Mapping of the *l(3)DTS4* Gene and Analysis of its Role in the Notch Pathway

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By

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ABSTRACT

MAPPING OF THE L(3)DTS4 GENE AND ANALYSIS OF ITS ROLE IN THE NOTCH PATHWAY

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l(3)DTS4 is an ethyl methanesulfonate (EMS) directed mutation that exhibits temperature sensitive dominant lethality and is one of the only mutations that expresses conditional dominant lethality in one dose in triploids (HOLDEN and SUZUKI 1973). *l(3)DTS4* is of particular note, not only because it is a dominant temperature sensitive lethal mutation, but also because our lab has shown it to have an unexpectedly dramatic interaction with two different Notch (N) alleles: N^{55e11} and N^{60g11} (JORDAN 2005). N^{55e11} */l(3)DTS4* and N^{60g11} */l(3)DTS4* heterozygotes showed significantly reduced wing notching in comparison to internal (N^{55e11} or N^{60g11}*/TM3, Sb*) controls (JORDAN 2005). A single copy of *l(3)DTS4* was sufficient to significantly prevent the wing notching caused by either Notch allele at the permissive temperature for *l(3)DTS4*. This indicates that *l(3)DTS4* plays a key role in the development of the outer wing margin, and its probable involvement in the Notch signaling cascade that regulates this type of cell differentiation.

The goal of this project was to map and identify the gene(s) disrupted by the *l(3)DTS4* mutation and to further understand their role in the Notch processes that determine outer wing growth. To this aim, complementation testing was used in order to create a deletion map indicating regions of possible interest. Two deletions, *Df(3L)ZP1* and *Df(3L)BSC113* were found to be non complementary to *l(3)DTS4.* That is, *l(3)DTS4*/*Df(3L)ZP1* and *l(3)DTS4*/*Df(3L)BSC113* heterozygotes had 100% fatality at all three tested temperatures. Together with polytene chromosome analysis, the complementation testing indicated that *l(3)DTS4* resides within a region from 67B3 – 67B5. Additionally, qualitative analyses were conducted in order to better understand the behavior of *l(3)DTS4.* Its effect on Serrate and dally were analyzed, and the development of *l(3)DTS4* homozygous embryos was also examined.

1. Introduction

The purpose of this thesis was to map and identify the gene that is responsible for the *l(3)DTS4* mutation. *l(3)DTS4* was first described by Suzuki (HOLDEN and SUZUKI 1973) in a screen of various 3rd chromosome *EMS* mutations. Later, *l(3)DTS4* was found to have a statistically significant, dramatic effect on wing notching in two different Notch heterozygotes, N^{55e11} and N^{60g11} (JORDAN 2005). This effect resulted in an almost complete rescue of wing notching (JORDAN 2005), suggesting that *l(3)DTS4* may interact with the Notch pathway. Of additional interest is the fact that *l(3)DTS4* is lethal over two copies of wild type alleles (HOLDEN and SUZUKI 1973) and that it is recessive lethal. As a result of the interaction between *l(3)DTS4* and the two tested Notch alleles, it was hypothesized that the *l(3)DTS4* mutation may map to a gene that is of particular function in the Notch signaling pathway.

The overall goal of these experiments was to further the understanding of *l(3)DTS4* and evaluate the potential for a possible role in the Notch signaling pathway. Identification of an unknown Notch protein partner would lend important information regarding the processes of this pathway and perhaps shed light on the development of the outer wing margin. Such a connection would be important, as its effects in the fly, from eye composition to wing formation, have been used as a model system for human development. RAMDASS explains how deregulation of Notch signaling has been

implicated in cervical cancers (RAMDASS *et al.* 2007) and BARTEN has postulated that beta amyloid aggregates implicated in the development of Alzheimers may be reduced using gamma-secretase inhibitors that can be partially separated from Notch inhibition (BARTEN *et al.* 2006). These are only two of the many current studies further investigating the therapeutic implications of the Notch pathway. Further research into this signaling system will undoubtedly shed more light on human conditions. *Drosophila melanogaster* is an ideal model system for Notch study and as more information regarding the *Drosophila* Notch pathway is uncovered it opens up new possibilities for its homologues.

1.1 The Notch Signaling Pathway

The Notch protein mediates cell to cell communication, involving gene regulation mechanisms that control multiple cell differentiation processes during both embryonic and adult life, making it both necessary in cell formation and determination, and essential for the development of nearly all multi-cellular animals (ARTAVANIS-TSAKONAS *et al.* 1999; BRAY 1998; BRAY 2006). Notch is a large single-pass transmembrane receptor protein with a large extracellular unit composed of transmembrane proteins, three Lin 12 Notch repeats and a linker region that connects to the transmembrane and intracellular domain (NICD) (ARTAVANIS-TSAKONAS *et al.* 1999; GO *et al.* 1998; LAI 2004). In *Drosophila*, the Notch receptor's two ligands are Delta and Serrate. Ligand binding is facilitated through the Delta/Serrate/*Lag2* (DSL) domain. Structural as well as functional

similarity to the DSL region in *Drosophila* has been substantiated in the corresponding homologues in various vertebrate models (MAINE *et al.* 1995).

Initiation of Notch signaling occurs outside of the cell when the extracellular domain of the Notch receptor interacts with the DSL region of a ligand on a neighboring cell, along with cooperative binding of the EGF repeats (BRAY 2006). Ligand binding promotes two proteolytic cleavages within Notch. The activity of the first cleavage is unclear, although it is known that this step is catalyzed by ADAM family metalloproteases (BRAY 2006). The second cleavage is mediated by γ-secretase (BRAY 2006). This cleavage ultimately results in the detachment of the NICD, so that it may translocate to the nucleus where it interacts with the DNA binding protein Suppressor of Hairless (*SuH*) and its co-activator, Mastermind (*Mam*) (BRAY 2006). Formation of the SUH–NICD–*Mam* ternary complex is necessary for activation of transcription from Notch target genes (KOVALL 2008). As explained by Bray (BRAY 2006), there are three different modes of Notch action: Lateral inhibition, lineage decisions and boundaries or inductive signaling.

Perhaps the best understood type of Notch signaling involves lateral inhibition. Lateral inhibition or lateral specification, is a mechanism by which an individual or group of cells may be singled out from a group of initially equivalent cells in order to undergo developmental changes. In this model, some signaling activity present in the original population of equivalent cells is either increased or decreased in the neighboring cells (ARTAVANIS-TSAKONAS *et al.* 1999). Notch signaling has been shown to selectively amplify or weaken differences within roughly equivalent cells, determining specific cell

fates (BRAY 1998). Several mechanisms are used in Notch dependent processes to regulate the ligand/receptor activity during lateral inhibition. One of these mechanisms, receptor turnover, results in the destabilization of Notch in the cell that is to become the signal sending cell. Additionally, regulation of Neuralized inhibitors that are expressed in activated Notch cells help reduce ligand activity in those cells. Upregulation of Notch target genes that encode repressor proteins inhibit cell fate promoting genes (BRAY 2006).

Notch receptors are involved in lineage decisions in various developmental scenarios, including hematopoiesis, T cell lineage decision and the differentiation into the neuronal or glial cell fate during neurogenesis (BRAY 2006). Lineage decisions arise through the asymmetrical inheritance of Notch regulators (such as Numb and Neuralized) between two daughter cells. Numb, along with the protein Sanpodo inhibits the Notch receptor through a mechanism involving endocytosis (LANGEVIN *et al.* 2005). Numb recruits both Notch and Sanpodo into endocytotic vesicles. Neuralized asymmetrically segregates into the same daughter cell as Numb, resulting in an increase of ligand activation (BRAY 2006).

Inductive Notch signaling describes signaling between boundaries or populations of cells. In this mode of Notch signaling, crosstalk occurs between two populations of cells and can establish either an organizer or result in the segregation of the two groups, resulting in distinct signaling populations (BRAY 2006). Fringe glycosyl transferases, and restricted ligand expression are two mechanisms that help to mediate inductive Notch signaling. Fringe glycosyl transferases affect boundary determination for inductive Notch

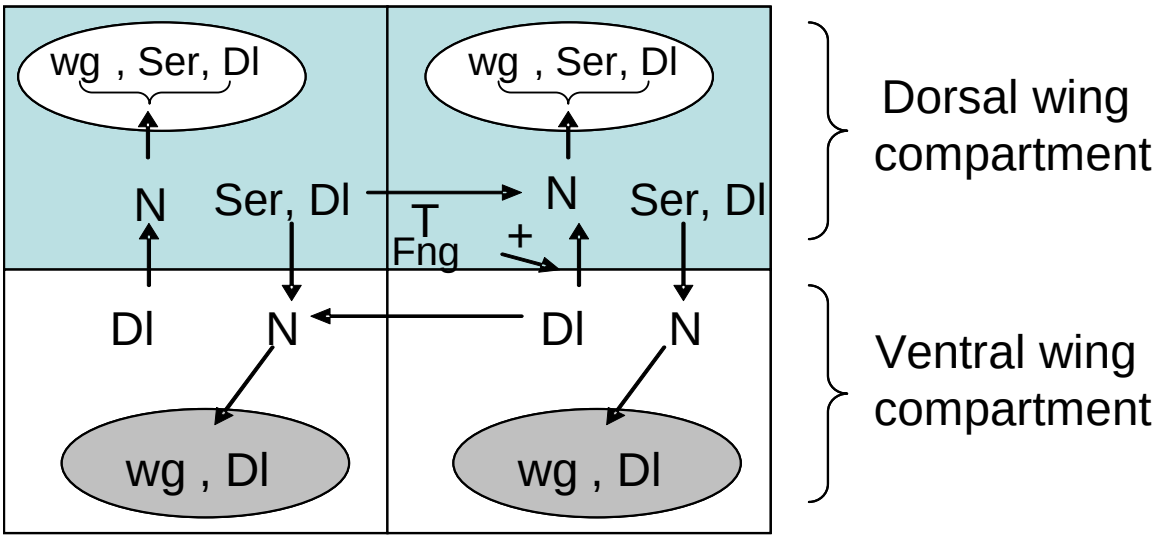
signaling through modifications to the Notch protein, altering its ligand specificity (CHENG *et al.* 2004). Restricted expression of ligands, restricted expression of Fringe and feedback regulation of Fringe expression can all contribute to differences between cell populations (CHENG *et al.* 2004), for example in the developing wing.

