THE FLORIDA STATE UNIVERSITY

COLLEGE OF ARTS AND SCIENCE

IDENTIFICATION OF ME31B FROM AN *IN VIVO* RNAI SCREEN AS A POTENTIAL REGULATOR OF NOTCH SIGNALING

By

MUHAMMED SOYLEMEZ

A Thesis submitted to the
Department of Biological Science
in partial fulfillment of the
requirements for the degree of
Master of Science

Degree Awarded:
Summer Semester, 2013

Muhammed Soylemez defended this thesis on June 24, 2013.
The members of the supervisory committee were:

Wu-Min Deng
Professor Directing Thesis

George W. Bates
Committee Member

James M. Fadool
Committee Member

Timothy Megraw
Committee Member

The Graduate School has verified and approved the above-named committee members, and certifies that the thesis has been approved in accordance with university requirements.

THIS THESIS IS DEDICATED TO MY FAMILY AND MY FRIENDS IN DENG LAB

ACKNOWLEDGMENTS

First of all I would like to express my deepest appreciation to Dr Wu-Min Deng for the useful comments, remarks and engagement through the learning process of this master thesis. Without his guidance, patience and persistent help this master thesis would not have been possible. I also would like to thank my committee members, Dr George W. Bates, Dr James M. Fadool and Dr Timothy Megraw for serving on my committee, giving comments and for providing direction during my studies. Besides, I would like to present my gratefulness to Dr Akira Nakamura from Kumamoto University, Kyusyu, Japan for his gifts that were incredibly important for my studies. In addition, my sincere thanks also goes to current Deng lab members; Yi-Chun (Jack) Huang, Gengqiang Xie, Yoichiro Tamori, Dongyu Jia, David Aponte, Zhiqiang Shu, Sheng-An Yang and Billy Palmer for their support and stimulating scientific conversation as well as making critical reviews of my thesis even in their busiest of times. Each of them taught me a lot. Particularly, I would like to send my special thanks to Dongyu Jia who spent a lot of time to help me.

Apart from the efforts of me, the success of this study depends also on the encouragement and guidelines of many others. I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this project and I am thankful to their help during the writing my thesis.

Last but not the least, I would like to thank my family and relatives especially my mother and father for supporting me spiritually.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The *Drosophila* somatic follicle cells are excellent for the study of cell-cycle regulation and cell differentiation. During oogenesis, the follicle cells go through three variations of cell cycle programs, the mitotic cycle, the endocycle and gene amplification. Notch signaling activation is required for the switch from the mitotic cycle to the endocycle (the M/E switch) and its downregulation is necessary for the switch from the endocycle to gene amplification (the E/A switch) in these cells. Recently, we have found that Broad, a zinc-finger transcription factor, is directly up-regulated by Notch signaling during the M/E switch in the follicle cells (Jia and Deng, unpublished data). During late oogenesis, Broad is also regulated by EGFR and Dpp pathways for chorionic appendage formation. To explore how these different signaling pathways regulate follicle cell differentiation and cell cycle switches, we performed an *in vivo* RNAi screen to examine the effect of induced knockdown of gene expression on Br expression during oogenesis. So far, 350 different RNAi lines have been screened and about 20 of them showed defects in either early or late Br expression in follicle cells. Knockdown of Me31B, a putative RNA helicase belonging to the DEAD-box family and component of ribonucleoprotein complex (RNP), resulted in disruption of the Br early expression pattern during the endocycle stages. In our studies with *Drosophila* have revealed that Hindsight and Cut, in follicle cells, Cut and Wg, in wing disc, are also regulated by *me31B*, suggesting a potential role of *me31B* in Notch signaling. In addition, here we report that *me31B* shows genetic interaction with Notch and acts upstream of the Notch signaling. Besides, experimental results also show that knockdown of *me31B* causes upregulation of Dl in follicle cells and cis-inhibition accordingly. Therefore, based on these findings I hypothesized that *me31B* potentially regulates Notch signaling by targeting Dl in follicle cells through miRNA pathway.

CHAPTER ONE

BACKGROUND

1.1 Oogenesis

1.1.1 Overview

Drosophila oogenesis was previously studied for its role in patterning the embryo, however it has since become a powerful model system for investigating many aspects of cell and developmental biology. Its usefulness can be attached partly to the genetic tractability of *Drosophila* as a model system; however, it is also one of the best intensively and successfully studied stages in the development of this model organism. A female *Drosophila* has two ovaries made up of approximately 16 ovarioles, each of which can be effectively considered as an independent egg assembly line. Each ovariole is tipped with somatic and germ line stem cells in the anterior region called the germarium, whose progeny are later on organized into egg chambers. Egg chambers bud off and mature as they pass down the ovariole, reaching the posterior as mature eggs competent for fertilization. Ovarioles usually contain six or seven contiguously more mature egg chambers (follicles), each separated by short chains of inter follicular stalk cells. Oogenesis generally takes a week and has been divided into 14 stages based on morphological criteria (Figure 1A-B).I will refer to stages 1–6 as early, stages 7–10 as mid, and stages 11–14 as late oogenesis. Stage one is budding of the egg chamber from the germarium, and stage 14 is the mature egg.

The germline stem cells exist at the far anterior of the germarium, a structure that harbors the germline and somatic stem cells (Figure 1B). These are divided asymmetrically to produce another stem cell and a daughter cell, which begins to differentiate. To form a cyst of 16 cells interconnected by cytoplasmic bridges known as ring canals, the daughter cell undergoes four mitotic divisions with incomplete cytokinesis. Only one of these 16 germ cells develops as an oocyte, whereas the rest of them become nurse cells, synthesizing nutrients and cytoplasmic components to be transported into the oocyte. The oocyte is the only cell within the cyst that will progress through meiosis. In fact, all cystocytes enter prophase of meiosis I, but a synaptonemal complex forms only in both cells with four ring canals and to a lesser degree in the two cells with three ring canals (Carpenter, A.T, 1975). Nevertheless, only the future oocyte will

complete meiosis, whereas the other cystocytes leave the meiotic program and finally start the endocycles characteristic for the nurse cells.

While the cysts are moving forward through the posterior region of the germarium, they interact with follicle cells. Cysts in the region 2a in germarium have not been fully enclosed by the follicle cells and still directly contact neighboring cysts [Horne-Badovinac and Bilder, 2005] When the cyst enter to region 2b, it turns to a one cell-layer disc and encompasses the whole width of the germarium, with oocyte-specific factors concentrated in the oocyte and a detectable microtubule-organizing center, which forms a polarized microtubule network that is polarized toward the oocyte and extends into all 16 cells through the ring canals.(Xu et al., 2012)

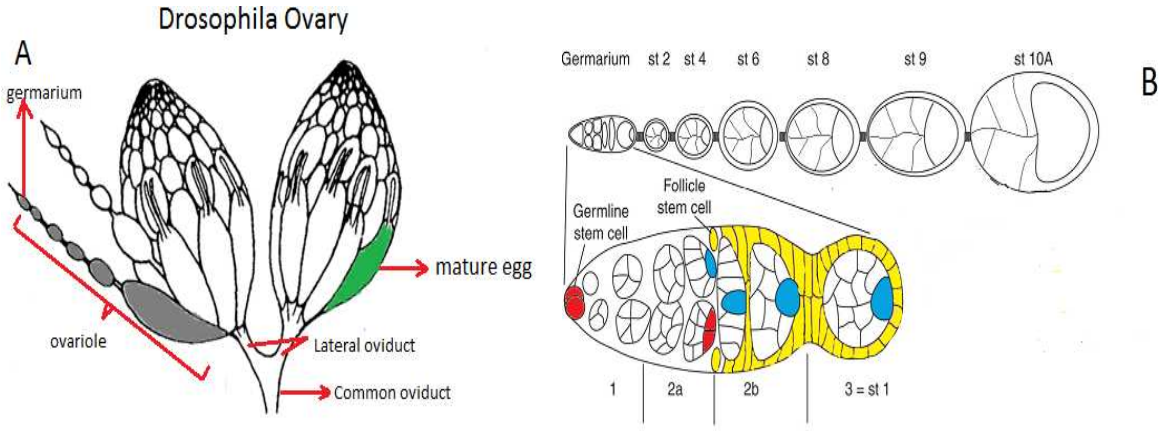


Figure 1. Drosophila ovary and oogenesis. (A) shows *Drosophila* ovary which generally consists of 16 ovarioles. (B) shows germarium and oogenesis. (A) was adapted from Miller, 1950 (B) Adapted From Roth and Lynch, 2009.

The somatic or follicle stem cells in the regions at 2a and 2b give rise to precursor follicle cells. Sixteen of the precursor follicle cells invade between the cysts and develop into polar cells and stalk cells, which play crucial role in follicle formation. The other precursor cells form an epithelial layer around the cyst, producing an egg chamber (Xu et al., 2012). The recently formed stalk cells separate the egg chamber from the germarium.

New egg chambers enter the larger, more posterior region of the ovariole, consisting of six to seven progressively older follicles when it buds from the germarium. Different kinds of cell-cell signaling events between the somatic and germline cells and between different populations of somatic cells control the formation, polarization and patterning of the egg chamber during the

course of 14 developmental stages (Poulton et al., 2008). During the early stage of oogenesis follicle cells proliferate and then the egg chamber starts to enlarge between stages 7–9. After that the oocyte in the follicle grows significantly, takes up yolk protein synthesized in the follicle cells, and occupies almost half the egg chamber by stage 10A. Finally during stages 10B to 14, nurse cells contribute maternal mRNAs and proteins to the oocyte by a cytoskeleton-based mechanism and transfer their cytoplasm into the oocyte to help it reach its large size. The follicle cells synthesize the vitelline membrane and then the eggshell over the oocyte. After the completion of the eggshell and the dumping of the nurse cell cytoplasm, the follicle cells and nurse cells undergo apoptosis, leaving behind the mature egg.(Xu et al., 2012).

1.2 Notch Signaling Pathway

The Notch pathway is one of the best-studied pathways in *Drosophila* oogenesis. Notch signaling is an evolutionarily conserved signaling network that is required for embryonic development, cell fate specification, and stem cell maintenance (*Artavanis-Tsakonas* et al., 1999; Ranganathan et al., 2011). Notch signaling plays important role in deciding cells fate and their activities through either activating or suppressing some important things like differentiation, proliferation, survival, and apoptosis (Bray, S.J, 2006; Fiuza and Arias. 2007). Notch itself is a transmembrane receptor protein that activate signaling by interacting another transmembrane ligand proteins such Delta (termed Delta-like in humans) and Serrate (termed Jagged in humans) on neighboring cells. Even though the fly genome includes only one Notch receptor, mammals have four Notch paralogs that show both redundant and unique functions. The extracellular domain has 29–36 tandem epidermal-growth-factor- (EGF-) like repeats, some of which are required for ligand interaction (Figure 2). For example, on the Notch receptor repeats 11–12 has duties to mediate efficient interactions with ligands available on neighboring cells (trans-interactions), while repeats 24–29 plays role in directing inhibitory interactions with ligands co-expressed in the same cell (cis-interactions) (de Celis and Bray, 2000). It is known that many times EGF repeats connect or link with calcium, which has a key role in determining the structure and affinity of Notch to its ligands (Cordle et al., 2008; Raya et al., 2004). In the Notch structure, EGF repeats are followed by an important negative regulatory component (NRR) consist of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD) (Figure 2). We may also indicate that at the tip of C-terminus Notch structure has conserved

proline/glutamic acid/ serine/threonine-rich motifs (PEST), containing degrons that mediate the stability of NICD. In addition, dNotch also contains the glutamine-rich OPA repeat (Figure 2).

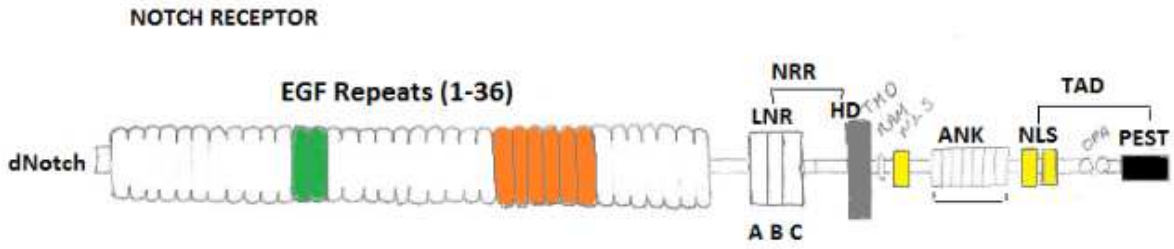


Figure 2. Structure of Notch receptor. While green EGF repeats make interaction with ligand in the neighboring cells (trans interaction), orange repeats make cis-interaction.

The canonical model requires the activation of the Notch receptor through a series of proteolytic events on binding to any of the Delta–Serrate–LAG2 (DSL) ligands. One important cleavage event to make signaling continue depends on γ-secretase-mediated cleavage of the Notch intracellular domain (NICD) from the cell membrane. This event causes the translocation of NICD into the nucleus, where it directly participates and works in association with an essential transcriptional complex with the DNA-binding protein Suppressor of Hairless (SU(H)) and the nuclear effector Mastermind (MAM), thereby activating transcription of target genes(Bray, S.J, 2006).

1.2.1 Notch Regulation in cis and in trans

The complexity of the network circuitry that modulates Notch signals has been well documented. However, a few issues of the pathway still remain to be addressed. One of the notable issues of the pathway is the recently recognized difference between *cis* and *trans* interactions among the ligands and receptors (D'souza et al., 2010). *Trans* interactions happen between a receptor that is expressed on one cell and the ligand on a neighboring cell, resulting in receptor activation. *cis* interactions takes place between ligand and receptor in the same cell membrane. Although we know much about the presence of *cis* interactions from genetic studies and experiments demonstrating co-localization of ligands and receptors on the same cell (Fehon et al., 1990), the

importance and function of these interactions has remained mysterious for some time. Recently, elegant studies have shown that this interaction is inhibitory, such that *cis* engagement of a surface-expressed receptor renders it refractory to *trans*-activating interactions. This 'simple' *cis–trans* relationship is necessary for the organism, as their equilibrium provides the directionality to the signal. However, the underlying mechanism to this phenomenon remains to be fully elucidated. Insight into the mechanism comes from imaging approaches combined with mathematical models in mammalian cell culture systems. These studies suggest that *cis* interaction generates a sensitive switch between mutually exclusive signaling states, a 'sending' state characterized by a high Delta/Notch ratio and a 'receiving' state characterized by a low Delta/Notch ratio. At the multicellular level, this switch can augment small differences between neighboring cells in the absence of transcription-mediated feedback. This would in turn enable the formation of sharp boundaries and lateral inhibition patterns that are seen during development (Sprinzak et al., 2010). Since all of the cells within an equivalence group originally express both the receptor and the ligand, the ratio of *cis–trans* interactions can be presumed to be an essential regulatory mechanism. Hence, the decision as to which of the two neighboring cells becomes the signal-sending versus the signal-receiving cell may be highly coordinated by the dynamic competition between *cis* and *trans* interactions

1.2.2 Notch Signaling During Oogenesis

Previous studies have shown that Delta-Notch signaling is required for many important aspects of oogenesis in *Drosophila* melanogaster. Some of these functions have been demonstrated genetically using mutant alleles of *Delta*, *Serrate* and *Notch* and by ectopic expression of *Delta* or constitutive activation of the *Notch* receptor.

Notch signaling is indispensible for Germ line Stem Cell (GSC) niche maintenance and formation. *Serrate* and *Delta* present on the surface of GSCs activates *Notch* in the somatic cells to form and maintain the GSC niche. In return, the niche induces and maintains stem cell fate (Ward et al., 2006). Ectopic expression or expanded activation of Notch signaling causes the formation of more cap cells and larger niches, thereby inducing ectopic or more GSCs. On the

other hand, decreased Notch signaling during niche formation leads to reduced cap cell number, niche size, and subsequently fewer GSCs (Song et al., 2007).

Notch signaling also regulates multiple facets of the differentiation of somatic follicle cells in the *Drosophila* ovary, including differentiation of the polar and stalk cells (Vachias et al., 2010). Loss of Notch in follicle cells or loss of Delta in the germ line results in fused egg chambers without polar cells (Grammont et al., 2001). Reduced expression or deletion of Delta in the follicle cells results in encapsulation of the cysts by the follicle cells, but not stalk formation. Constitutive expression of Notch results in more polar cells and long stalks between the egg chambers. It has been previously shown that formation of polar and stalk cell fates depends on different levels of Notch activation. On one hand the future polar cells have high-level Notch activation, resulting from a germline Delta signal. On the other hand, stalk cells show low-level Notch activation, which comes from a Delta signal in the polar cells. A recent study has shown that Delta-Notch signaling is required for lateral migration of follicle stem cell daughters across the ovariole as well as for follicle stem cell replacement (Nystul and Spradling, 2010).

During oogenesis, as an egg chamber develops at the end of stage 6, epithelial follicle cells switch from the mitotic cell cycle to the endoreplication cycle, also called the endocycle (duplication of DNA without cell division). Delta from the germ line cells and Notch from the somatic follicle cells are needed for this switch (Deng et al., 2001). At stage 6 of oogenesis, somatic Notch interacts with its ligand Delta (Dl) in the neighboring germline cells (Fehon et al., 1990). This interaction results in the detachment of the ectodomain and exposure of an extracellular metalloprotease site (S2), which consequently becomes susceptible to cleavage by transmembrane proteases of the ADAM/TACE (A Desintegrin And Metallopeptidase/ Tumour necrosis factor A Converting Enzyme) family (Mumm et al., 2000; Nicholas et al., 2007). As a result of this S2 cleavage, the remaining membrane-tethered Notch fragment further experiences two intramembranous cleavages (S3/S4 cleavages), by gamma-secretase, a membrane protein complex containing presenilin as the catalytic component (Wolfe, M.S. 2006). The intracellular domain of Notch is then released and translocated into the nucleus where it acts as a co-activator of the transcription factor, Suppressor of Hairless (Su(H)) to regulate gene expression (Struhul and Adachi, 1998).

Cut is a one of the downstream target genes regulated by Notch signaling. Its expression and function have been described in several developmental stages in *Drosophila*. In wing imaginal

discs, it is induced by Notch signaling at the dorsal-ventral (DV) boundary to maintain the DV border (de Celis and Bray, 1997; de Celis et al., 1996). In addition, *Cut* is also present in follicle cells during the early stages (i.e., the mitotic cycle). It is required for egg-chamber encapsulation and coordination of the germline and follicle-cell differentiation (Jackson and Blochlinger, 1997). During the mitotic cycle/endocycle switch (the M/E switch), *Cut* expression is downregulated by the Notch pathway in follicle cells and this downregulation is necessary for the proper entry of follicle cells into the endocycle and for their differentiation (Sun and Deng, 2005).

Hindsight (Hnt), a *Drosophila* homolog of mammalian *Ras Responsive Element Binding protein (RREB-1)*, is another target of Notch signaling (Yip et al., 1997; Zhang et al., 1999). It has been shown that *Hnt* is upregulated by Notch signaling and facilitates the Notch-dependent downregulation of *Cut*, *String*, and *Hedgehog* signaling in follicle cells, thus promoting the M/E switch and cell differentiation (Sun et al., 2008). Based on similar studies, it has been shown that *Hnt* is also required for the proper switch from endocycle to gene amplification stages.

1.3 Maternal Expression at 31B (Me31B)

Maternal expression at 31B is a RNA helicase protein that belongs to DEAD-box family. DEAD box proteins were first identified in the late 1980s as a group of NTPases that are similar in sequence to the elF4A RNA helicase (Gorbalenya et al., 1989). In this study, these proteins (p68, SrmB, MSS116, vasa, PL10, mammalian eIF4A, yeast eIF4A) play a role in RNA metabolism and share several common elements (Linder et al., 1989). Among these elements, nine common sequences, Q-motif, motif 1, motif 1a, motif 1b, motif II, motif III, motif IV, motif V, and motif VI, found to be conserved among species, which is an important criterion of the DEAD box family (Linder et al., 1989). Motif II is also known as the Walker B motif and contains the amino acid sequence D-E-A-D (asp-glu-ala-asp), which gave this family of proteins the name “DEAD box”(Linder et al., 1989).

Helicases are enzymes that separate duplex oligonucleotides in an energy-dependent manner and are essential in all aspects of DNA and RNA metabolism. Analysis of their amino acid sequences, whether DNA or RNA helicases, revealed that they have conserved sequences, and allows them to classified five major super families (SF1- SF5) (Koonin, E.V, 1993).

As stated above, Me31B is an RNA helicase and can be isolated during a differential gene library screen for expression in *Drosophila melanogaster* during oogenesis but not embryogenesis. The in situ studies showed a greater RNA expression in the nurse cell cytoplasm than in the oocyte and assumed that a transcription burst occurs around stage 6/7 oogenesis (DeValoir et al., 1991). The name Me31B gene actually comes from its maternal expression (ME) and its localization to the 31B region of the chromosome II (31B). Nakamura *et al*., showed that Me31B protein forms a cytoplasmic RNP complex with oocyte-localizing RNAs and Exuperantia, a protein that is known to be involved in RNA localization. Loss of Me31B in early oogenesis causes premature translation of oocyte-localizing RNAs within nurse cells without affecting their transport to the oocyte. In the early *Drosophila* egg chamber that lacks Me31B, at least two mRNAs, *OSK* and *Bicaudal-D* mRNAs, are prematurely translated in nurse cells and their transport to the oocyte occurs in an independent manner. These results suggest that Me31B mediates translational silencing of RNAs during their transport to the oocyte and provides insights that RNA transport and translational control are linked through the assembly of RNP complexes (Nakamura et al., 2001).

Even though now we know that me31b is found in follicle cells, Nakamura et al., 2001 claimed that when distribution of endogenous Me31B was examined, there was no detectable signal observed in somatic follicle cells at any stage of oogenesis, whereas it is first seen as a low level in region 2B of the germarium and remains concentrated in the oocyte in mid-oogenesis. In the nurse cell cytoplasm of the early egg chamber, Me31B signal is also detected at a low level and becomes more evident from stage 5-6 when Me31B expression is drastically increased. In addition, Me31B is frequently enriched around nurse cell nuclei and later accumulates at the posterior pole of stage 10 oocytes. However, this posterior accumulation is transient, as revealed by uniform distribution of the signal in cleavage embryos. By the cellular blastoderm stage, Me31B becomes undetectable in the entire embryonic region and no zygotic expression of Me31B was detected during embryogenesis (Nakamura et al., 2001).

Previously, studies showed that Me31B is associated with dFMR1/FMRP in neuronal mRNPs regulate dendritic morphogenesis in sensory neurons, and, like its human homolog RCK, is required for optimal miRNA function (Barbee et al., 2006; Chu and Rana, 2006). In addition, the *Drosophila* homolog Me31B is found in maternal sponge bodies where it known to repress translation of many granules associated mRNAs (Nakamura et al., 2001). Taken together, these

observations indicate to a role for Me31B in the repression of mRNAs localize in dendrites, their aggregation into transport mRNA, and in control of locally translated synaptic mRNAs such as CaMKII, which regulate synaptic plasticity (Hillebrand et al., 2010).
Recently miRNA based regulation has been shown to be important for the control of spin growth in hippocampal neurons (Schratt et al 2006) and to be target of protein-degradative pathways involvement in long-term memory formation in Drosophila (Ashraf et al., 2006). Thus Me31B will be important in modulating miRNA function pertinent to development of functional neuronal plasticity (Barbee et al., 2006). More generally, because Me31B homologs in yeast and mammals have been shown to function in P body formation in somatic cells (Andrei et al., 2005; Coller, R. Parker, 2005) the requirement for Me31B in miRNA function provides evidence of efficient miRNA-based repression in varied cell types and biological contexts (Barbee et al., 2006).

1.4 miRNA Regulation

MicroRNAs (miRNAs) are short non-coding RNA sequences that control gene expression transcriptionally or post-transcriptionally by either repressing or degrading target RNA (Bartel*et al*., 2004). MiRNAs are now known to play a significant role in regulatory mechanisms in various organisms, for instance development timing, host-pathogen interactions, as well as cell differentiation, proliferation, apoptosis and tumorigenesis. Likewise, as a regulatory element, miRNA itself is coordinately modulated by multifarious effectors when carrying out basic functions, such as SNP, miRNA editing, methylation and the circadian clock. It has been shown that an individual miRNA is able to control the expression of more than one target mRNA and that each mRNA may be regulated by multiple miRNAs (Rhoades et al., 2002). The interaction of miRNAs with mRNAs is usually highly conserved among species and it will localize about about 6 to 8-nt at the 5’ terminus of mRNAs. Even a slight change in sequence may alter its target. It has also been suggested that the location of the central loop in the miRNA:mRNA duplexes may play a key role in affecting the efficiency of gene regulation mediated by miRNAs (Ye et al., 2008). Because one miRNA might have more than one target and each target gene might be regulated by more than one miRNA, further experiments such as gain-of-function and loss-of-function are still needed, to determine how many of these predicted targets are genuinely

targeted by a certain miRNA. For those experiments in which RNAi is used, making those predictions for miRNA target is important. In this way more accurate results can be obtained.

1.4.1 Biogenesis of miRNA

Two main events occur in the process of mature miRNA formation in animals which is generally 21-21 nt in length and non-coding RNA. Firstly, developing miRNA transcripts (pri-miRNA) are processed into a short stem-loop structure ~70-nucleotide precursors (pre-miRNA) by the help of Drosha. Secondly, this pre-miRNAs are cleaved to generate ~21–25-nucleotide mature miRNAs (Lee et al., 2009). Pri-mRNAs are known to localize either within the introns of other genes or in intergenic regions and are transcribed by the host gene or by their own promoter. In addition, certain miRNAs are clustered in polycistronic transcripts, indicating that these miRNAs are coordinately regulated during development (Lagos-Quintana et al 2003).

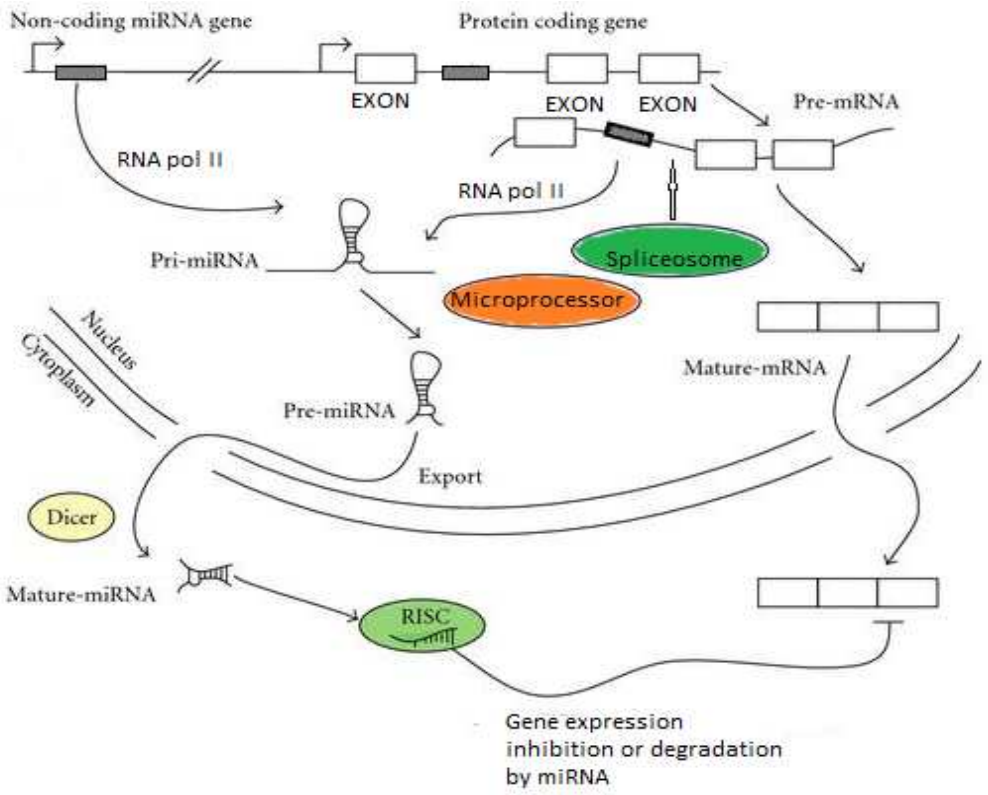


Figure 3.miRNA biogenesis pathway and its effect on gene expression. (Adapted from Shomron and Levy, 2009)

However, little is known about the transcriptional regulation of pri-miRNAs. After mature miRNA is produced, this short RNA loses one of its strands while the other is loaded onto an Argonaute-containing RNA-induced silencing complex (RISC) which plays a role in mediating gene silencing. When the miRNA attaches to its target gene, regulation takes place mainly through mRNA degradation or translational inhibition (Bushati and Cohen, 2007)(Figure 3).

1.5 Aims of this Project

My Master research focuses on identification of Me31B from an *in vivo* RNAi screen as a potential regulator of Notch signaling. In chapter two I will describe the RNAi screening that I made to explore new components in the Notch signaling pathway and I will explain why Me31B is a potential regulator of Notch signaling. In chapter three, there will be different kind of experiments to identify and characterize Me31B. In last chapter, I will correlate Me31B with Notch signaling through the miRNA pathway and I will show some experimental results to make clear the process.

CHAPTER TWO

RNAI SCREENING

2.1 Introduction

RNA interference (RNAi) is an RNA dependent gene-silencing process that is managed by the RNA-induced silencing complex (RISC) and it is initiated by short double-stranded RNA (dsRNA) molecules. The RNAi machinery knocks down but does not eliminate the RNA targets of dsRNA in a sequence-specific manner, in response to endogenous or exogenously introduced dsRNAs (Fire et al., 1998; Zamore et al., 2000). Since the RNAi technique has been well developed, it has become very popular and widely used for studying gene function. By using the RNAi method, scientists have an incredible tool with which to understand human biology as well as molecular and genetic mechanism of human diseases. With using RNAi, almost any gene product can be selectively down regulated and, due to the genome-wide RNAi libraries, this process can be accomplished in a high throughput and unbiased manner. During the past several years, a number of genome-scale RNAi high-throughput screens (HTSs) have been done in both *Drosophila* and mammalian cultured cells to study different biological processes, including signal transduction, cancer biology, and host cell responses to infection and so on. Based on the results from these screens, scientists have identified new components of these processes and, importantly, have also gained more insights into the complexity of biological systems, forcing new and innovative approaches to understanding functional networks in cells.

When the normal function of a gene is required for a given function, RNAi knockdown may lead to a phenotype detectable in an assay that tests that function, either directly or indirectly. Mainly, two major classes of RNAi are known – small interfering RNAs (siRNA) and micro-RNAs (miRNA) (Chapman and Carrington, 2007) (Figure4). siRNA fragments are generated by Dicer from dsRNA precursors which can be both endogenously or exogenously. The miRNA pathway starts in nucleus (endogenously) from processing of non-coding pri-miRNA by a complex of Drosha and DGCR8 proteins to produce ~70-nt pre-miRNAs. Then pre-miRNAs are transported to the cytoplasm and they are cut by Dicer to make miRNA fragments. One strand of short dsRNA products (known as a guide RNA) is then incorporated into Argonaute 2 (Ago2) and the RNA-induced silencing complex (RISC) for selecting target mRNAs by base pairing for

degradation. In miRNA, the mature miRNA recognize target size (generally in the 3'-UTR) and base pairing between guide and target mRNA leads to translational inhibition. One extremely important thing for our studies here we should indicate is that miRNA binding to target mRNA may also lead to mRNA target degradation in processing (P)-bodies (de Fougerolles et al., 2007).

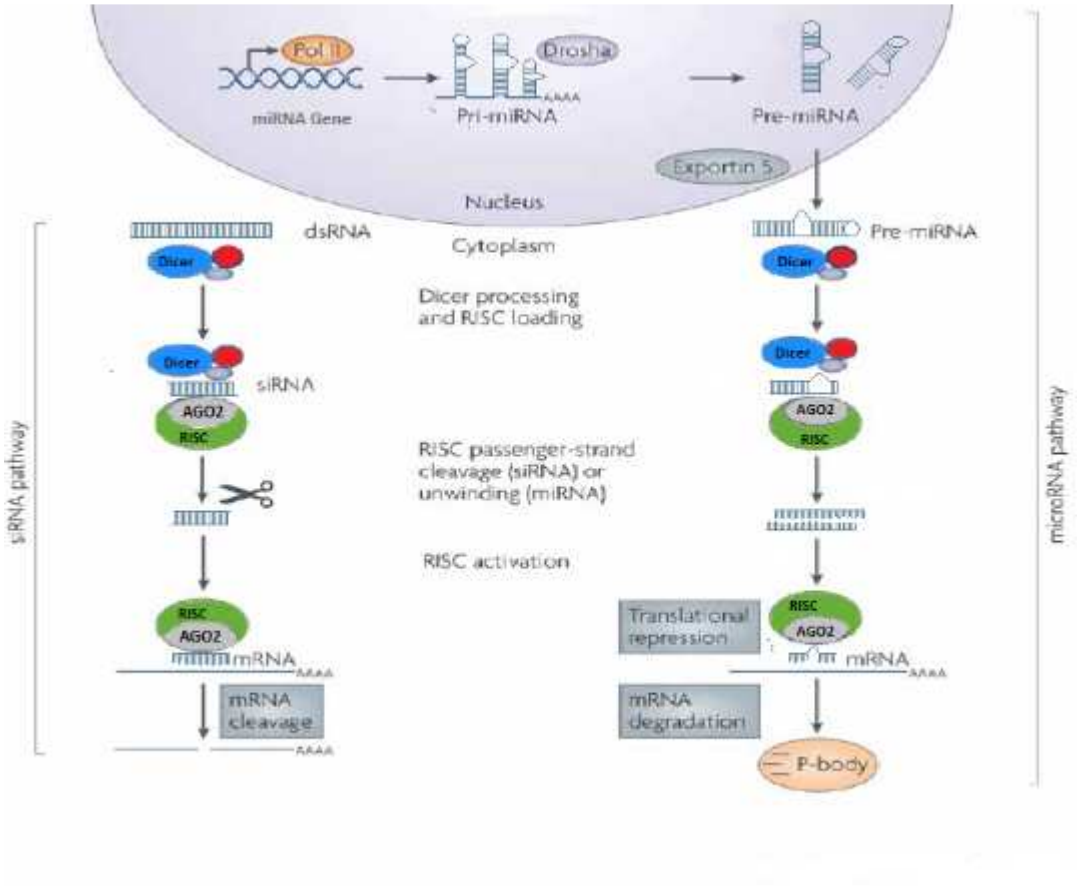


Figure 4. The process of RNAs in miRNA and siRNA pathway. Pri-miRNA is processed by Drosha in nucleus and pre-miRNA by Dicer in cytoplasm. siRNA start as a dsRNA endogenously or exogenously and degraded by dicer to 21-21 nucleotide length. And then miRNa and siRNA are incorporated into RISC. The siRNA guide strand recognizes target sites to direct mRNA cleavage (carried out by AGO2). miRNa does not have perfect match. The mature miRNA recognizes target sites (typically in the 3'-UTR) in the mRNA, leading to direct translational inhibition. On the other hand binding miRNa to mRNA can be also resulted to going P bodies and resulting mRNA degradation (Adapted from deFougerolles et al., 2007)

In this chapter, I show that Maternal expression of 31B (Me31B) in somatic follicle cells is important for proper Notch signaling and downregulation of Me31B affects Notch activation at the switch from Mitotic cycle to Endocycle. To explore how different signaling pathways

regulate follicle cell differentiation and cell cycle switches, we performed an *in vivo* RNAi screen to examine the effect of induced knockdown of gene expression on Br, a zinc-finger transcription factor that is directly up-regulated by Notch signaling, expression during oogenesis. So far, 350 different RNAi lines have been screened and about 20 of them showed defects in either early or late Br expression in follicle cells. Br mRNA is expressed in follicle cells in a dynamic pattern. Its expression is first detected in all follicle cells at stages 6/7. During the stage 10A all the columnar cells, except dorsal anterior follicle cells, contain the *br* transcript. However, only two groups of dorsal-lateral anterior follicle cells express *br* mRNA during stage 10B, marking dorsal appendage secreting cells (de Celis et al., 1996). (Figure 5A). Knockdown of Me31B, a putative RNA helicase belonging to the DEAD-box family, resulted in disruption of the Br early expression pattern during the endocycle stages, small nuclei and some apoptotic cells.

2.2 Results

We discovered different genes including *me31B* that their loss-of-function show different phenotypes in different stages of oogenesis in this genetic screen. Using the flip-out technique with RNAi, we are able to induce targeted interfering RNAs in random patches of somatic cells in the developing egg chamber. RNAi can be induced in a stage and tissue specific fashion, in this experiment in somatic follicle cells. The GAL4 protein regulates the transcription of the Upstream Activating Sequences (UAS), a region similar to an enhancer element. The target gene after UAS could then be expressed as an inverted repeat under control of the yeast upstream activating sequences (UAS), which are binding sites for GAL4. When the Gal4 ‘driver’ fly is crossed with the UAS-RNAi ‘responder’ stock, the resulting progeny have both components of the system, and thus the target gene is silenced in those tissues in which GAL4 is expressed. This technique allows us to quickly screen for genetic interactions of interest. We crossed UAS-RNAi males with virgin flies of genotype: *hsFlp,GFP:stau;;act>CD2>Gal4>RFP/TM3*. The crossed flies were maintained in yeast vials at 25 degrees. When sufficient eggs were laid, parent flies were transferred to another vial. New progenies rose in the same condition that their parents were in. Three or four days after they come out from the pupa, they were exposed to heat shock in 37 C degree two times for 50 minutes. Two days after heat shock in 29 degrees, their ovaries were dissected. Egg chambers were stained with primary antibody (anti-Br antibody), then

secondary antibody (Alexa Fluor m488) and DAPI. And then they were examined under fluorescent microscope (Zeiss LSM 510 confocal microscope) and photographed. The genes that showed phenotypes from the screen are listed in Table 1.

Table 1. List of genes and phenotype found in RNAi screen.

Name of Gene	Stage	Apoptosis	Up-regulation of Br	Down-regulation of Br	Other phenotype
Lethal(1)Bb	10B	_	_	+	**
RpS26	14	_	_	_	**
Prosα7	6-8	+(9)	_	+	*
Me31B	7-8	+	_	+	©
D1	10A	_	+	+	©
E2f	8-9	+	+	_	**
Cap	10A	_	_	+	©
snRNP-U1-70K	_	_	_	_	§
Cbp80	10A	+	_	+	©
logs	10A	_	_	+	_
Rps94	10B	_	_	+	_
CG3605	10A	+	_	+	©
amalgam	_	+	_	_	_
Dlg1	10B	_	_	+	_
Fs(1)K10	7-8	_	_	+	_
Ftz-fs1	_	_	_	_	©
msk	10B	_	_	+	©
Tor	10A	+	_	+	_
pic	10A	+	_	+	*
SmE	10A	_	_	+	**

**: Defective egg chamber and Defective dorsal appendage. ©: Defect in dorsal appendage
*: Defective egg chamber §: Fused egg chamber

From Table 1, around loss-of-function of 20 different genes showed phenotypes either about Br expression pattern or other defect. While some Br expression phenotypes are seen in middle stages, some of them in late stages suggesting that most likely those genes are under control of

different signaling pathways. Previous research showed that late expression pattern of *br* was shaped by Epithelial Growth Factor Receptor (EGFR) and Decapentaplegic (Dpp) pathways. In addition, our lab recently found that early upregulation of Br protein was regulated by Notch signaling (unpublished data). Thus, Br staining was applied to study the interactions between multiple pathways and candidate genes. As we previously mentioned, in wild type, Br expression starts at stages 6/7 and its expression is seen in all middle stages. When it comes to stage 10 follicle cells all over the oocyte are stained except the dorsal anterior region when we do Br immunostaining. At stage 12, 2 groups of lateral-dorsal anterior follicle cells are heavily stained but the other follicle cells in the dorsal and ventral sites are stained weakly (Tzolovsky et al., 1999). Expression in the posterior region becomes gradually weaker and at the last step expression is only seen around the dorsal appendage associated follicle cells.

To understand Table 1 much more, we can describe some genes in more details. One of them is *Pros*α*7* (proteosome α 7 subunit) which is a protein coding gene and its loss-of-function impacts Br expression from stage 7/8. Br expression shows downregulation at these stages although in wild type Br is always expressed in middle stage. Meanwhile, many follicle cells undergo apoptosis in mutant clones.

Another one is *Dlg1*, a neoplastic tumor suppressor gene. Normally, Br expression is weaker in the ventral site of egg chamber than dorsal site but *UAS-dlg1 IR* line showed relatively more downregulation at the stage 10B at the ventral site. Since this stage is considered as a late stage of oogenesis, the phenotype is more likely associated with the EGFR pathway.

One another gene that showed interesting phenotype is *Tor* (Target of Rapamycin). It is a protein coding gene and loss of its function showed down regulation of Broad at the stage 10A. The cells in the clones are small and some apoptotic cells are seen in the Tor KD line.

The last but not least one is *me31B*. Throughout the remaining part of this study we mainly focus on it. In me31B knockdown follicle cell clones, we found that Br expression fails to be upregulated around stage 7-8 (Figure 5B) and we saw defect in dorsal appendage in late stages. Besides, some follicle cells show apoptosis and egg chamber degeneration were detected in Me31B knocked down egg chambers.

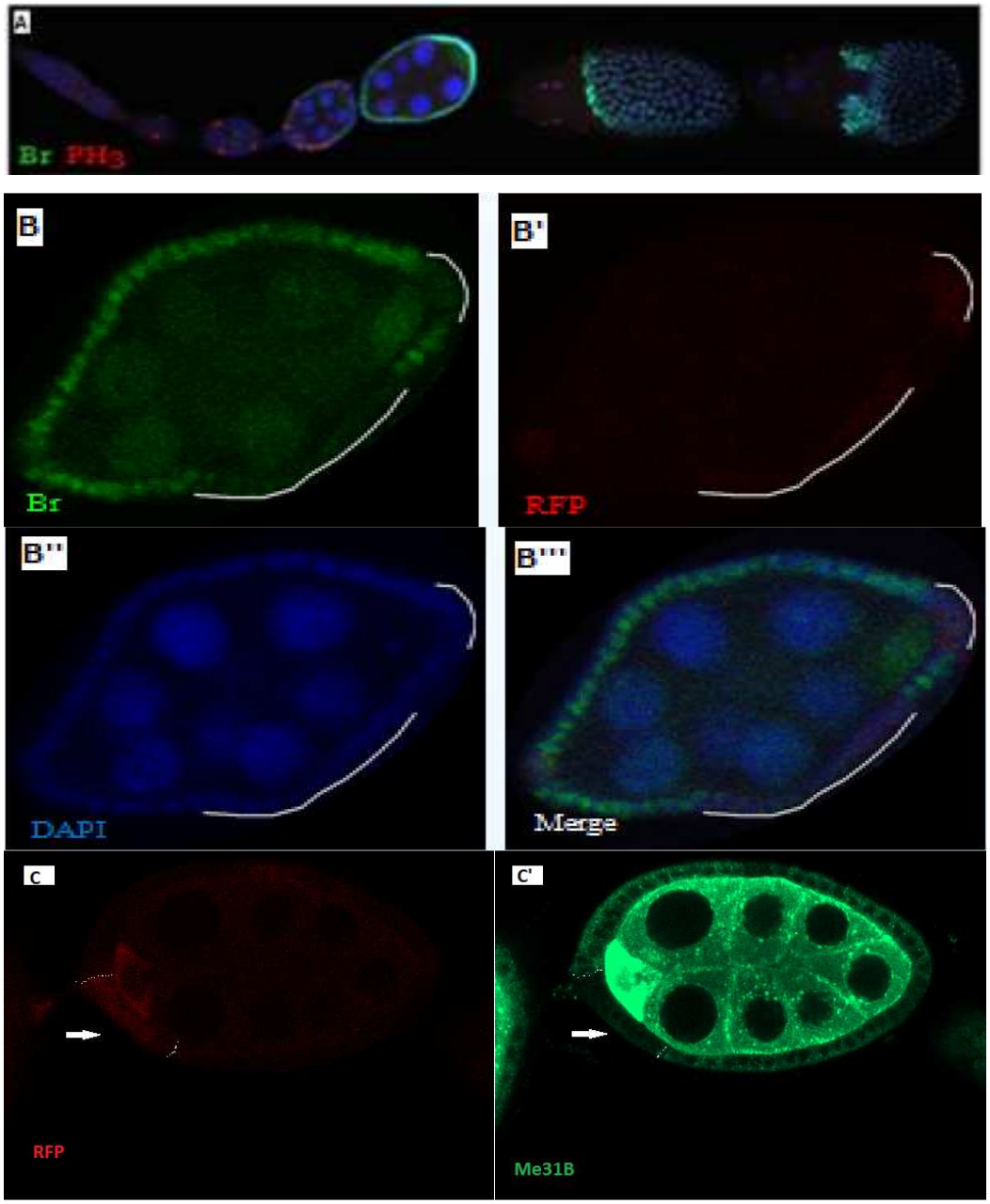


Figure 5. Me31B and Br expression in *me31B* KD RFP clones.(A) Br expression pattern in somatic follicle cells during Drosophila oogenesis. Expression starts with switch from mitotic cycle to endocycle. PH3 indicates those follicle cells still in mitotic cycle. Br expression start around stage 6/7 and during whole middle oogenesis all follicle cell express it in wild type. (B) Br is down regulated in RFP clones (B') in middle stage (7/8) when Me31B is knock down. (B'') Nuclei showed with DAPI in clones are smaller than the others. RFP clones (C) shows Me31B reduced expression (C').

2.3 Discussion

Because we are particularly interested in Notch signaling, we chose to study Me31B. Two main reasons made us dwell upon it. One of them is early broad downregulation, the other one is the small nuclei seen in clones.

From our laboratory's recent studies, we know that early upregulation of Br from stages 6/7 is specifically regulated by Notch signalling. Thus, defects in Br expression relate the candidate gene to Notch signalling. From our screening project, we saw that Me31B RNAi KD follicle cell clones showed early Br downregulation in stage 7/8 even though in wild type Br should be expressed in all middle stages of oogenesis.

On the other hand, as downregulation of Broad is important to show a problem in Notch signalling transduction, the smaller nuclei phenotype in those somatic clone cells is equally important to indicate a Notch signaling defect. As we know during the *Drosophila* oogenesis, follicle cells undergo three different modes while they are developing. They are mitotic cycle, endocyle and gene amplification stages (Deng et al., 2001). In Mitotic cycle cells have complete mitotic division. This means that when the cell and nucleus reach the certain size, it triggers the division of cell and nucleus. In this way the nucleus never becomes bigger than that certain volume. However, because in endocycle stage cells skip M phase of mitosis, hypothetically after each S phase nucleus becomes bigger and bigger. In Me31B RNAi egg chambers, the nuclei in the RFP- marked clones (Figure 5B'') are smaller than the neighbour WT cells suggesting that in the clones those cells still undergo mitotic cycle, failing to switch properly to endocycle. Because those RFP clones have not switched to endocycle phase, it suggests that in the Me31B KD somatic clones Notch signaling is not active even though the egg chamber is already in middle stage. Besides, to show Me31B IR line is not off target, we made immunostaining of Me31B with mouse anti-Me31B antibody (gift from Dr Akira Nakamura). In the clone (Figure 5C) