# ROLE OF GALECTIN IN THE REGULATION OF NEURAL EXCITABILITY BY *DROSOPHILA* SIALYLTRANSFERASE

An Undergraduate Research Scholar Thesis

By

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# ABSTRACT

Role of galectin in the regulation of neural excitability by *Drosophila* Sialyltransferase.

(May 2013)

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Galectins are major sugar binding proteins that can be blocked from interactions with its sugar epitopes, galactose, by sialylation. Previous research from Panin lab has showed that sialylation affects the neural excitability. Since galectins and sialylation are so intimately linked, this study examines whether galectin has a role in neural excitability. To test the hypothesis, possible mutant fly lines with galectin gene deletion were created with hobo deletion method. Next, we will screen for the fly lines that contain galectin gene deletion. Then, we will conduct functional and behavioral analysis of the galectin mutants to investigate if neurological functions of galectin mutant flies are compromised. Finally, the galectin mutant flies will be crossed with sialyltransferase mutants to examine whether the defect is rescued or amplified. The results will shed light on the molecular and genetic mechanism of galectin in the nervous system and the mode of interaction of galectin with sialylation.

# DEDICATION

This is dedicated to my lovely parents Mr. Bhairav Prasad Manandhar and Mrs. Bijaya Laxmi Manandhar.

# ACKNOWLEDGEMENTS

I would like to thank Department of Biochemistry and Biophysics for giving me an opportunity to carry out necessary research work and to use departmental resources. I would like to extend my deep gratitude to Dr. Vlad Panin whose ever-present help and stimulating suggestions helped me during the time of research. I would also like to thank Panin lab members for their help and support without which I would not have been able to complete this research project.

# CHAPTER I

# INTRODUCTION

Sialic acids, electronegatively charged monosaccharides, are mainly found as terminating branches of N-glycans, O-glycans and glycosphingolipids. Present mostly on the cell surfaces, sialic acids can modify shape, size, hydrophilicity and the net charge of glycoconjugates. Due to this, sialic acids affect a host of biological processes that include immunity, cell signaling, ligand binding and neural excitability. Role of sialic acid in immunity and cell signaling has been well studied and is mostly attributable to its terminal position in glycoconjugate that makes it an ideal candidate for antigen presenting [1] (Figure 1). It is the third function of sialic acid that is of particular interest for our research. Sialic acid can play a dual role of providing the motif for ligand binding or act as biological mask to affect the ligand binding. Galectin is one of the major sugar binding proteins that can be blocked from interactions with its sugar epitopes, galactose, by sialylation. It has been reported that α 2,6 sialylation prevents the accessibility of galectin to galectin-reactive glycoepitopes [2] (Figure 2).

Galectins belong to evolutionarily conserved lectin family and is characterized by carbohydrate recognition domain that shows particular affinity for β-galactoside containing oligosaccharides. Through the carbohydrate recognition domain-glycans interaction, galectins interact with extracellular and cell-surface glycoconjugates to induce apoptosis, mediate cell-cell interaction, induce cell migration and attenuate immune response [4]. Although galectin's role in immune cell response and apoptosis has been extensively studied, role of galectin in the nervous system remains largely ignored.

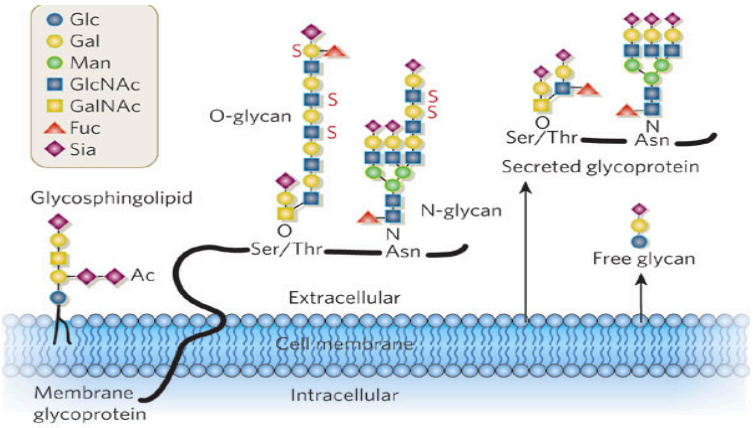


Figure 1: Sialic acids are located at non-reducing end of glycans attached to glycoconjugates and appear largely on cell surface of mammals and other vertebrates [3].

