* is a conserved regulator of *Notch* Signaling. Nature Genetics.1998;19:74-78.
13. De Celis J.F., Garcia-Bellido A., Bray S. Activation and Function of *Notch* at the dorsoventral boundary in the *Drosophila* wing imaginal disc. Development;1996;122:359-369.
14. Brothers, V.M., Tsubota S.I., Germeradd S.E., Fristron J.W. Rudimentary locus of *Drosophila melanogaster*: partial purification of carbamylphosphate synthase-aspartate transcarbamylase-dihydroorotase complex. Biochem.;1978;16:321-332.

15. Tsubota Stuart, Ashburner Michael, Schedl Paul. P-Element-Induced Control Mutations at the *r* Gene of *Drosophila melanogaster*. Molecular and Cellular Biology;1985;5:2567-2574.
16. Falk D. Genetic Mosaics of the *rudimentary* locus of *Drosophila melanogaster*: a genetical investigation into the physiology of pyrimidine synthesis. Dev. Biology;1977;58:134-147.
17. Wojcik E., Murphy A. M., Fares H., Dang-Vu K., and Tsubota S. I.. *Enhancer of rudimentary, e(r),* a highly conserved *enhancer of the rudimentary gene*. Genetics; 1994; 138:1163-1170.
18. Roykhman M., Ibach S., Harding D., Wolff B., Vowels M., Gelsthorpe M. E., Wojcik E. J., Rizzo N. P. and Tsubota S. I. The Generation and Analysis of Deficiencies within a small genomic region on the X chromosome of *Drosophila melangaster* containing two genes, *enhancer of rudimentary* and CG15352. Fly; 2007.4:245-250.
19. Gelsthorpe, M., Pulumati M., McCallum C., Dang-Vu K., Tsubota S. I., The putative cell cycle gene, *enhancer of rudimentary*, encodes a highly conserved protein found in plants and animals. Gene; 1997.186:189–195
20. Wan C., Tempel Z., Liu J., Wang B.C., Rose R.H. Structure of the conserved transcriptional repressor *enhancer of rudimentary* homolog. Biochemistry; 2005;44:5017-5023.
21. Arai R., Kukimoto-Niino M., Uda-Tochio H., Morita S., Uchikubo-Kamo T. Crystal Structure of an *enhancer of rudimentary* homolog (ERH) AT 2.1 Angstroms resolution. Protein Science;2005;14:1888-1893.
22. Li H., Inoue M., Yabuki T., Aoki M., Seki E. Solution structure of the mouse *enhancer of rudimentary* protein reveals a novel fold. Journal of Biomol;2005;4:329-334.
23. Engels W. R., Berg and M. Howe. P elements in Drosophila. Mobile DNA.1989;437-484.
24. O'Hare K., Rubin G. Structure of P transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. Cell; 1983;34:25-35.
25. Mullins, M. C., D. C. Rio and G. M. Rubin. *Cis*-acting DNA sequence requirements for P element transposition. Genes and Development; 1989; 3:729-738.
26. Rio, D. C. Molecular mechanisms regulating Drosophila P element transposition. Annu. Rev. Genetics; 1990;24:543-578.
27. Kaufman, P. D. and D. C. Rio. Drosophila P-element transposase is a transcriptional repressor in vitro. Proc. Natl. Acad. Sci. U. S. A; 1991;88:2613-2617.
28. Kaufman, P. D. and D. C. Rio. P element transposition in vitro proceeds by a cut-and-paste mechanism and uses GTP as a cofactor. Cell; 1992;69:27-39.
29. Engels W.R. Hybrid dysgenesis in *Drosophila melanogaster*: rules of inheritance of female sterility. Genetics;1979;33:219-236.
30. Preston, C. R.,W. R. Engels. P element-induced male recombination and gene conversion in Drosophila. Genetics; 1996;144(4):1611-22.
31. Preston, C. R., Sved J. A.,Engels W. R.. Flanking duplications and deletions associated with P-induced male recombination in Drosophila. Genetics; 1996;144:1623-1638.

32. Dr. Stuart Tsubota, The College at Brockport, Department of Biology, personal communication
33. Lindsley D., Grell E. 1972. Genetic Variations of *Drosophila melanogaster.* Carnegie Institution publications no 627. Carnegia Institution of Washington, Washington, D.C.
34. Lindsley D., Zimm G.G. 1992. The Genome of *Drosophila melanogaster.* Academic Press, San Diego.
35. David Marcey and Nathan Silva. *Green Fluorescent Protein.* [Online]. Available: http://www.clunet.edu/BioDev/omm/gfp/frames/gfptx.htm
36. Cody Chris W., Prasher Douglas C., Westler William M., Prendergast Franklyn G., Ward William W. Chemical structure of the hexapeptide chromophore of the Aequorea green-fluorescent protein. Biochemistry; 1993;32:1212-1218.
37. Rubin, G.M. (1996) Around the genomes: The Drosophila genome project *Genome Research* 6, p71-79. Berekely Drosophila Genome Project.
38. Stacey, K. A. 1994. Recombination. In: Kendrew John, Lawrence Eleanor (eds.). *The Encyclopedia of Molecular Biology.* Oxford: Blackwell Science, 945–950.
39. Dr. Stuart Tsubota, The College at Brockport, Department of Biology (unpublished results)
40. Flybase, 2010. The *Drosophila* Database. Available from the World Wide Web at the URL http://morgan.harvard.edu
41. GenBank, 2010. NIH Genetic Sequence Database. Available from the World Wide Web at the URL http://www.ncbi.nlm.nih.gov/genbank/