1.2 The Role of Notch in Wing Development

The development of the *Drosophila* wing is a good mechanism by which to study Notch function. It involves a variety of processes such as cell growth, proliferation, pattern formation and differentiation that are to some extent all under the genetic control of the Notch protein (BENITEZ *et al.* 2009). The wing is sensitive to genetic manipulation, and aberrations in gene expression or pattern are identifiable in wing morphology in a way that is informative about the developmental process (MOLNAR and DE CELIS 2006).

The wing develops from the wing imaginal disc, which is an epithelial tissue that changes during larval development to give rise to the wing, with approximately 50,000 structured cells (WIDMANN and DAHMANN 2009). As development continues to adulthood, the wing imaginal disc produces four components of Drosophila physiology: the wing blade, the thorax cuticle, and the distal and proximal hinge (WIDMANN and DAHMANN 2009). Of particular interest to this project is the development of the wing blade, as notching at the distal ends of the wing tip result from abnormal Notch signaling across the Dorsal Ventral (DV) compartment boundary.

The Notch signaling pathway plays an integral role in the development of the *Drosophila* wing margin (BENITEZ *et al.* 2009; DE CELIS and BRAY 1997; DE CELIS *et al.* 1996), being essential in the determination of the DV boundary, wing vein formation and bristle patterning. It is thought that Notch activation affects DV organization through a variety of signaling interactions at the DV boundary; Notch signaling occurs between two populations of cells and can establish organization and segregation of the groups (BRAY 2006). Notch is activated at the interface between the dorsal and ventral field of cells, and acts to keep the two populations distinct and separate. Notch activity determines which boundary cells are responsible for growth coordination and wing patterning. Notch activation at the DV interface (Figure 1) is reliant on signaling between cells of adjacent groups, not within their own populations (BRAY 2006). The gene Delta *(Dl)* is expressed by both dorsal and ventral cells in response to Notch activation, but this signaling is affected by the presence of Fringe (*Fng*), resulting in better signaling to the dorsal cells (MAJOR and IRVINE 2005). *Fng* increases Notch activation by *Dl* and reduces Notch activation by Serrate (HAINES and IRVINE 2003). Activated Notch in the dorsal cells interact with the gene Apterous to promote Serrate expression, causing Notch activation on the ventral side of the boundary and creating an asymmetry between the two populations (Figure 1). This expression is blocked from signaling to other dorsal cells by Fringe, and so must only signal across the compartment boundary to ventral cells where it activates Notch synergistically (Figure 1) (MAJOR and IRVINE 2005).



redrawn from (MAJOR and IRVINE 2005)

Figure 1. Gene Expression at the DV Compartment boundary of the developing wing, This schematic depicts the relationship between Notch activation and Dorsal - Ventral compartmentalization. *Dl* is expressed by both dorsal and ventral cells in response to Notch activation. This signaling is enhanced to dorsal cells through the presence of *Fng*. Serrate expression is blocked in dorsal cells by *Fng* and thus is limited to signaling back across the compartment boundary to ventral cells where it activates Notch synergistically.

Another signaling pathway that plays a key roles in the specification, growth, patterning and morphogenesis of the region of the wing disc that becomes the wing blade is the *Wnt/*Wingless pathway (WIDMANN and DAHMANN 2009). Wingless is initially expressed in a set of cells within the wing disc during mid second instar development (WU and COHEN 2002). By late second instar development, signaling between the DV compartments results in the expression of Wingless in cells adjacent to the DV boundary (WILLIAMS *et al.* 1993). At later stages, third instar larval development produces Wingless gene expression at both the distal and proximal hinge, producing a Wingless protein gradient to induce long range target gene expression in surrounding cells (WIDMANN and DAHMANN 2009).

Wingless expression results in an increase in wing disc pouch size, which then defines the wing primordium, which is required for wing growth (WIDMANN and DAHMANN 2009). Clonal analysis indicates that Wingless signaling acts to facilitate wing disc pouch growth by inhibiting apoptosis (WIDMANN and DAHMANN 2009). Malfunction of the gene Wingless is obvious in the wing malformations that arise. Loss of Wingless signaling during early larval development results in partial or complete loss of wing structures, coupled with transformation to dorsal structures (WIDMANN and DAHMANN 2009). Conversely, increased expression of Wingless in the notum can result in the formation of extraneous wing like structures (WIDMANN and DAHMANN 2009).

Additionally, Notch plays a role in the development of wing veins in the Drosophila wing by controlling lateral inhibition in order to determine the final vein width (JOHANNES and PREISS 2002). Widths of the veins that run along the wing are

precisely defined and depend on Notch activity. Mutation of Notch function result in a direct change in wing vein width – a decrease in Notch activity increases the width of the vein, whereas an increase does the opposite (BRAY 1998). In that process, Delta activates Notch in order to release the NICD, which then binds with Supressor of Hairless leading to transcriptional activation of effector genes Struhl and Artavanis (JOHANNES and PREISS 2002). This forces competent precursor cells into the inter-vein fate. Hairless acts against Notch signaling in this process, inhibiting activation of downstream effector genes and interfering with wing vein refinement (JOHANNES and PREISS 2002). The specification of cells adjacent to and bordering the wing vein is a precise selection, with a corresponding physiological change (BRAY 1998).

The Notch pathway is necessary not only for proper wing formation in Drosophila, but is used to determine cell fates and to regulate pattern formation throughout all stages and types of Drosophila development: flight muscles during metamorphosis (SOLER and TAYLOR 2009), the regulation of cell fates in the developing pronephros (kidney precursors) (MCLAUGHLIN *et al.* 2000), sensory bristles (JOSHI *et al.* 2006) and is essential at numerous stages of the development of the Drosophila eye (COOPER and BRAY 1999; HERRANZ and MILAN 2008; STRUTT *et al.* 2002). The Notch pathway is of interest for many reasons, including the fact that its dysfunction results in a tremendous variety of developmental defects and adult pathologies (LAI 2004).

Taken together, it is evident that the Notch regulatory pathway is continuously required for cell to cell communication during tissue differentiation. These communications result in intracellular signals that are involved in multiple processes in

Drosophila melanogaster, such as pigment cell differentiation and larval central nervous system development, as well as wing pattern formation and development (TAKIZAWA *et al.* 2003). Because *l(3)DTS4* has been shown to affect wing notching in a temperature dependent manner (JORDAN 2005), a likely hypothesis is that this mutation affects a gene that codes a protein partner that interacts either directly or indirectly in the Notch pathway.

The goal of this study was to use deletion mapping to map and identify the molecular region covered by the *l(3)DTS4* deletion in order to locate a potential Notch pathway protein partner. Additionally, further effects of the *l(3)DTS4* deletion were characterized by more narrowly identifying the stage at which *l(3)DTS4* homozygotes express embryonic lethality and quantifying the degree to which *l(3)DTS4* affects Serrate expression, and interacts with Dally (two members of the Notch pathway)

2. Methods

2.1 Complementation Tests

Deletions for complementation testing were chosen through the deficiency table provided in FlyBase (TWEEDIE 2009). Deficiencies were chosen by their proximity to the proposed cytological location of *l(3)DTS4:* 66B3 – 66 B11 (DEAK *et al.* 1993). Males from these stocks were crossed to female virgin *l(3)DTS4/Tm3, Sb* flies, and the progeny were scored in order to determine whether or not complementation had occurred by scoring for the survival of the various possible progeny classes. Because both the deletions and mutation were lethal in homozygotes, failure of complementation would be shown by the complete absence of "wild-type" appearing flies (i.e without visible markers such as *Sb*). The complementation tests were done at three different temperatures, 22.0°C, 25.5°C and 27.0°C because *l(3)DTS4* is a temperature sensitive mutation, and its effects on lethality and wing notching vary with temperature (JORDAN 2005). All fly stocks were obtained from the Bloomington Stock Center and maintained in standard yeast, cornmeal vials.

Ten virgin female *l(3)DTS4/TM3 Sb*^{1} were collected and placed in ten separate vials with males containing the genotype of interest (1 male/female pair per vial). After one week, the parents were removed. After emergence, all F_1 progeny were scored and

the numbers of each observed phenotype evaluated for statistically significant variations from Mendelian inheritance

Because these mating schemes utilized recessive lethal mutations, all stocks carried an easily identifiable balancer, either: *TM3 Sb* (Stubble bristles), *TM6b Tb* (Short, wider abdomen, extra humeral bristles), *TM6c* (Stubble bristles) or *TM2* (Abnormal halteres). As a result, failure of complementation would be indicated by the complete absence of "wildtype" appearing progeny (progeny that do not carry either balancer); proof that flies heterozygous for both *l(3)DTS4* and the deficiency were non viable. Conversely, complementation would be apparent by Mendelian inheritance among the F_1 progeny, with all phenotypes visible including wild type (Figure 2).

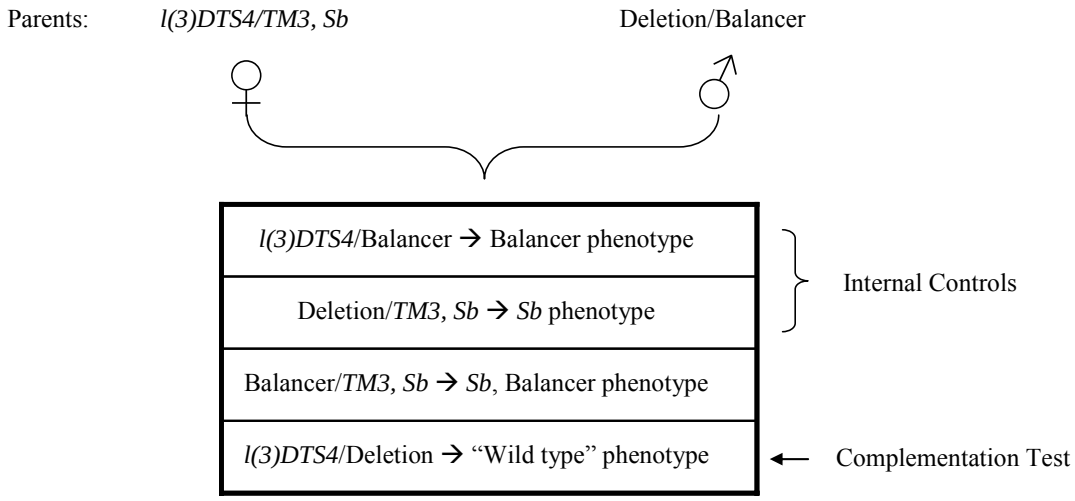


Figure 2. Deletion Mapping Scheme

This figure shows the deletion mapping scheme for *l(3)DTS4*. *l(3)DTS4/TM3, Sb* virgin females were crossed to males heterozygous for the chosen deletion and an easily identifiable balancer. Mendelian inheritance predicts four different progeny. *l(3)DTS4*/Balancer will bear the balancer phenotype, and can be easily distinguished from the other three progeny. Deletion/*TM3, Sb* will always have the *Sb* phenotype. Balancer/*TM3, Sb* will have both the balancer and *TM3, Sb* phenotypes. *l(3)DTS4/*deletion does not carry any identifiable markers, and will appear to be wild type, without *Sb* or the balancer phenotype. *l(3)DTS4/*deletion provides the complementation test, as survival of these flies suggest that the chosen deletion does not cover *l(3)DTS4*. *l(3)DTS4/*balancer serves as an internal control for *l(3)DTS4/*deletion and deletion/*TM3, Sb* serves as an internal control for deletion/*l(3)DTS4* (see section 2.3).

2.2 Statistical Analysis

All phenotypic distributions were analyzed according to standard statistical methods. In statistics, a null hypothesis (H_0) is an explanation that is provided in order to explain the behavior of a given set of data. For the purposes of this experiment, three null hypotheses were taken into consideration. One was the expected Mendelian inheritance among the F_1 progeny, the second was the amount of viable progeny of deletion/*l(3)DTS4* to *l(3)DTS*/balancer, and the third compared the amount of deletion/balancer to deletion/*l(3)DTS4*. By comparing *l(3)DTS4*/Balancer to *l(3)DTS4*/deletion, we were able to control for the dominant lethality of *l(3)DTS4*, and by comparing deletion/*l(3)DTS4* to deletion/balancer, we were able to control for the dominant lethality of the deletion. This was done because the dominant lethality of *l(3)DTS4* will affect both balancer and mutation alike, and either the deletion or *l(3)DTS4* may have partial semidominant lethal character which could cause a false positive in the first statistical test (Mendelian inheritance).

The internal controls served as a numerical standard that could be used to assess the significance of the complementation test. A possible genetic interaction would be indicated by complete absence or significant underrepresentation of the progeny bearing both the *l(3)DTS4* gene and the mutation in comparison to the internal controls. The significance of such a reaction was tested using an a priori chi squared test (χ^2).

An *a priori* chi squared (χ^2) test uses the values that were obtained from the experiment and compares them for adherence to the null hypotheses. There were three criteria that were analyzed statistically for adherence to the null hypothesis: whether or not the progeny distributed according to Mendelian inheritance, whether the ratio of *l(3)DTS4*/balancer was comparable to *l(3)DTS4/*deletion and whether deletion/balancer was comparable to *l(3)DTS4/*deletion. These were evaluated by subtracting the number of expected progeny from the observed, squaring that value and dividing it by the number of expected to obtain the χ^2 squared value (JONES 2008).

In order to allow acceptance or rejection of the null hypotheses, the significance of the statistical test was evaluated by determination of a two tailed *p*-value. In statistical hypothesis testing, the *p*-value is the probability of obtaining a result at least as far from expectations as the one that was actually observed, given that if the null hypothesis were true. For example, if the *p* value were less than 0.05, then the results are only at most 5% likely to be as far from expectations as observed, provided that the null hypothesis were true.

2.3 Polytene Chromosome Analysis

Polytene chromosomal analysis of *w; +/+; Df(3L)ZP1/+* larvae was done following the standard protocol provided in Drosophila Protocols (KENNISON 2000). *w;*

+/+; Df(3L)ZP1/TM6, Tb flies were crossed to "wild type" *w; Canton S* flies and maintained at 22.0°C on yeasted media. After approximately six days, *w; +/+; Df(3L)ZP1/+* climbing 3rd instar larvae were chosen by selecting against the *Tb* (tubby) phenotype. These larvae were placed in a 7% saline solution for approximately 1 minute in order to ensure swelling of the appropriate glands due to the hypertonic environment. The salivary glands were extracted by grasping the larvae firmly by the mouth hooks and the tail end with dissecting tweezers and separating the two. The salivary glands were easily identifiable as long sausage shaped appendages connected at the top by a thin membranous joint. The joint connecting the glands was severed allowing removal of the glands which were then exposed to 45% acetic acid for approximately 30 seconds ï 1 minute in order to ensure proper disruption of the nuclei and fixation of chromatin. The salivary glands were then moved to a cover slip containing one drop of staining solution, composed of:

3 parts acetic acid

2 parts lactic acid

1 part H_2O

1 g orcein per 50 mL

for several minutes. The slide was carefully lowered onto the cover slip, inverted, and then the cover slip tapped in place in a spiral pattern in order to spread the chromosomes. Additionally, a sharp probe was used to agitate the cover slip back and forth in order to disperse the dye across the cover slip and ensure proper spreading of the chromosomes.

The slides were analyzed immediately with a compound microscope with phase contrast optics, and further spreading could be done if warranted. If slides were to be kept for longer than 24 hours, the edges were sealed with clear nail polish. Images were taken and sharpened using SPOT Advanced Imaging Software (Diagnostic Instruments, Inc. Sterling Heights, MI).

2.4 Wing Images

In order to capture the effect that *l(3)DTS4* has on wing serration, *l(3)DTS4/Tm3, Ser* was crossed to *w; +/+; Dr/Tm3, Ser* in order to produce progeny with both the normal *Ser* phenotype and the reduced *Ser* phenotype seen in *l(3)DTS4/Ser* heterozygotes. Because *l(3)DTS4* has such a dramatic affect on wing serration, *l(3)DTS4/Tm3, Ser* flies had an obvious reduced *Ser* phenotype, with many of the flies appearing wild type. Conversely, the *Dr/Tm3, Ser* flies were observable through normal *Ser* appearance and *Dr* (dominant eye phenotype):

l(3)DTS4/Tm3, Ser → reduced *Ser*

Dr/Tm3, Ser → *Ser* and *Dr*

This cross allowed for an internal control of *Ser* expression. Wings were removed from both *l(3)DTS4/Tm3, Ser* and *Dr/Tm3, Ser* flies and mounted on glass slides with

Permount. Images were photographed with a compound microscope with phase contrast optics.

2.5 Analysis of Df(3L)ZP1/N^{55e11} and Df(3L)ZP1/N^{60g11} Wing Notching

In order to determine the effect, if any, that *Df(3L)ZP1* has on wing notching, the amount of wing notching present in *Df(3L)ZP1/N^{55e11}* and *Df(3L)ZP1/N^{60g11}* heterozygotes at both 22°C and 25.5°C were analyzed and compared to internal controls. Either male *N^{60g11}/Fm7a* or *N^{55e11}/Fm7a* flies were crossed to female *Df(3L)ZP1/TM3, Sb*. The progeny each had identifiable phenotypes with the following distribution:

N^{60g11} or *N^{55e11}/Df(3L)ZP1* → Notched wings

N^{60g11} or *N^{55e11}/TM3, Sb* → Notched wings, *Sb* bristles

Df(3L)ZP1/Fm7i → Bar eyes

Fm7i/ TM3, Sb → Bar eyes, *Sb* bristles

The *N^{60g11}* or *N^{55e11}/TM3, Sb* progeny served as internal controls for the amount of wing notching present on *N^{60g11}* or *N^{55e11}/Df(3L)ZP1* heterozygotes. The amount of wing notching was determined by assessing the distance between wing veins two and three a value of one, then applying this number to the total length of wing notches present on each wing of the fly. This value was divided by two, and the average amount of wing notching per wing was measured for each genotype and compared to internal controls.

2.6 Analysis of Embryonic Lethality

The stage at which *l(3)DTS4* homozygotes died was assessed using *l(3)DTS4/Tm3, Ser, ActGFP* flies so that the homozygous *l(3)DTS4* embryos could be differentiated by the lack of *GFP*. Male and female *l(3)DTS4/Tm3, Ser, ActGFP* flies were kept on grape juice agar media for varying lengths of time in order to evaluate the surviving progeny. Mendelian inheritance predicted three progeny genotypes. *l(3)DTS4/TM3, Ser, ActGFP* have one copy of *GFP* and would fluoresce under the appropriate illumination. *l(3)DTS4* homozygotes do not carry a copy of *GFP* and would show no *GFP* fluorescence. *TM3, Ser, ActGFP* homozygotes carry two copies of *GFP* and should fluoresce more brightly than the *l(3)DTS4/TM3, Ser, ActGFP* embryos. Consequently, three different fluorescence values were expected: bright *GFP* fluorescence from the *TM3, Ser, ActGFP* homozygotes, intermediate *GFP* fluorescence from the *l(3)DTS4/TM3, Ser, ActGFP* heterozygotes, and minimal autofluoresence from the *l(3)DTS4* homozygotes. However, only two different fluorescence values were observed: intermediate *GFP* fluorescence indicated *ActGFP* heterozygotes, and auto fluorescence indicated *l(3)DTS4* homozygotes. We concluded that *TM3, Ser, ActGFP* homozygotes did not live to the embryonic stage because there was only one type of *GFP* fluorescence seen. All tests were conducted at room temperature (approximately 23°C) on standard grape juice agar collection media:

750 ml of H_2O

20 g of agar

250 ml of concentrated grape juice

6.25 g of sucrose

The period of egg laying collection times varied from 1 to 3 hours and the number of live embryos was recorded from 10 to 20 hours post lay for all three time trials.

3. Results

3.1 l(3)DTS4: Area of Maximum Likelihood

In addition to the proposed cytological location provided by FlyBase (DEAK *et al.* 1993), an area of maximum likelihood was chosen for the location of *l(3)DTS4*, based on the recombination data provided by Holden and Suzuki (HOLDEN and SUZUKI 1973). Using roughoid (*ru*) as the leftmost map location, with a map location of 3 - 0.0 cM (61F8) and hairy (*h*) as the rightmost map location, with a map location of 3 - 26.5 cM (66D10), the number of right single, left single and double recombinants, along with the parental type progeny were as follows:

right single recombinants = 15 + 17 = 32

left single recombinants = 200 + 188 = 388

double recombinants = assume zero

parental-type progeny = 570 + 744 = 1314

It is important to note that in the original experiment there were no right single recombinants or double recombinants. These numbers were estimated using the proportions of phenotypic frequencies that were observed at the two different temperatures (22 °C and 29 °C). Comparison of the two temperatures was done in order to observe the *l(3)DTS4* phenotype, which is temperature sensitive lethality. The double

recombinants were not detectable or distinguishable from the parental stock because a double recombination of two marker phenotypes at the right and left ends puts them back at the same chromosome (same phenotype as non recombinant progeny). In order to account for the introduction of error from this estimation, these numbers were then reduced by half. By using numbers from different temperatures you increase the probability of error, and by reducing the numbers you increase the error bars to reflect the additional error that was caused by the subtraction across temperatures. Although the reduction by half may not fully account for the additional error, it acknowledges the fact that the error exists, and is a way of examining the data rationally, giving:

left single = 16

right single = 194

double = 0

parental = 657

The maximum likelihood / mapping function analysis of the half data set gives a maximum likelihood location of DTS4 at 25.0 cM, with an upper 95% confidence limit of 25.6 cM, and a lower 95% confidence limit of 24.1 cM (Figure 3) (JONES 2008).

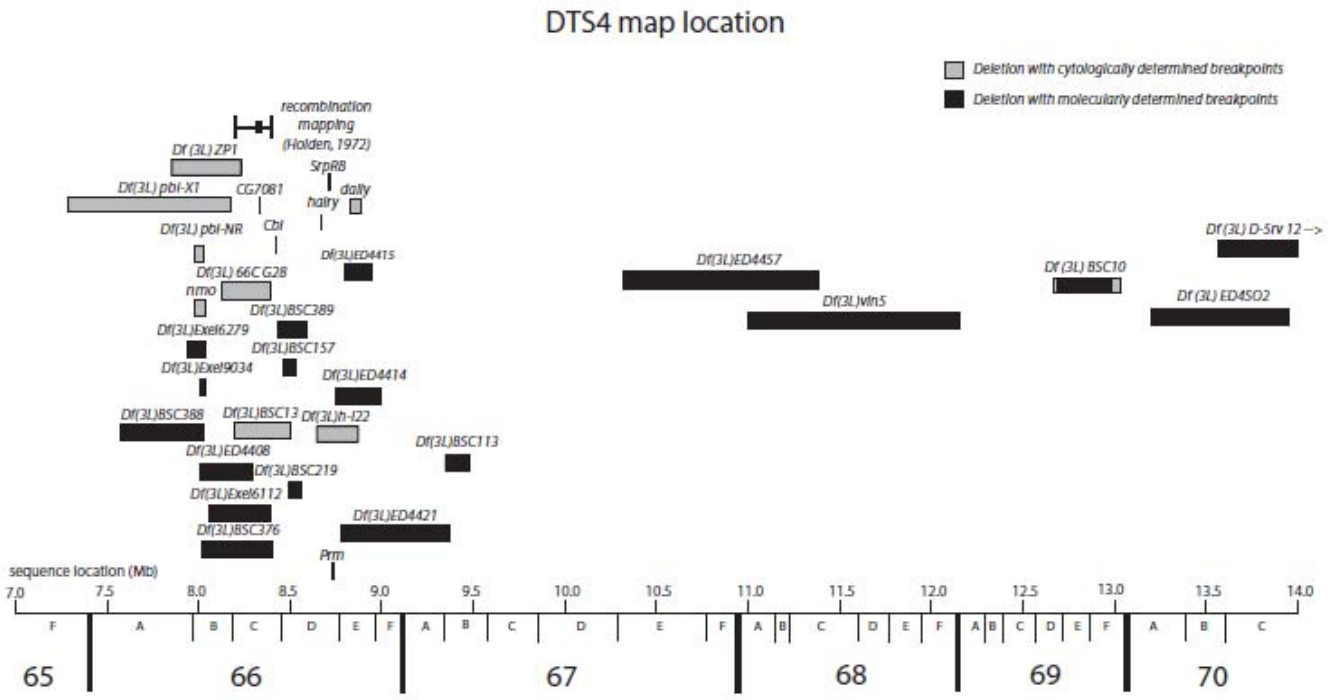


Figure 3. l(3)DTS4 Deletion Map

This figure shows the deletions and lethal mutations that were compared for complementation to *l(3)DTS4*. Black bars represent deletions or known lethal mutations with known molecular breakpoints and gray bars represent deletions or known lethal mutations with known cytologic breakpoints. The area of maximum likelihood as predicted from Holden and Suzuki's recombination data is shown at the top left of the figure. All of the deletions and lethal mutations in this map do complement *l(3)DTS4* except for *Df(3L)ZP1* and *Df(3L)BSC113.*

3.2 Deletion Mapping

Generally in deletion mapping, heterozygotes show the mutant phenotype, which in this case would be lethality, if covered by the deletion. Because *l(3)DTS4* is homozygous lethal, we suspected that it should be hemizygous lethal, even though it is a gain of function mutation. Many gain of function mutations are neomorphs, rather than hypermorphs that could survive over a deletion. Previous reports that *l(3)DTS4* died over various deletions also suggested that deletion mapping would be an effective way to further localize it (MAROY 1997). Using the proposed cytological location for *l(3)DTS4* provided by DEAK (DEAK *et al.* 1993)and the area of maximum likelihood determined from the recombination data of HOLDEN and SUZUKI (HOLDEN and SUZUKI 1973), a region of interest on the left arm of the 3rd chromosome of *Drosophila melanogaster* was chosen as the basis for the selection of lethal alleles for complementation testing (Figure 3). We achieved complete deletion coverage for the region 65F3 to 67B3 (Figure 3). Within this region, only two deletions, *Df(3L)ZP1* and *Df(3L)BSC113,* died over *l(3)DTS4*. Both *Df(3L)ZP1/ l(3)DTS4* and *Df(3L)BSC113* heterozygotes exhibited 100% lethality at all three temperatures tested: 22.0°C, 25.5°C and 27.0°C. These observations were statistically significant with a p value < 0.001 (Table 1, Table 2). Different temperatures were tested to control for the dominant temperature sensitive lethal activity of *l(3)DTS4*. One other deletion showed a temperature sensitive interaction with *l(3)DTS4* (see Section 3.6).

Table 1. *l(3)DTS4/Df(3L)ZP1* Complementation Test

Genotype	Number of progeny (Temp)			Phenotype
	(22.0°)	(25.5°)	(27.0°)	
w; l(3)DTS4/Df(3L)ZP1*	0	0	0	Normal wings, normal bristles (Complementation test)
w; l(3)DTS4/TM3, Sb, Ser*	418	382	183	Stubble bristles, Does not appear *Ser*
Df(3L)ZP1/Tm3, Sb				Stubble bristles
TM3,Sb, Ser/Tm3, Sb	0	0	0	dead
P value (mendelian)	<0.0001	<0.0001	<0.0001	

This table shows the progeny that resulted from a cross between female *w*; l(3)DTS4/TM3, Sb*[1] and male *Df(3L)ZP1/TM3, Sb, Ser*. Only one phenotype was visible in the resulting progeny, *Sb* bristles and normal wings. It was determined that *w*; l(3)DTS4/Df(3L)ZP1* flies were not present due to the lack of any flies with wild type bristles. It was determined that *TM3,Sb, Ser/Tm3, Sb* flies were not present due to the lack of any flies that were Ebony as well as *Sb* and *Ser*. Because *l(3)DTS4* reduces *Ser* expression, *Df(3L)ZP1/Tm3, Sb* and *w*; l(3)DTS4/TM3, Sb, Ser* flies were indistinguishable from each other. This eliminates the possibility of using internal controls, thus statistical significance was assessed using adherence to Mendelian inheritance.

Table 2. *l(3)DTS4/Df(3L)BSC113* Complementation Test

Genotype	Number of progeny (Temp)			Phenotype
	(22.0°)	(25.5°)	(27.0°)	
w; l(3)DTS4/Df(3L)BSC113*	0	0	0	Normal body, normal bristles (Complementation test)
w; l(3)DTS4/TM6B, Tb*	77	81	44	Tubby body
Df(3L)BSC113/Tm3, Sb	69	83	46	Stubble bristles
TM3,Sb/Tm6B, Tb	45	52	38	Ebony, stubble, Tubby
P value (mendelian)	<0.0001	<0.0001	<0.0001	
P value (Internal Control 1)	<0.0001	<0.0001	<0.0001	
P value (Internal Control 2)	<0.0001	<0.0001	<0.0001	

This table shows the progeny that resulted from a cross between female *w*; l(3)DTS4/TM3, Sb*[1] and male *Df(3L)BSC113/TM6B, Tb*. Three phenotypes were visible in the resulting progeny. It was determined that *w*; l(3)DTS4/Df(3L)BSC113* flies were not present due to the lack of any flies with wild type bristles and body. The resulting progeny were assessed for adherence to Mendelian inheritance, and the *P* value (mendelian) was determined at all three temperatures. The *P* value (Internal Control 1) was determined by comparing the number of *w*; l(3)DTS4/Df(3L)BSC113* flies to *w*; l(3)DTS4/TM6B, Tb*, the *P* value (Internal Control 2) was determined by comparing the number of *Df(3L)BSC113/Tm3, Sb* flies to *w*; l(3)DTS4/Df(3L)BSC113* (See section 2.3).

The cytological locations provided for the two deletions that failed to complement *l(3)DTS4* were as follows: *Df(3L)ZP1* was given as 66A17-66A20;66C1-66C5 (MAROY 1997), based on a personal communication to FlyBase citing the unpublished results of a polytene chromosome analysis. *Df(3L)BSC113* was given as 67B1 – 67B5, based on the Berkeley Drosophila Genome Release 5 estimated cytology of aberrations with sequence defined breakpoints. Because cytologically defined breakpoints are inherently less accurate, further testing was done in order to verify the location of *Df(3L)ZP1*. Deletions with molecularly defined endpoints were tested against *Df(3L)ZP1* and the only one that was heterozygous lethal was *Df(3L)BSC113/Df(3L)ZP1* (Table 3); indicating that a region closer to 67B1 – 67B5 is more likely for *Df(3L)ZP1.* In order to further evaluate the discrepancies observed in the location for *Df(3L)ZP1*, polytene chromosome analysis was done in order to determine the location of the deletion.

Table 3. Df(3L)ZP1/ Df(3L)BSC113 Complementation Test

Genotype	Number of progeny (Temp)			Phenotype
	(22.0°)	(25.5°)	(27.0°)	
Df(3L)ZP1/Df(3L)BSC113	0	0	0	Normal body, normal bristles (Complementation test)
Df(3L)ZP1/TM6B, Tb	41	32	39	Tubby body
Df(3L)BSC113/Tm3, Sb, Ser	37	29	43	Stubble bristles, Ser wings
TM3,Sb, Ser/Tm6B, Tb	35	30	38	Ebony, stubble, Tubby body, *Ser* wings
P value (mendelian)	<0.0001	<0.0001	<0.0001	
P value (Internal Control 1)	<0.0001	<0.0001	<0.0001	
P value (Internal Control 2)	<0.0001	<0.0001	<0.0001	

This table shows the progeny that resulted from a cross between female *w*; Df(3L)ZP1 /TM3, Sb, Ser* and male *Df(3L)BSC113/TM6B, Tb.* Three phenotypes were visible in the resulting progeny. It was determined that *Df(3L)ZP1/Df(3L)BSC113* flies were not present due to the lack of any flies with wild type bristles and body. The resulting progeny were assessed for adherence to Mendelian inheritance, and the *P* value (mendelian) was determined at all three temperatures. The *P* value (Internal Control 1) was determined by comparing the number of *Df(3L)ZP1/Df(3L)BSC113* flies to *Df(3L)ZP1/TM6B, Tb,* the *P* value (Internal Control 2) was determined by comparing the number of *Df(3L)BSC113/Tm3, Sb, Ser* flies to *Df(3L)ZP1/Df(3L)BSC113* (See section 2.3).

3.3 Polytene Chromosome Analysis

In order to clarify the apparent discrepancy between the provided cytological location for *Df(3L)ZP1* and our deletion mapping results, polytene chromosome analysis was done on *w; +/+; Df(3L)ZP1/+* flies. The location of the *Df(3L)ZP1* deletion was claimed to be approximately 66A17-20;66C1-5 (MAROY 1997). If so, this would be a relatively large deletion, and one that should be easily identifiable. Upon examination of 3L, a large deletion in the 66A17-20;66C1-5 was found not to be present, as there was no deletion loop in this region. However, an inversion loop was seen with a left breakpoint at approximately 67A 10 - 11 and a right breakpoint at approximately 67B 6 - 7 on 3L (Figure 4). Deletion loops and inversion loops are easily distinguished as an inversion results in a loop that is of equal thickness to the rest of the chromosome arm. Deletion loops are half as thick because they contain only one chromatid. They often detach from the homologue and are noticeably thinner than the rest of the chromosome arms.

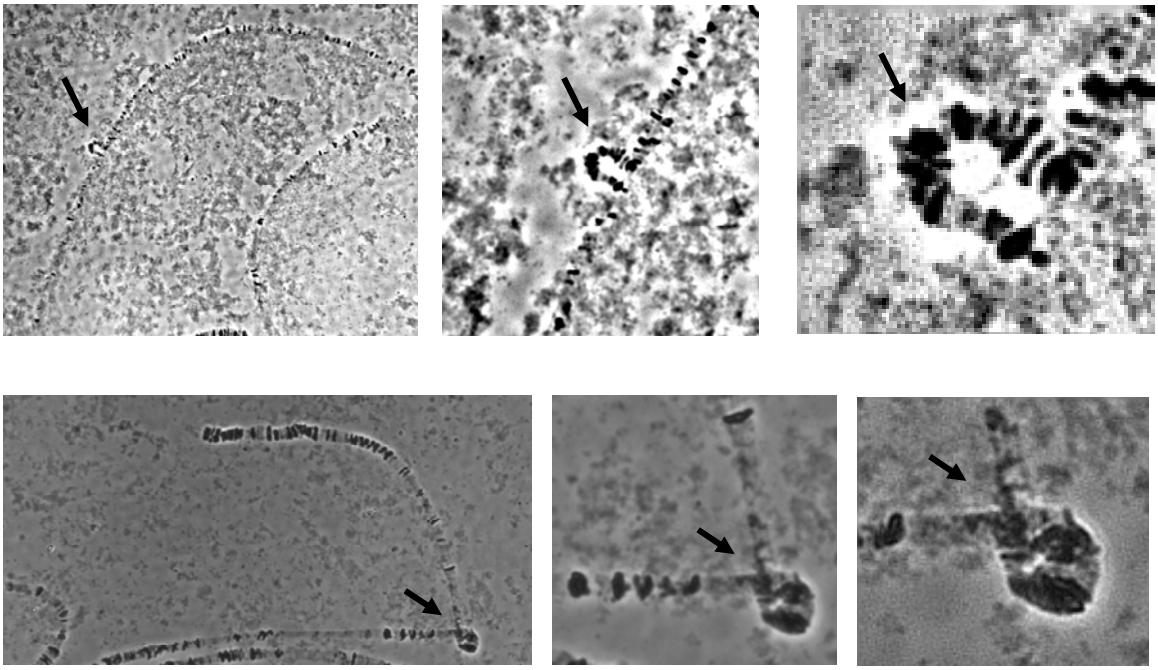


Figure 4. Inversion Loop on Polytene Chromosome 3L

This figure shows 3L polytene chromosome arms. The top three panels are increasingly zoomed in images of the inversion loop seen with a left breakpoint at approximately 67A 10 - 11 and a right breakpoint at approximately 67B 6 – 7. The bottom three panels are also of the same inversion loop seen at 3L, but these are from a different slide.

Using the location identified in the polytene chromosome analysis, additional complementation tests were conducted using all available deficiencies spanning this region. Complete failure of complementation was observed for the *Df(3L)BSC113* deficiency over a copy of either *l(3)DTS4* or *Df(3L)ZP1* (Table 2, 3). These additional deletion mapping experiments indicated that *l(3)DTS4* is either wholly or partially contained within 67B3 – 67B5 (Figure 3), and clarified the location of the right breakpoint determined in polytene chromosome analysis.

Because *Df(3L)ZP1/Df(3L)BSC113* dies (Table 3), the right breakpoint of *Df(3L)ZP1* appears to break within a lethal gene, presumably *l(3)DTS4*. Deletion mapping has indicated that *l(3)DTS4* is not covered by the region deleted by *Df(3L)ED4421* (Figure 2, Figure 5). As a result, many of the genes deleted by *Df(3L)BSC113* can be eliminated as potential candidates for *l(3)DTS4,* leaving eight potential genes (Figure 5). Genes indicated within this region include several with unknown functions, but also proteins involved in binding and even peripheral nervous system development (Figure 5) (Table 4). Further complementation testing with lethal alleles for the most likely candidates could illuminate which is most likely to be affected by the *l(3)DTS4* mutation.

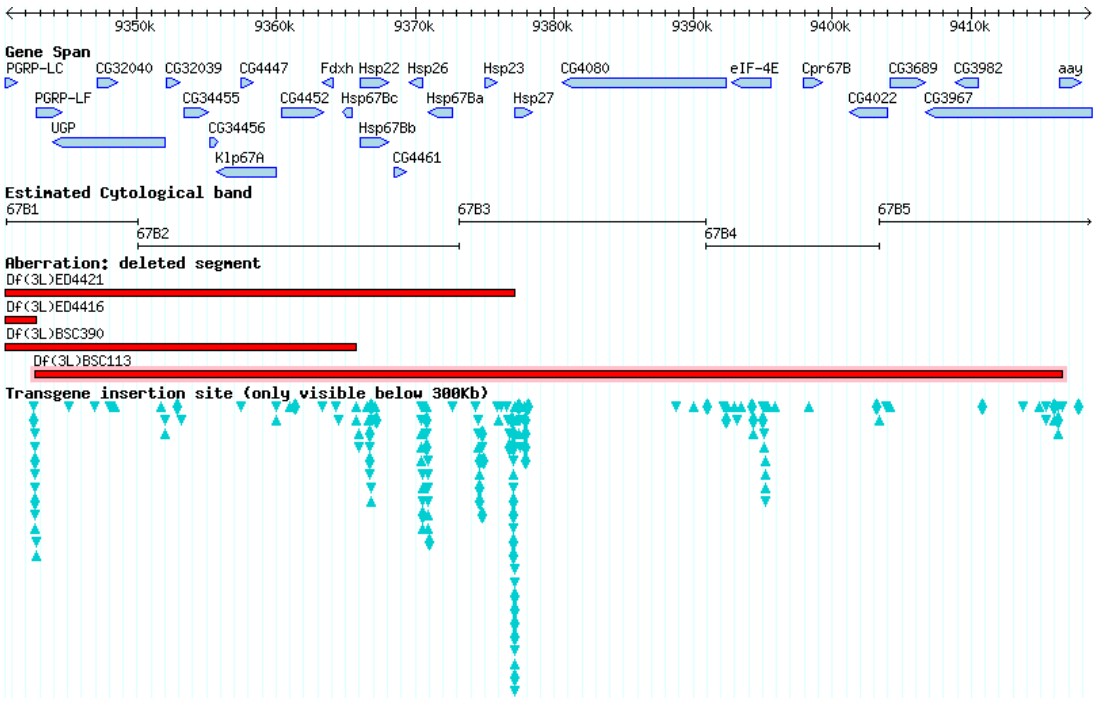


Figure 5. Cytological Map of Df(3L)BSC113

This figure shows the cytological location of *Df(3L)BSC113* along with the genes that are covered by this and neighboring deletions. Our mapping experiments have indicated that *l(3)DTS4* lives over one copy of *Df(3L)ED4421*, eliminating gene candidates that are covered by this region. This reduces the number of gene candidates to eight: CG4080, eIF-4E, Cpr67B, CG4022, CG3689, CG3967, CG3982 and aay.

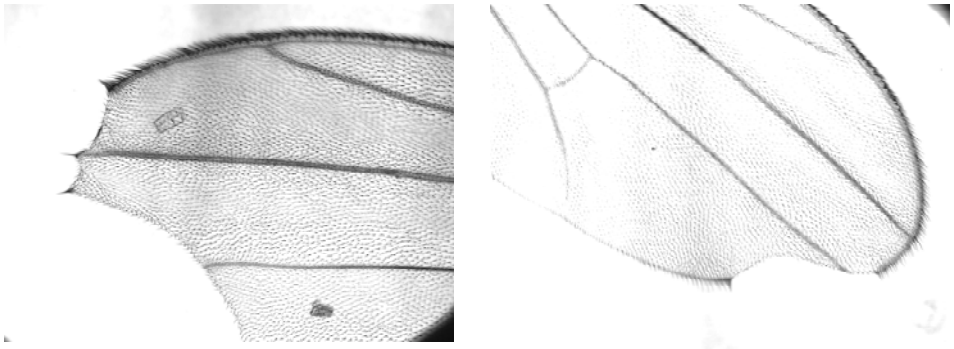
Table 4. Possible *l(3)DTS4* Gene Candidates

Name	Location	Function
CG4080	67B3-67B4	zinc ion binding
eIF-4E	67B4-67B4	translation initiation factor activity; RNA cap binding; SUMO binding.
Cpr67B	67B4-67B4	structural constituent of chitin based cuticle.
CG3689	67B5-67B5	hydrolase activity, mRNA cleavage.
CG4022	67B4-67B5	Unknown
CG3982	67B5-67B5	hydrolase activity
CG3967	67B5-67B5	Unknown
astray	67B5-67B5	phosphoserine phosphatase activity; L serine biosynthetic process; axon guidance, peripheral nervous system development

This table shows the list of eight potential gene candidates for *l(3)DTS4*. Their cytological locations and known functions are listed. They were chosen based on their coverage by *Df(3L)BSC113*, and narrowed down to these eight by eliminating genes that were covered by both *Df(3L)ED4421* and *Df(3L)BSC113* (Figure 5).

3.4 Wing Serration

An interesting observation that contributes to the function of *l(3)DTS4* in wing formation is the lack of wing serration in flies bearing the genotype *l(3)DTS4/Ser*. Figure 6 shows the qualitative extent to which *l(3)DTS4* reduces the amount of wing notching in flies bearing one copy of the *Serrate* gene (Figure 6). In comparison, *Df(3L)ZP1* had a negligible effect on the level of serration (See Section 3.6).



+/+; +/TM3, Ser +/+; l(3)DTS4/TM3, Ser

Figure 6. Wing Serration

This figure shows the reduction of *Ser* seen in flies heterozygous for *l(3)DTS4/TM3, Ser* in comparison to flies that are *+/Tm3, Ser*.

3.5 Analysis of Embryonic Lethality

In order to determine more about the lethality of *l(3)DTS4*, a timed egg lay was conducted in order to measure the time at which *l(3)DTS4* homozygotes die. At each time set, two populations of embryos were observed: those that brightly fluoresced (*l(3)DTS4/ Tm3, Ser, ActGFP*) and those that autofluoresced (*l(3)DTS4*). Continual development (versus death) was distinguished by longer body size. At each time interval the number of embryos at the developmental stage was counted. As time progressed it was clear that the *l(3)DTS4* homozygote population were not growing in body size, or reproducing any of the embryonic developmental stages that are indicative of proper *Drosophila* embryonic growth (HARTENSTEIN 1993). It was determined that at 23°C, homozygous *l(3)DTS4* embryos between 17 – 20 hours old had a 97.01% death rate, 18 – 20 hours old a 100% death rate and 19 – 20 hours old a 100% death rate (Table 5). This indicates death at or before stage 17 of embryonic development (SWEETON *et al.* 1991), which is consistent with the Notch lethal sensitive periods (SHELLENBARGER and MOHLER 1975). At stage 17, head involution is complete and the embryonic surface has completed development. The cuticle gets thicker and cuticle specializations, such as ventral denticles, dorsal trichomes, basiconical sensilla, trichoid sensilla, and the cephalopharyngeal skeleton secreted by the involuted head epidermis, become visible. Additionally, abdominal segments 8 and 9, antennomaxillary complex, anal plate, clypeolabrum. posterior spiracle and the thoracic segment all reach nearly final developmental stages (HARTENSTEIN 1993).

Table 5. l(3)DTS4 Embryonic Lethality

Age (hours)	*l(3)DTS4/TM3, Act GFP, Ser (#)*	*% Lethality Heterozygotes*	*l(3)DTS4 (#)*	*% Lethality Homozygotes*
9 – 12 hrs	*67*	*0*	*10*	*70.15*
10 – 12 hrs	*41*	*0*	*5*	*75.61*
11 – 12 hrs	*27*	*0*	*4*	*70.37*
13 – 16 hrs	*67*	*0*	*5*	*85.07*
14 – 16 hrs	*41*	*0*	*3*	*85.37*
15 – 16 hrs	*27*	*0*	*3*	*77.78*
17 – 20 hrs	*64*	*4.48*	*1*	*97.01*
18 – 20 hrs	*40*	*2.44*	*0*	*100*
19 – 20 hrs	*25*	*7.41*	*0*	*100*

This table shows the number of *l(3)DTS4/TM3, Act GFP, Ser* and *l(3)DTS4* embryos present at each time trial. The white rows represent a single experiment that was collected for three hours, the light gray rows represent a single experiment that was collected for two hours and the dark gray rows represent a single experiment that was collected for one hour. The percent lethality was determined by using Mendelian Inheritance to predict that there should be twice as many heterozygous progeny as homozygous. The percent lethality for both *l(3)DTS4/TM3, Act GFP, Ser* and *l(3)DTS4* was determined by subtracting the number of flies observed for either genotype from the number of flies expected for either genotype, dividing this number by the number observed and multiplying it by 100. The number of flies expected was based off of the first counts for each experiment (9 – 12 hrs, 10 – 12 hrs and 11 – 12 hrs).

3.6 Analysis of Df(3L)ZP1/N^{55e11} and Df(3L)ZP1/N^{60g11} Wing Notching

After observation of both *Df(3L)ZP1/N^{55e11} and Df(3L)ZP1/N^{60g11}* heterozygotes in comparison to internal controls, it was determined that *Df(3L)ZP1* did not have a statistically significant effect on wing notching on either Notch allele (N^{60g11} or N^{55e11}) (Table 6). Comparing the average amount of wing notching for *N^{55e11}/Df(3L)ZP1* to the average amount of wing notching for *N^{55e11}/Tm3, Sb* at both tested temperatures (22 °C and 25.5 °C) failed to show a statistically significant difference. Likewise, the difference between the notching present on the wings of flies with the *N^{60g11}/Df(3L)ZP1* genotype compared to the amount on *N^{60g11}/Tm3, Sb* was not significant (Table 6).

Table 6. Wing Notching in $N^{60g11}/Df(3L)ZP1$ and $N^{55e11}/Df(3L)ZP1$ Heterozygotes

	Average Wing Notching	
Genotype	*22 °C*	*25.5 °C*
$N^{55e11}/Df(3L)ZP1$	*0.66*	*0.67*
N value (# flies)	*43*	*68*
std dev	*0.26*	*0.29*
p value	*N.S.*	*N.S.*
$N^{55e11}/Tm3, Sb$	*0.62*	*0.66*
N value (# flies)	*52*	*70*
std dev	*0.3*	*0.31*
p value	*N.S.*	*N.S.*
$N^{60g11}/Df(3L)ZP1$	*0.68*	*0.68*
N value (# flies)	*39*	*34*
std dev	*0.36*	*0.32*
p value	*N.S.*	*N.S.*
$N^{60g11}/Tm3, Sb$	*0.67*	*0.66*
N value (# flies)	*46*	*51*
std dev	*0.36*	*0.34*
p value	*N.S.*	*N.S.*

This table shows the average amount of wing notching present at the distal tip of the fly wing for $N^{55e11}/Df(3L)ZP1$ and its internal control $N^{55e11}/Tm3, Sb$ and $N^{60g11}/Df(3L)ZP1$ and its internal control $N^{60g11}/Tm3, Sb$. Averages were obtained by averaging the amount of wing notching present on both wings of each fly and dividing that value by two to get an average value per wing.

3.7 The Interaction of l(3)DTS4 with Dally

The interaction of *l(3)DTS4* with dally was first observed through a statistically significant temperature sensitive interaction between *l(3)DTS4* and *Df(3L)hi-22* (Table 7). At 25.5°C and 27°C, *l(3)DTS4*/*Df(3L)hi-22* heterozygotes had a nearly 100% lethality. In order to further examine this observation, complementation testing was done for all genes covered by the *Df(3L)hi-22* deletion, with all available lethal alleles. The only lethal allele to show some lethality over one copy of *l(3)DTS4* was dally^{06464} (Table 8). Statistically significant temperature sensitive interactions were seen at 27°C, but not at 22°C or 25.5°C. These observations, however, must take into account the fact that the nature of this dally allele has been described as semi-lethal (BERKELEY DROSOPHILA GENOME PROJECT 1993), lethal recessive (SPRADLING 1999), viable (LIN 1999) and partially lethal (NAKATO 2002). This makes complementation testing less straightforward. Also, unlike the other complementation tests, dally^{06464} was not compared to internal controls because *l(3)DTS4* suppressed the *Ser* phenotype (Table 8). As a result, the *p* values that are provided in Table 8 are for adherence to Mendelian inheritance only.

Table 7. *l(3)DTS4/Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p} Complementation Test

Genotype	Number of progeny (Temp)			Phenotype
	(22.0°)	(25.5°)	(27.0°)	
w; l(3)DTS4/Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p}	29	5	11	Normal wings, kinked bristles (Complementation test)
w; l(3)DTS4/TM3, Ser*	48	70	142	Reduced *Ser* wings, normal bristles
TM3, Ser/Tm3, Sb^{1}	0	0	0	dead
Df(3L)h – i22 Ki^{1}*rn*$^{roe\text{-}1}$*p*^{p} */ Tm3, Sb*^{1}	39	95	141	Extreme stubble (pink peach eyes in females)
P value (Mendelian)	N.S	<0.0001	<0.0001	
P value (Internal Control 1)	<0.05	<0.0001	<0.0001	
P value (Internal Control 2)	N.S	<0.0001	<0.0001	

This table shows the progeny that resulted from a cross between female *w*; l(3)DTS4/TM3, Sb*^{1} and male *Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p}*/TM3, Ser*. Three phenotypes were visible in the resulting progeny. *w*; l(3)DTS4/ Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p} flies were visible due to the *Ki* gene carried with the *Df(3L)h – i22* deletion. These flies had kinked bristles. *w*; l(3)DTS4/TM3, Ser* flies had normal bristles and a reduced *Ser* phenotype (Figure 6). *TM3, Ser/Tm3, Sb*^{1} flies would have been observable in flies with Ebony body, serrated wings and stubble bristles, but these flies were not present. *Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p} */ Tm3, Sb*^{1} flies were both stubble and kinked, resulting in an "extreme" stubble phenotype. These flies were distinguishable by the almost complete lack of dorsal bristling. The resulting progeny were assessed for adherence to Mendelian inheritance, and the *P* value (mendelian) was determined at all three temperatures. The *P* value (Internal Control 1) was determined by comparing the number of *w*; l(3)DTS4/Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p} flies to *w*; l(3)DTS4/TM3, Ser*, the *P* value (Internal Control 2) was determined by comparing the number of *Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p} */ Tm3, Sb*^{1} flies to *w*; l(3)DTS4/Df(3L)h – i22 Ki*^{1}*rn*$^{roe\text{-}1}$*p*^{p} (See section 2.3).

Table 8. l(3)DTS4/P{PZ}dally^{06464} P{PZ}l(3)87Df^{06464} ry506 Complementation Test

Genotype	Number of Progeny (Temp)			Phenotype
	(22.0°)	(25.5°)	(27.0°)	
w/+; l(3)DTS4/ P{PZ}dally^{06464} P{PZ}l(3)87Df^{06464} ry506*	51	54	17	Normal wings, normal bristles (Complementation Test)
w/+; l(3)DTS4/TM3, ry^{RK} Sb^{1} Ser^{1}*	66	93	260	Reduced *Ser* phenotype, *Sb* bristles
P{PZ}dally^{06464} P{PZ}l(3)87Df^{06464} ry506/ TM3, Sb^{1}				Normal wings, *Sb* bristles
TM3, Sb^{1}/TM3, ry^{RK} Sb^{1} Ser^{1}	0	0	0	Ebony, *Ser* wings, *Sb* bristles (dead)
P value (Mendelian, χ^2, d.f. = 1)	N.S.	N.S.	<0.001	

This table shows the progeny that resulted from a cross between female *w*; l(3)DTS4/TM3, Sb^{1}* and male *P{PZ}dally^{06464} P{PZ}l(3)87Df^{06464} ry506/*TM3, ry^{RK} Sb^{1} Ser^{1}. *w*; l(3)DTS4/P{PZ}dally^{06464} P{PZ}l(3)87Df^{06464} ry506* flies were observed as "wild type" appearing flies with normal wings and normal bristles. It was determined that *TM3, Sb^{1}/TM3, ry^{RK} Sb^{1} Ser^{1}* flies were not present due to the lack of any flies that were Ebony as well as *Sb* and *Ser*. Because *l(3)DTS4* reduces *Ser* expression, flies with normal or reduced *Ser* wings and *Sb* bristles were counted together, giving one value for both *w*/+; l(3)DTS4/TM3, ry^{RK} Sb^{1} Ser^{1}* and *P{PZ}dally^{06464} P{PZ}l(3)87Df^{06464} ry506/TM3, Sb^{1}* flies. This eliminates the possibility of using internal controls, thus statistical significance was assessed using adherence to Mendelian inheritance.

4. Discussion

4.1 Deletion Mapping and Polytene Chromosome Analysis

The interaction between *l(3)DTS4* and *Df(3L)BSC113* provided a location for *l(3)DTS4* that was supported by sequence addresses mapped to chromosome bands by the Drosophila Genome Project. In order to understand these two results, polytene chromosome analysis was done on *Df(3L)ZP1*.

Polytene analysis of *Df(3L)ZP1* heterozygotes identified an inversion loop in the region disrupted by *Df(3L)BSC113*. Further examination of the aberration indicated a left breakpoint at approximately 67A 10 - 11 and a right breakpoint at approximately 67B 6 - 7 on 3L, which is well to the right of *Df(3L)ZP1's* previously reported location. Analysis of the 3L chromosome indicate no possible deletion in the region provided by MAROY (MAROY 1997). Such a deletion would have been easily visible as the homologue would have separated or looped at this location, and been half as wide as the rest of the polytene. The provisional breakpoints determined from polytene chromosome analysis (Figure 4) were clarified with complementation data (Figure 7), indicating that *Df(3L)ZP1* is at least disrupted in 67B 3 – 67B 5; the right breakpoint of the inversion loop overlaps *Df(3L)BSC113* and also breaks within *l(3)DTS4*.

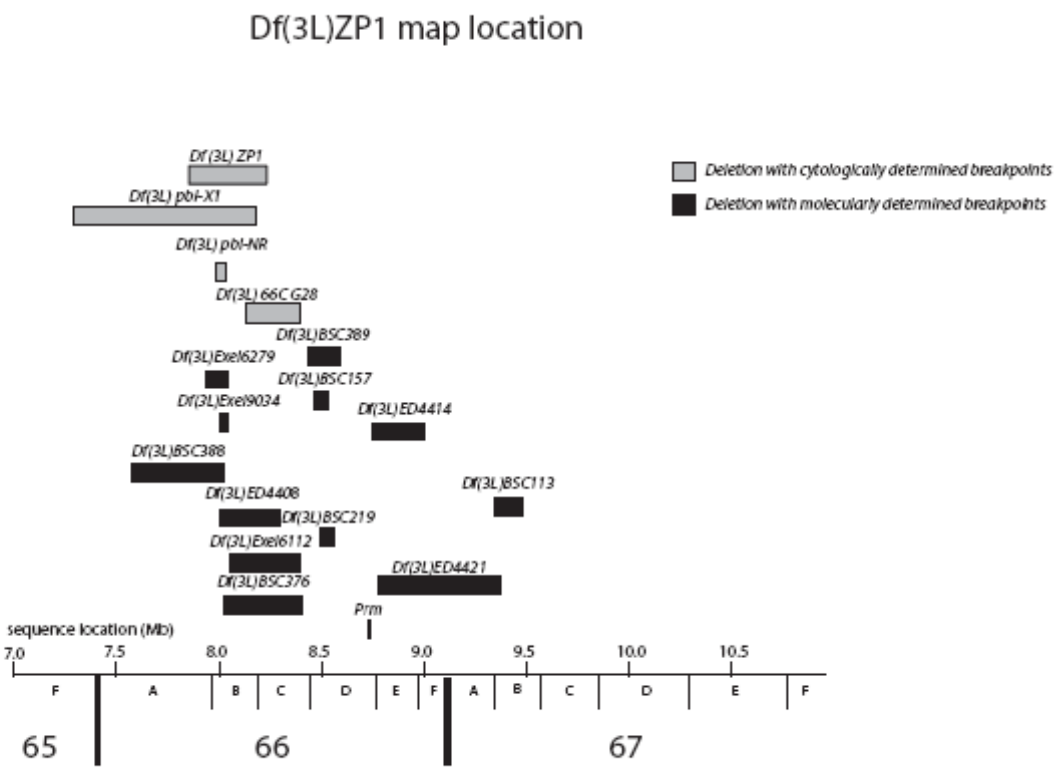


Figure 7. Df(3L)ZP1 Deletion Map

This figure shows the deletions that were compared for complementation to *Df(3L)ZP1*. Black bars represent deletions with known molecular breakpoints and gray bars represent deletions with known cytologic breakpoints. The only deletion in this map to fail to complement *Df(3L)ZP1* was *Df(3L)BSC113.*

4.2 l(3)DTS4 Area of Maximum Likelihood

The cytological location identified through deletion mapping and polytene chromosome analysis in this project was not completely covered by the area of maximum likelihood that was calculated from the results provided by Suzuki (HOLDEN and SUZUKI 1973). A weakness of the Suzuki/Holden method was that they were scored at different temperatures. Because *l(3)DTS4* is temperature sensitive lethal, the scoring of progeny requires inferring lethality by comparing results from two temperatures (22 °C and 29 °C). This method is not precisely accurate because the crossover rate is quite different at the two different temperatures (LINDSLEY and ZIMM 1992). As a result, the location of the 95% confidence limits may have been affected (LINDSLEY and ZIMM 1992).

The identified cytological location of 67 B3 – B5 corresponds to a recombination map location of 28.9 for 67 B5 (TWEEDIE 2009) which indicates that our area of maximum likelihood was at most 3.5 cM away from the right most map location covered by *Df(3L)BSC113*. We widened the confidence limits to reflect the error introduced by estimating recombinants (See Section 3.1), however it is likely that we were conservative in our estimate. Although roughoid and hairy have been cloned, and their cytological locations are known exactly, nevertheless their published recombination map locations are still approximations. Uncertainty in the true value of the marker locations would tend to increase our 95% confidence limits with respect to other landmarks. So we should draw both sets of confidence limits on our maps for internal use - the wider limits (with the half data set) should be regarded as including a rough estimate of total uncertainty,

including uncertainty in marker locations. Another factor to consider is that Drosophila shows nearly complete interference over short map distances. This would tend to make the mapping function under estimate the true map distance over very short distances. Additionally, stocks maintained as permanently heterozygous (as *l(3)DTS4* is) may accumulate recessive lethal mutations (TEMIN 1964), which may have affected the results.

4.4 Wing Serration and dally Interaction

It is evident that *l(3)DTS4* has a startling physiological effect on wing pattern formation. The statistically significant gain of function phenotype that is evident in flies bearing a single copy of *l(3)DTS4* over *Ser* and the two Notch alleles (N^{55e11} and N^{60g11}), indicate a possible neomorphic function of *l(3)DTS4*. *Ser* is involved in wing pattern formation, and causes a phenotype similar to that seen in the N^{55e11} and N^{60g11} flies, so the observation that flies heterozygous for *l(3)DTS4* and *Ser* further supports the notion that *l(3)DTS4* plays a role in the *Notch* signaling cascade governing this facet of wing development. An interesting observation was the fact that *Df(3L)ZP1* does not affect wing serration or notching in N^{55e11} or N^{60g11}, presumably because the deletion causes a loss of function. The dally interaction and the lethal temperature sensitive period are both consistent with *l(3)DTS4* being a member of the Notch pathway (SHELLENBARGER and MOHLER 1975). Further research will identify the exact gene responsible for this dramatic observation, and its potential role in the Notch signaling pathway.

4.5. Further Studies

Analysis of the genes disrupted by *Df(3L)BSC113* indicate 8 candidates for *l(3)DTS4* (Table 4). Testing lethal alleles of these genes against both *l(3)DTS4* and *Df(3L)ZP1* could possibly determine the gene responsible for the *l(3)DTS4* mutation. Alternatively the right breakpoint of *Df(3L)ZP1* should break within the *l(3)DTS4* gene and could be mapped by long range PCR. As more deletions with molecularly defined breakpoints are provided to the Bloomington and other stock centers, complete coverage of the map provided in Figure 2 will be attainable. Based on the complementation testing, deletion mapping, polytene analysis and the interaction with *Ser* and Notch, *l(3)DTS4* may be a member of the Notch pathway or a related pathway. Based on the gene list (Table 4), there are eight likely candidates, culled from the thousands of possibilities that existed before. Many of them have unknown or incompletely known function, and so their potential role in the Notch pathway may tell us something novel. Although the *l(3)DTS4* gene has not yet been identified, a lot more is now known about this unique mutation's location and function.

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