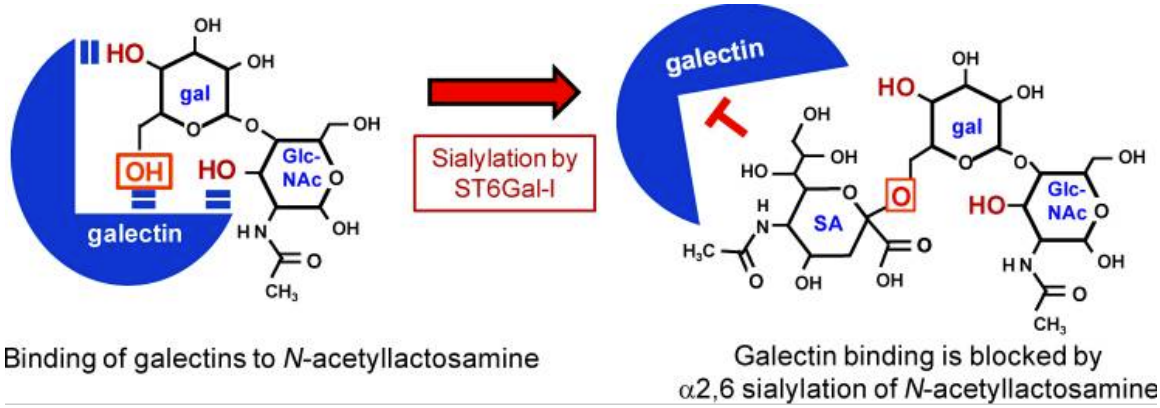


Figure 2: α 2,6 sialylation prevents the accessibility of galectin to galectin-reactive glycoepitopes.

Previous research from Panin lab has uncovered novel function of α-2,6 sialylation in nervous system. α-2,6 sialyltransferase activity was discovered to regulate neuronal excitability and affect neuromuscular junction development [5] (Figure 3). Because α-2,6 sialylation blocks galectin binding, we hypothesize that galectin, in conjunction with sialyltransferase, play a pivotal role in nervous system. Interestingly, in-situ hybridization of galectin mRNA in Drosophila during

embryogenesis reveals that throughout the developmental stages, mRNA expression is concentrated in central nervous system and musculature [3].

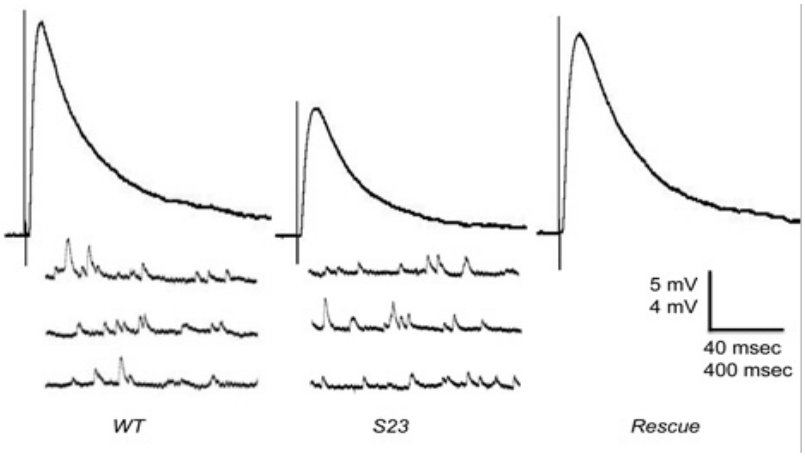


Figure 3: Evoked excitation junction potential amplitude is decreased in *DSiaT*(S23) mutants. The amplitude is restored on the *DSiaT* rescued flies.

In this research project, we use *Drosophila* as a model organism to generate a galectin mutant by hobo-hopping deletion method. By performing behavioral assays, we hope to investigate whether galectin has a role in neural excitability. If it has a role in neural excitability, we will combine galectin mutant with *DSiaT* mutant and perform various functional assays to gain deeper insight into the mode of interaction of galectin with sialic acid in the regulation of neural excitability.

# CHAPTER II

# METHODS

**Hobo hopping deletion**

Deletion-generator technology that exploits the properties of double transposable element will be used to produce gene deletions. The double element includes hobo, a deleter transposable element that is bracketed by white and yellow genetic markers that correspond to white eye and yellow body phenotypes respectively. As shown in Figure 1, the whole construct, including hobo, white and yellow markers is inserted in the carrier transposable P element. This P transposable element with hobo deleter was introduced near the site of gene of interest. Next, this double element when activated by hobo transposase, will induce a local hop of the hobo element, which is followed by recombination. This results in the deletion of the region between the initial and final position of hobo. Depending on the loss of the genetic marker, we can track the direction of the local hop. In our case, we will be selecting for the deletion of yellow body marker as our gene of interest is located on the right side of the double element. Depending on how far the hop has taken place, we will screen for single and double mutant for galectin genes [6].

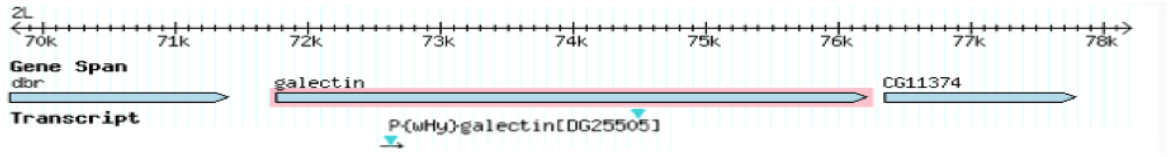


Figure 4: Genomic map of *Drosophila* showing the position of hobo element and galectin gene. http://flybase.org/cgibin/gbrowse/dmelstocks/?ref=2L;start=70421;stop=74421;nav4=1;plugin=

For the cross, stocks containing hobo in galectin P{wHy} and hobo transposase In(2LR)/ cyo,P{hs-H\T} were ordered from Bloomington Fly Stock Center, Indiana. These stocks were used as parents to generate galectin mutant progenies.

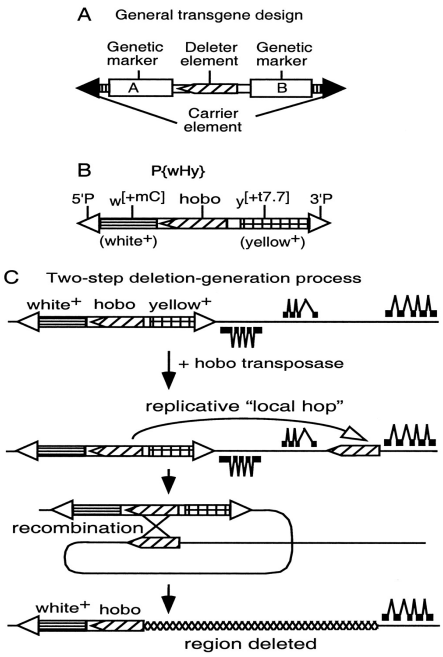


Figure 5**:** Basic Strategy of Hobo Local hopping mediated deletion [6].

**Elimination of Hobo confined deletion**

Genomic DNA of the candidate flies was extracted using REDExtract-N-Amp Tissue PCR Kit from Sigma-Aldrich. Although only the flies with loss of yellow gene marker were selected from the cross, it is possible that the local hops spans only yellow body marker gene. Thus, these flies with this type of local hop will lose the yellow marker but will not have the deletion we seek. In such cases, 3' end of P element will be retained. To eliminate these events, a PCR test based on the retention of both P element ends was done. The expected amplified product is about 360 base pairs in length and only those flies with the retention of 3' end of P element will give the

expected bands. The primers used were pendout2 (5'-CGACGGGACCACCTTATGTT-3') and wHy_genomic_primer1 (5'-TTCCATCCACCACCTCGATG-3'). The products of the PCR reactions were run on 1.2% agarose gel along with the controls.

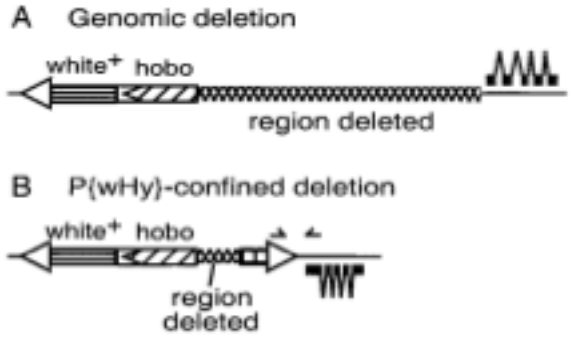


Figure 6: Schematic showing hobo confined deletion and genomic deletion[6].

**Deletion mapping using the inverse PCR**

The inverse PCR protocol of J. Rehm available at reference [7] was used to screen for the fly lines containing galectin mutants. The restriction enzyme used was AluI, and the primers were Ph-EA1 5'-GGGCATAATCTATTTCGCTTTCT- 3' and Ph3-2 5'-CGAGTATTTTGTGTGCCGCAAGT-3'. The flies, which didn't give PCR product in the above PCR test to eliminate the hobo confined deletion test, were tested using this method. This was done using PCR, gel-electrophoresis, and DNA sequencing at the end. As Ph-EA1 and Ph3-2 did not give the expected results, another set of primers Hobo forward 5'-CTAAAGAACCACGGATTCTGATA GAC-3' and Hobo reverse 5'-GATCGTTGACTGTGCGTCCACTCA-3' were also used to amplify the ligated DNA containing hobo element.

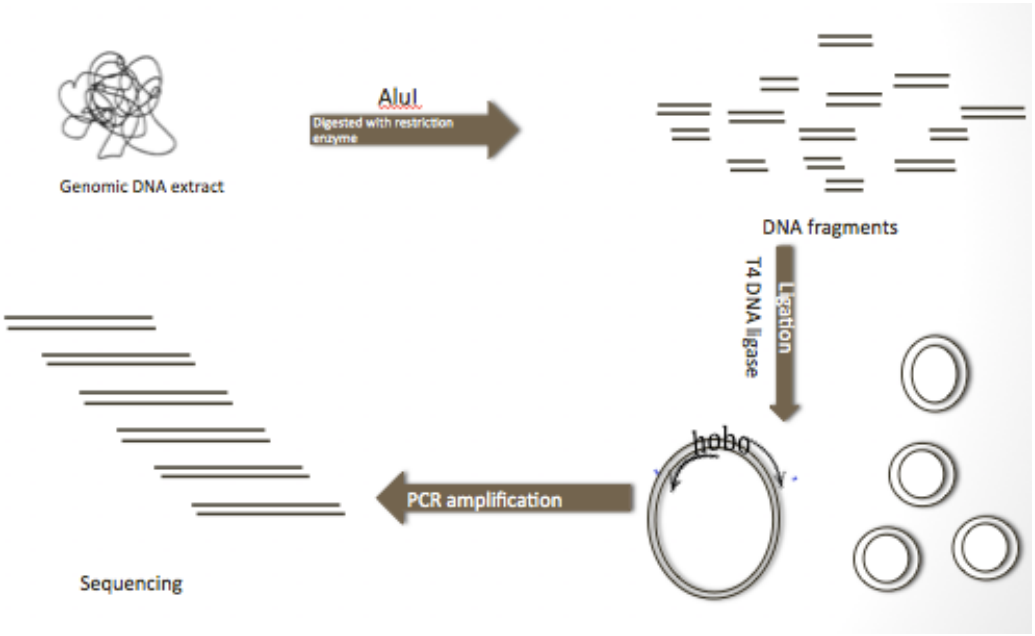


Figure 7: Schematic for inverse PCR.

**Deletion mapping using the linear PCR**

For the linear PCR, the primers used were Ph3-2 5'-CGAGTATTTTGTGTGCCGCAAGT-3' and GalI_gnmc-rev 5'-CTAAAGAACCACGGATTCTGATAGAC-3'. One of the primers is within the hobo region while the other primer lies at the end of galectin gene.

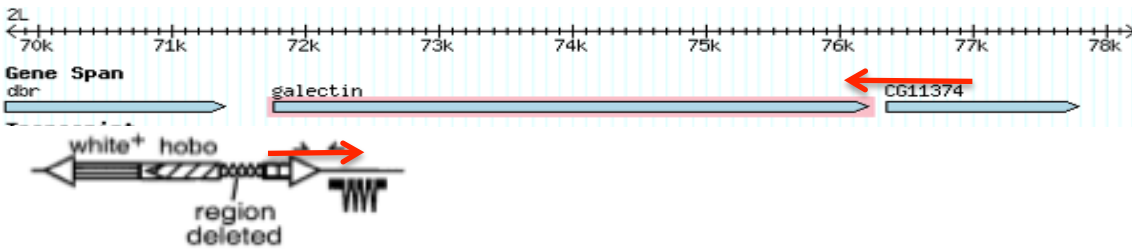


Figure 8: Schematic for linear PCR.

**Generation of galectin and DSIAT double mutant**

Once the galectin mutant has been discovered from the possible mutant fly lines, the galectin mutant will be crossed with DSIAT mutant to create a double mutant. As the double mutant does not have both functional galectin and DSIAT, behavioral assays such as heat shock paradigm, larval crawling pattern and ability of the fly to right itself will elucidate the mode of interaction between galectin and sialylation pathway.

# CHAPTER III

# RESULTS

**Hobo hopping deletion using P{wHy} hybrid transposable element**

Stocks containing hobo in galectin P{wHy} and hobo transposase In(2LR)/ cyo,P{hs-H\T} were crossed according to the hobo hopping deletion protocol [2]. The progenies were scored for yellow body, red eyes and either bristle or curly wings but not both. As the galectin gene lies to the right hand side of the hobo-construct, by scoring for the flies of these phenotypes, we are selecting the flies that represent the jumping of hobo transposable element in the right hand direction.

As the hobo hopping in the right direction was a very scarce event in the first trial, the original protocol of hobo hopping method was modified to include more frequent and prolonged heat shock. The flies were subjected to heat shock for one hour every day until they hatched instead of 30 minutes every other day, as it was suggested in the original protocol. This modification was included to increase the expression of the hobo transposase. Thus, there is more likelihood that the local hopping will take place. The efficiency was improved to 1.05% and 40 flies with the sought phenotypes were obtained out of 3,815 flies scored.

Out of the 40 candidate flies obtained, 5 flies did not produce any progenies and hence, could not be further tested. For the remaining 35 flies, stable lines were generated by crossing each individual candidate flies with yw; Bl/Cyo flies. The progenies from the first cross were then scored for curly wings, red eyes and yellow body. Finally, they were self-crossed to create stable

mutant fly lines corresponding to each unique recombination event.

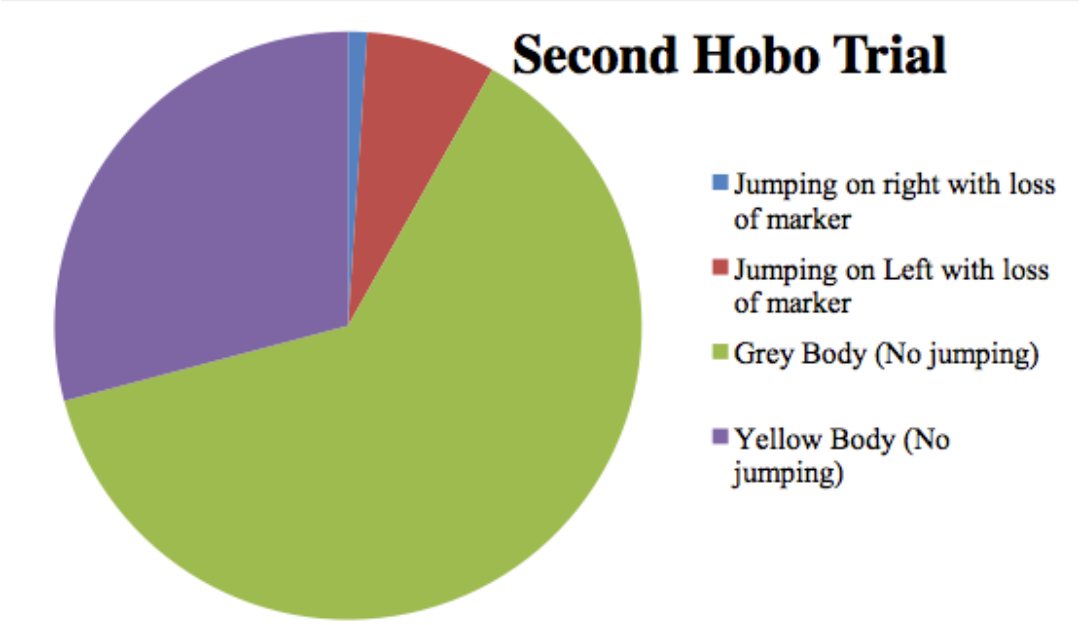


Figure 9: Summary of the second round of hobo hopping trial. Another undergraduate researcher, Niraj KC, carried out the experiments and the results are from his experiment.

**Elimination of Hobo Confined Deletion**

In the hobo-confined deletion, the local hop that deletes part of yellow gene but does not extend beyond the P{wHy} transgene. In these candidate flies, yellow gene is inactivated since part of the yellow gene is deleted. Thus, these flies will give the expected *yellow-mutant* phenotype during the scoring process but are not the candidate flies that we want for further analysis, as the local hop does not extend far enough to cover the galectin gene on the right hand side.

The fly lines with hobo-confined deletion retain both P-ends as the deletion is confined to P{wHy} transgene. The fly lines that gave an amplification product using primers that correspond to 5' and 3' end of P-end were eliminated. Out of the 37 fly strains tested (2 from

first hobo cross and 35 from second), 4 strains showed an amplified band of about 360 base pairs and 33 did not (Figure 8). The candidates that were eliminated using the method were nkc7, nkc25, nkc4 and nkc5. The positive control used during this test was the original hobo in galectin (yw; P{wHy}) stock which contains the original hobo P{wHy} hybrid transposable element. The negative control used was a wild type fly that lacks the hobo transposable element. Hence, no band was observed in the negative control.

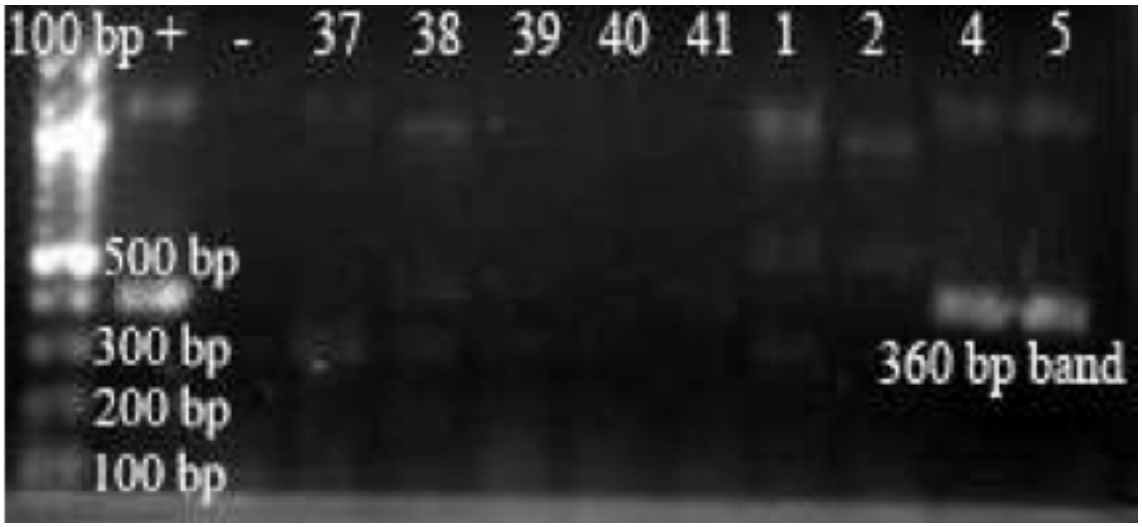


Figure 10: Gel electrophoresis of PCR products to eliminate hobo-confined deletion. Here, nkc4 and nkc5 show amplification product and were eliminated. Another undergraduate researcher, Niraj KC, carried out this experiment and the results are from his experiment.

**Deletion mapping using the inverse PCR**

Genomic DNA was extracted as described in Chapter II (methods) followed by the enzymatic digestion with AluI. The digested DNA was self-ligated and PCR amplified using Ph-EA1 and Ph3-2. The gel pictures, showing the progression of this process, are shown in Fig. 11. The inverse PCR was carried out for all 33 fly strains. However, the inverse PCR did not give a specific amplification product for all the candidate flies. PCR conditions were adjusted to

improve the resolution of the DNA bands. Yet, the non-specific amplification problem persisted.

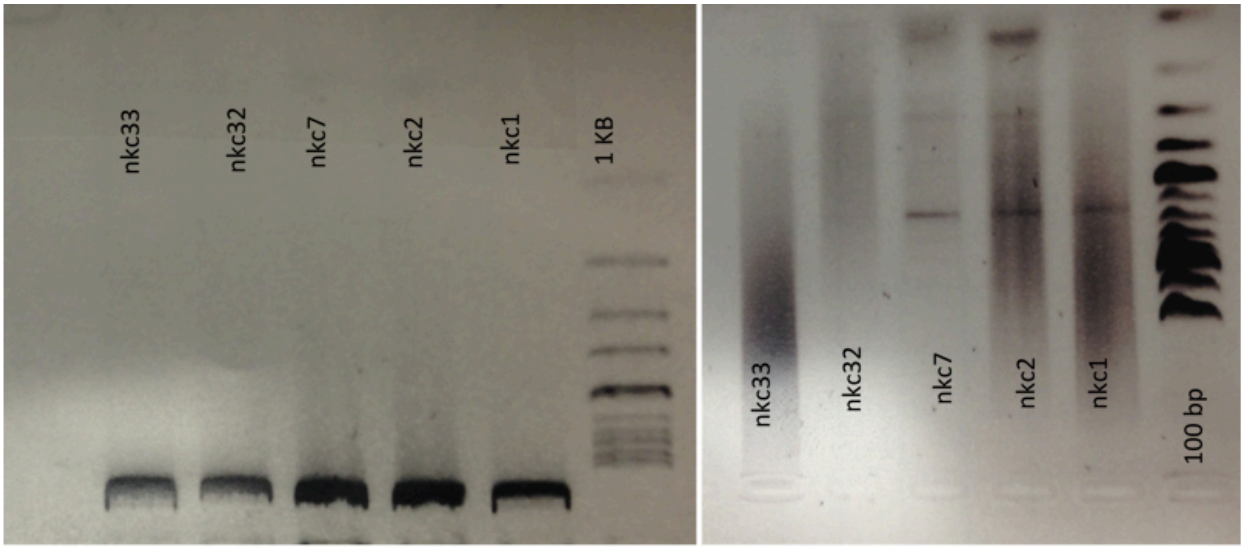


Figure 11: Inverse PCR to map deletion events. From left to right: a) DNA extraction of nkc1, nkc2, nkc7, nkc32 and nkc33. b) PCR amplification after restriction digest and ligation.

This led us to think that the problem existed in the ligation step of the inverse PCR protocol. Ligation is very sensitive to the temperature and impurities. First, ligation was carried out at various temperatures to determine if it would give a specific PCR amplification product. The parent stock containing hobo in galectin p{wHy} was used as the positive control for these experiment. In the parent stock, hobo in galectin has not undergone any local hop thus it's amplification product can be precisely estimated by looking for the AluI restriction sites within the P{wHy} transgene. The expected amplification product is 683bp long. The PCR still failed to give a specific amplification product. Next, we tried phenol/chloroform precipitation of DNA to reduce the impurities during DNA extraction process. For the parent stock containing hobo in galectin P{wHy}, inverse PCR gave the expected amplification product of 680 bp. However, for the candidate flies, the problem of non-specific amplification persisted. I am currently in the

process of optimizing PCR conditions to get a specific amplification product for candidate flies.

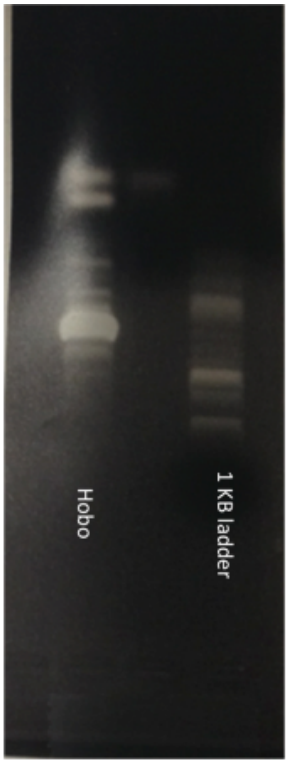


Figure 12: Inverse PCR to map deletion event for Hobo in galectin.

Since ligation is very likely the problematic step that is leading to non-specific amplification, increasing the efficiency of ligation could overcome this problem. The current enzyme AluI we are using to digest the DNA extract is a blunt-end enzyme. Blunt end ligation efficiency has been known to be low. Thus, using a different sticky end enzyme to digest the DNA extract might lead to better results.

Another possibility for non-specific amplification may be that hobo has undergone multiple recombination events leading to hobo being present in more than one copy. During the cross between the parent stocks, heat shock was prolonged to increase the hobo transposase

expression. The increased hobo transposase expression might have led to multiple recombination events resulting in deletion as well as duplication. In such case, inverse PCR will give non-specific amplification, as multiple DNA products will be amplified by same set of primers.

**Deletion mapping using linear PCR**

Supplementing the inverse PCR, the linear PCR was also carried out in order to map the deletion. As the primers are located at hobo end and at the end of galectin gene, the candidate flies that have galectin gene intact will give the product of 3Kb band. Any candidate fly that gives PCR amplification product less than 3Kb might be potential mutant fly containing deletion of galectin gene region close to hobo. Linear PCR does not involve ligation step. However, like inverse PCR, the linear PCR amplification product was non-specific as shown in figure 9. Ideally, wild type(MH) and control(MH) would not give any PCR products while the candidate mutant flies would give specific amplification product. Different PCR conditions were tried to get specific amplification. The possible reason for this may be that the primers being incompatible. Furthermore, linear PCR is an inefficient method to precisely map the deletion region. Thus, further optimization of linear PCR was not pursued.

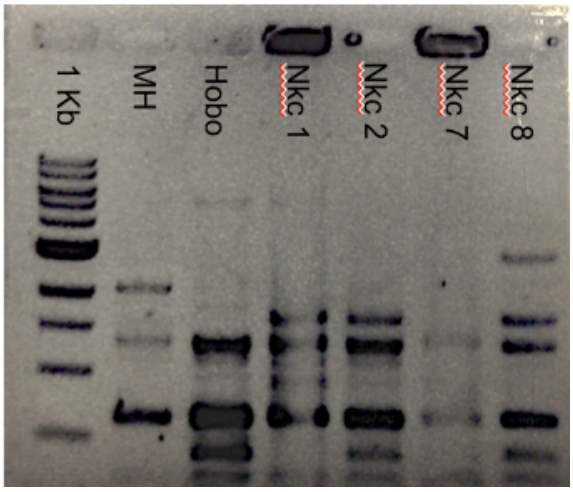


Figure 13: Linear PCR to map the deletion events.

# CHAPTER IV

# SUMMARY AND CONCLUSIONS

In this project, using hobo hopping deletion method, we have generated thirty-three potential mutants that may include the deletion of the galectin gene. Employing various genetic and molecular tools, we have eliminated the non- candidates from the total mutant pool.

For the remaining of the research period, I will be optimizing the inverse PCR conditions for candidate lines and verifying the deleted region. Depending upon how far the hobo transposable element has hopped to the right (Figure 5), I might get the deletion of one galectin gene or of both galectin genes or none. Once the fly line containing galectin gene deletion has been verified, we will conduct functional and behavioral analysis of the galectin mutants to investigate if neurological functions of galectin mutant flies are compromised. Next, the galectin mutant flies will be crossed with sialyltransferase mutants to examine whether the defect is rescued or amplified. The results will shed light on the molecular and genetic mechanism of galectin in the nervous system and the mode of interaction of galectin with sialylation.

As hobo-hopping deletion can produce varying-length deletions, there is a potential of creating various splicoform-specific mutants for galectin gene which provide a tool to investigate functions of different galectin splicoforms. Our strategy might generate hypomorphic alleles of galectin which will be also useful for further genetic approaches, such genetic interaction studies with other galectin genes and genes of the sialylation pathyway. These mutant forms of galectin may be valuable in elucidating the role of galectin in different pathways in addition to the

sialylation pathways. Moreover, galectin has been known to be intimately involved in immune response and cell signaling. Further research on galectin mutant may be of great medical value.

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