ABSTRACT

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"Genetic Analysis Of Two Mutations That Affect Neural-Specific Glycosylation"

(Under the Direction of Dr. MICHAEL TIEMEYER)

Complex carbohydrate expression regulates cell-cell interactions that can affect development and normal tissue function. Human genetic disorders that affect glycosylation often result in mental retardation and an overall failure to thrive. In *Drosophila melanogaster*, the HRP-epitope is an example of a neural specific N-linked oligosaccharide that provides opportunities for identifying mechanisms that control glycosylation and identifying mutations that affect glycosylation. Screening for new genes necessary for HRP-epitope expression has begun to identify important regulatory factors by generating mutants. One mutant, called *sff,* and another mutant, called MS-16, have decreased levels of expression of the HRP-epitope in neural tissue. In addition, the adult mutants show abnormal behavioral characteristics that include failure to escape in a sufficient amount of time and the tendency to congregate at the bottom of the vial even when there is a possibility to escape. By following the HRP-epitope expression in embryos from crosses of mutants to chromosomal deletions, we will characterize the genetic loci that interact with *sff* on the second chromosome and perform a genetic mapping of the mutation in MS-16 on the second chromosome. Additionally, we will seek how these mutations interact with the *tollo* pathway, which has previously been shown to modulate expression of the HRP-epitope. After an analysis of the phenotypes associated with the functions of *sff* and MS-16, we will then be able to assess neuromuscular development, embryonic neurological development, viability, fertility, and adult behavior.

INDEX WORDS: Neural-specific glycosylation, *Drosophila melangaster* mutations, HRP-epitope, Honors Program, The University of Georgia, Center for Undergraduate Research Opportunities

GENETIC ANALYSIS OF TWO MUTATIONS THAT AFFECT NEURAL-SPECIFIC GLYCOSYLATION

by

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Footprints

One night a man had a dream. He dreamed He was walking along the beach with the LORD. Across the sky flashed scenes from His life. For each scene He noticed two sets of footprints in the sand. One belonging to Him and the other to the LORD.

When the last scene of His life flashed before Him, he looked back at the footprints in the sand. He noticed that many times along the path of His life there was only one set of footprints. He also noticed that it happened at the very lowest and saddest times of His life.

This really bothered Him and He questioned the LORD about it. LORD you said that once I decided to follow you, you'd walk with me all the way. But I have noticed that during the most troublesome times in my life there is only one set of footprints. I don't understand why when I needed you most you would leave me.

The LORD replied, my precious, precious child, I Love you and I would never leave you! During your times of trial and suffering when you see only one set of footprints, it was then that I carried you.

Thank you Shelia A. Fennell and Keith M. Fennell for also being in my life to carry me when I was troubled. Thank you Keith M. Fennell, Jr. for allowing me to see that I can be everything you think I am. I LOVE YOU!

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CHAPTER 1
INTRODUCTION

In all stages of life, there are special cell-surface glycans that mediate interactions between cells and define cellular identities within complex tissues. In neural tissue, specific glycans can have a pivotal affect on cellular responses. When cells differentiate and form organized tissues, glycan expression can change, leading to the generation of glycosylation profiles that are tissue and cell-type specific. Glycosylation involves the enzyme-catalyzed process of attaching carbohydrate groups to polypeptides. Protein glycosylation functions in cell signaling and cell adhesion and recognition of cells by other cells. As a result, glycosylation generates complex carbohydrate expression that when altered, can result in mental retardation and human genetic disorders such as muscular dystrophy, and congenital disorders of glycosylation (CDG). Thus, regulation of N-glycosylation and O-glycosylation pathways impacts development and behavior (Blake et. al. 2002).

N-glycosylation

One form of carbohydrate modification, N-glycosylation, influences protein translocation, folding, and secretion. In addition, it also influences protein conformation and protein-protein interaction (Helenius & Markus 2004). Biosynthesis begins in the rough endoplasmic reticulum, where the glycosylation process occurs cotranslationally. Congenital disorders of glycosylation arise from defects in N-glycosylation pathways that are found in the cytosol, endoplasmic reticulum, and the Golgi apparatus (Jaeken 2003). The cytosol and organelles aide in the assembly and processing pathways that produce glycoproteins and other glycoconjugates needed

to maintain functional tissues. Many CDGs are caused by defects in N-linked oligosaccharides that are added to many proteins and, as a result, are multisystemic.

O-glycosylation

Another form of carbohydrate modification, O-glycosylation is no less complex in its functions. Biosynthesis occurs predominately in the Golgi apparatus. Unsuccessful O-linked post-translational modification of alpha-dystroglycan causes many forms of muscular dystrophies (2003). If dystroglycan is not appropriately modified, it is unable to bind to its extracellular matrix ligands, resulting in disruption of cortical layering, fusion of cerebral hemispheres, and granule cells that have migration defects within the central nervous system (Hewitt et. al. 2003).

Tollo Expression of HRP-epitope

Tollo, a member of the *Drosophila* Toll-like Receptor family, is expressed on embryonic ectodermal cells and induces tissue specific glycosylation. This is important because cell-surface glycans control cell-cell interactions and cellular identities within complex tissues at all stages of life (Seppo, Matani, Sharrow, & Tiemeyer 2002). If glycan expression is not regulated, mental retardation, muscular dystrophies, and immune disorders can occur. In *Drosophila*, Tollo induces adjacent neurons to express neural-specific N-linked oligosaccharides called HRP- eptitopes.

The HRP-epitope structure is detected within *Drosophila melanogaster* in the form of an alpha three-linked fucose on N-linked glycans. It is expressed within the *Drosophila melanogaster* nervous system and a small subset of non-neural tissues such as the garland gland, the anal pads, and the posterior hindgut (See Figure 1). Ongoing genetic screening has identified

new mutations that regulate HRP- epitope expression. Embryos were scored for the loss of the epitope because it is assumed that mutations in genes can alter the HRP-epitope expression by affecting N-linked glycosylation (See Figure 2). Thus, with the expression of the tissue specific and developmentally regulated HRP-eptiope, one can genetically analyze glycan expression *in vivo*. (Seppo, Matani, Sharrow, & Tiemeyer 2002).

MS16-2 Mutant

One mutant, named MS16-2, was generated by a random ethylmethane sulfonate (EMS) mutagenesis screening. It exhibited decreased levels of HRP expression and behavioral deficiencies in negative geotaxis. Decreased levels of HRP-epitope expression are detected in early embryogenesis and its expression is not restored until late in the embryonic development. The MS16-2 mutants will have its 2nd chromosome mapped by using well-defined deletion lines. By observing heterozygous embryos we can discover interactions with various genetic loci.

sff Mutant

A second mutant, named *sff*, was also generated by an EMS mutagenesis screening. The name, sugar-free frosting, is derived from the fact that there is an observable, but minimal residual HRP-epitope staining in its embryos. *Sff* mutants exhibit decreased levels of HRP expression and behavioral deficiencies in negative geotaxis. When compared to wild type, *sff* mutants don't have normal response to gravity that aides them in moving rapidly up the vial to escape. *Sff* was mapped to a location on the 3rd chromosome using deletion lines with well-known chromosomal breakpoints. Thus, the 2nd chromosome will now be mapped to discover

novel interactions between *sff* and genes found in defined deletion intervals. Embryos shall be monitored for phenotypic variations and loss of the HRP-epitope.

CHAPTER 2
MATERIALS AND METHODS

Setting Up Crosses

The first step of this experiment was to cross the *Drosophila melanogaster* w^{1118}*;sff* with a Bloomington 2nd chromosome deficiency kit and MS16-2 with the same deficiency kit. To accomplish this, approximately six males from the w^{1118}*;sff* stock or six males from the MS16-2 stock were crossed with approximately ten females from the Bloomington 2nd chromosome deficiency lines. Each day, the virgin w^{1118}*;sff* and MS16-2 females were separated and put into new food vials that were kept at room temperature.

Collection of Embryos

The next step was to collect embryos from the w^{1118}*;sff* and MS16-2 crosses. All of the flies with the same cross were placed in collection tubes that contained apple juice agar and a spot of yeast paste. The tubes were then placed in a 25°C incubator overnight. The following day, the flies were transferred to a new collection tube because there was an insignificant amount of eggs that were laid the first night. Following the second collection that was set up on the second day, the embryos were collected from the apple juice plates and prepared for HRP embryo staining.

HRP-epitope Staining

In order to collect the embryos and perform HRP staining, 500µl of 4% formaldehyde in PBS and 500µl of heptane was prepared in an eppendorf tube and placed on the nutator to equilibrate.

The embryo tubes were then filled halfway with 50% bleach and vortexed briefly at a setting of four. When four minutes passed, the embryos were decanted onto Nitex, washed with water, and transferred to the eppendorf tubes containing the embryo fixative/PBS mixture. The lower (fixative) phase was then removed after the embryos were nutated at room temperature for ten minutes. When the fixative was completely removed, 500µl of methanol was added in order to facilitate the cracking of the embryonic shell. After the shell cracked, the heptane and half of the methanol phase were removed. The embryos were then washed twice for one minute each in methanol.

In order to rehydrate the embryos to prepare for HRP staining, the embryos were washed in PBS + 0.3% Triton-X 100 (washing solution) three times for ten minutes each. The PBS-TX 100 was removed and the embryos were blocked for four hours in wash solution and 0.1% BSA + 5% normal goat serum (blocking solution). Primary antibody was later added in blocking solution and the embryos were left on a nutator at 4°C overnight.

The next day, the primary antibody was removed and the embryos were washed in the washing solution twice for five minutes and twice for thirty minutes, and were then followed by a thirty-minute block in blocking solution. The secondary antibody, consisting either of Jackson Labs HRP goat antibody or AlexaFluor Goat antirabbit IgG (488), was then added to the blocking solution and allowed to nutate for two hours and was followed by another seventy minute wash cycle. If AlexaFluor was used, Dapi was also included with the secondary antibody.

To develop the color of the embryos using conjugated peroxidase, the embryos were left to sit

in 500µl DAB/wash solution for approximately five to ten minutes. 5µl of 3% peroxide was added and allowed to develop the color for another five to ten minutes under close supervision so as not to overstain. The peroxide was promptly removed and the embryos were allowed to nutate in washing solution three times for five minutes each. The wash solution was removed and 50% glycerol/PBS was added to bottom of the tube. When the embryos sunk back to the bottom of the tube, 70% glycerol/PBS was put in the tube the same way and placed into the cold room until they were ready to be transferred to slides by cutting the tip of a 200µl pipettor and pipetting 25µl to a clean glass side.

CHAPTER 3
RESULTS

The 2nd chromosome deficiency kit contains one hundred ten lines. Of these lines, eighty-eight have been stained and approximately twenty-six percent are interesting because they show abnormal morphology, lethality, and other abnormal phenotypic characteristics when crossed to MS16-2. In many cases, observed lethality is seen in younger embryos before the time at which the HRP-epitope is being expressed. It is unknown why the embryos experience early death. When compared to wild type staining, there were no HRP deficient (HRP-) or MS16-2 phenotypes; all of the dead embryos and embryos with odd phenotypic characteristics were positive for the HRP-epitiope. The progeny with these phenotypes are described below.

MS16-2

In homozygous MS16-2 stained embryos, the younger embryos showed lack of staining. However, the older embryos showed faint staining of the central nervous system and garland gland (See Figure 2). After staining, the embryos of MS16-2 x deletion 6780 and MS16-2 x deletion 6507 were shown to exhibit lethality (See Figure 3). This overall failure to thrive was also observable in the deletion homozygotes. The embryos of MS16-2 x deletion 6507 had abnormal phenotypic characteristics that included an abnormally shaped gut and twisted body.

Additionally, crosses of MS16-2 x deletion 2583 gave embryos in which the neurons in the young embryos are ectopic; they are located towards the periphery. These embryos also exhibited lethality and abnormal morphology.

Mapping of MS16-2

We have begun to map the genes based on the twenty-six percent of deletion lines that we located on the 2nd chromosome and deemed interesting (See Figure 4). Deletion line 7531 resides on chromosomal segment 39E7 while deletion lines 6780, 757, and 6866 reside on chromosomal segments that spans from 54E5 to 56D10. Deletion lines 6780 and 757 are virtually side by side to each other while 757 and 6866 overlap each other on chromosomal segment 56C4. This particular segment contains the *windbeutel* gene, designated *wbl*, which is a requirement for dorsoventral patterning in *D. melanogaster*.

w^{1118};sff

Deletion mapping was performed on the 3rd chromosome of *sff*. There were four regions that were identified to interact with the mutation although there was only one region that had a strong interaction. For this reason, the 2nd chromosome of *sff* was mapped in order to identify novel interactions between the crosses of *w^{1118};sff* and deletion intervals.

Due to technical difficulties with the use of DAB (3-3' Diaminobenzidine tetrahydrochloride) and conjugated peroxidase, there are some lines that must be restained with fluorescent antibodies. Nevertheless, the embryos of *w^{1118};sff* x deletion 567, *w^{1118};sff* x deletion 6647, and *w^{1118};sff* x deletion 8469 exhibited reduced HRP staining when compared to the complete body staining of the wild-type looking embryos that were observed on the same slide (See Figure 5). This is interesting because the normal expectation is for the heterozygote progeny to have wild type embryo staining.

CHAPTER 4
DISCUSSION

The goal of this experimental screening was to use defined deletions in order to characterize the genetic loci on the 2nd chromosome that interact with *sff* and perform a genetic mapping of the mutation in MS-16. After an analysis of the phenotypes associated with crossing *sff* and MS-16 to these deletions, it was discovered that the *windbeutel* gene resided in chromosomal segment 56C4. This gene functions within the follicle cells of the ovary and plays a role in modification and folding of proteins that are secreted from the follicle cells and aides in establishing the dorsoventral pattern (Konsolaki & Schupbach 1998). Mutant alleles of the *windbeutel* gene can cause various degrees of lethality and phenotypic abnormalities due to loss of function. Interestingly, it is suggested that the *windbeutel* gene functions in post-translational modification of proteins. Thus, it can control N-linked glycosylation by triggering modifications that take place in the endoplasmic reticulum, providing us with a definitive reason to perform crosses of MS16-2 and defined deletions in order to discover novel interactions.

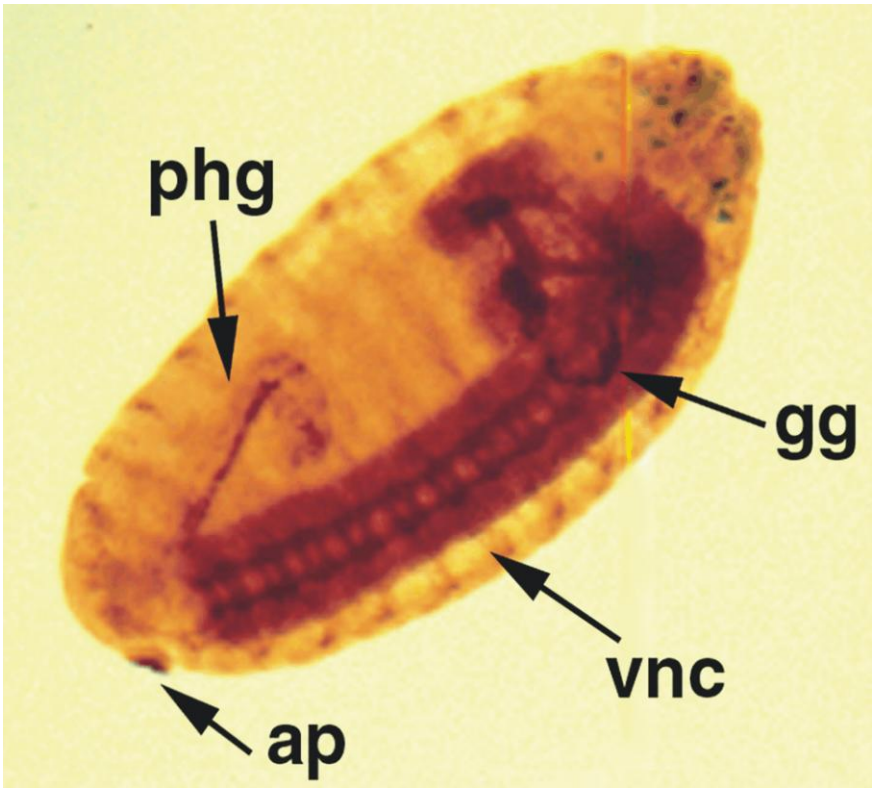
The mutations reveal genomic regions that either contain or interact with MS16-2. Although we are unable to see HRP^- or MS16-2 phenotypes, we noticed that lethality could possibly occur before the expression of the HRP-epitope. Incidentally, it is unclear if MS16-2 affects glycosylation on a pathway that is separate from the pathway that it regulates by the neural-specific HRP-epitope or the same pathway.

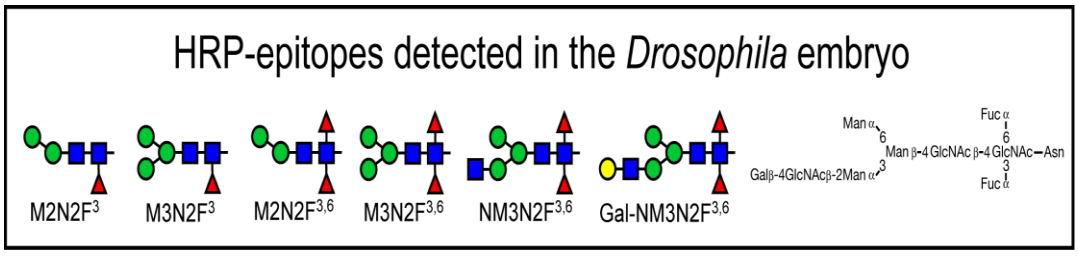
Due to the technical difficulties of HRP-epitope staining with DAB, there are problems

determining the severity of the reduction of HRP-epitope expression. We can only determine if HRP-eptiope expression is present or not, making it difficult to decide if the N-linked olgliosaccharide is actually totally gone or just greatly reduced. This question must eventually be answered by using fluorescent antibody staining in the place of DAB.

There are many directions in which these experiments can go in order to answer certain questions about glycosylation. Future plans include using finer mapping techniques to identify genes. This will be done by using smaller and smaller deletions in order to determine deficiencies within a certain region.

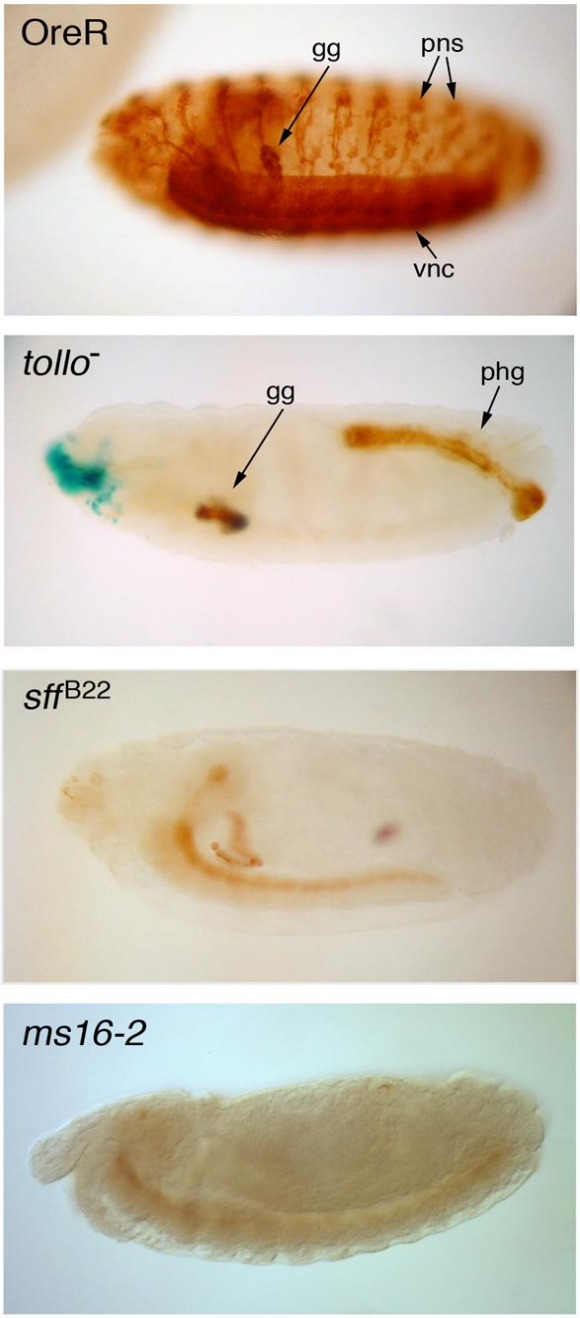
Figure 1: The HRP-epitope Expressed on Neurons and Small Subset of Non-neural Tissues





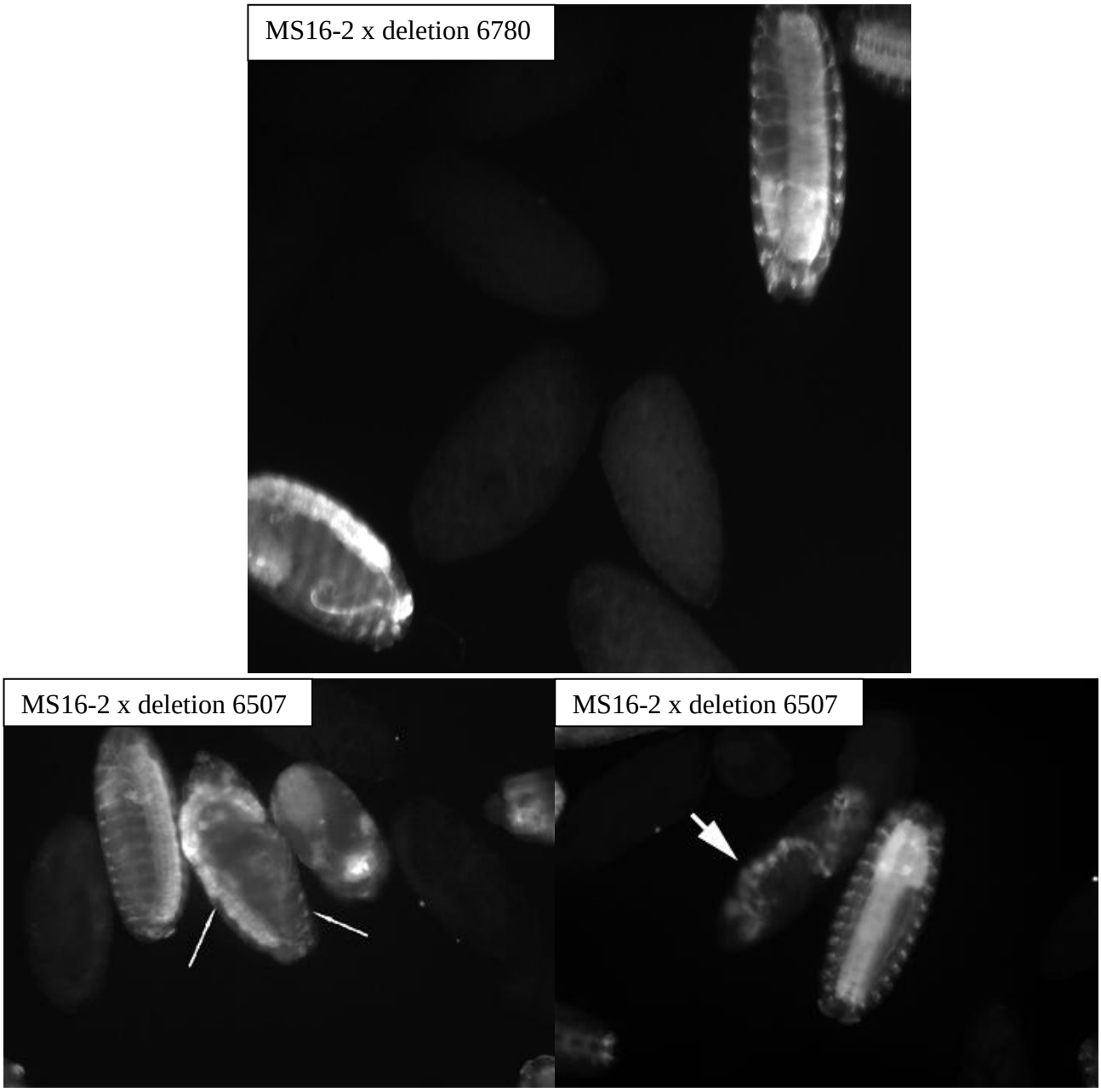
The HRP epitope is an N-linked oligosaccharide that is controlled by the Tollo gene; its structure is detected within *Drosophila melanogaster* in the form of an alpha three-linked fucose. It is expressed within the *Drosophila melanogaster* nervous system and small subset of non-neural tissues such as the garland gland (gg), the anal pads (ap), and the posterior hindgut (phg). The ventral nerve cord is also shown (vnc).

Figure 2: HRP Staining for OreR, Tollo^{-}, *sff*^{B22}, and MS16-2



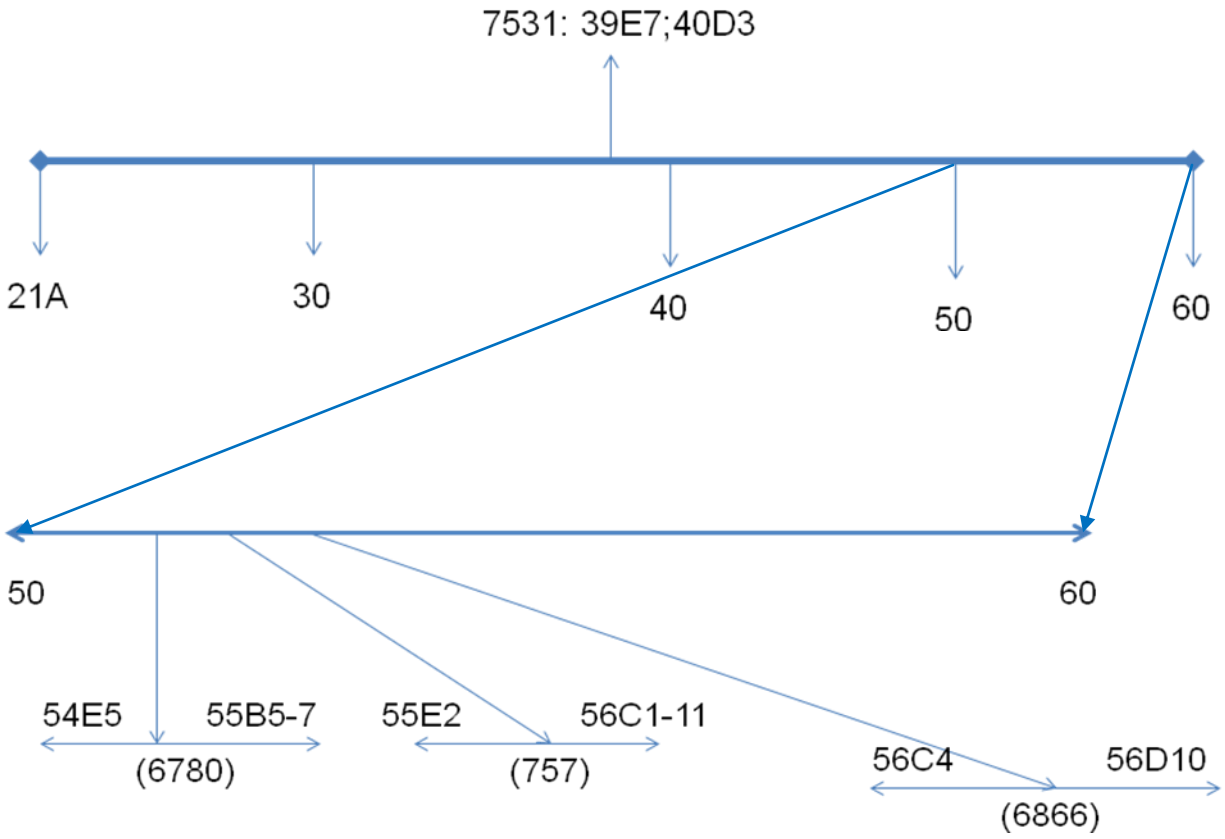
Based on wild type staining of Ore R, *sff* has residual HRP staining while MS16-2 has virtually no staining at early developmental stages (stages 13 and 14 shown).

Figure 3: Fluorescent Staining of MS16-2 Crossed with Deletion 6780 and Deletion 6507



The heterozygote embryos exhibited lethality. The embryos of MS16-2 x deletion 6507 had abnormal phenotypic characteristics that included an abnormally shaped gut and twisted body (shown with arrows).

Figure 4: Mapping of 2nd Chromosome Deficiency



7531, 6780, 757, and 6866 are some of the interesting deletion lines that has been mapped. Chromosomal segment 56C4 contains the *windbeutel* gene that is required for dorsoventral patterning in *D. melanogaster.*

Figure 5: HRP-epitope Staining of Deletion Lines Crossed with w^{1118};*sff*

The heterozygote embryos had light HRP staining, showing that there was a partial loss of the HRP-epitope. The reference staining are the embryos that resemble wild type staining. Both of the staining took place in the same time frame and was stained with DAB (3-3' Diaminobenzidine tetrahydrochloride).

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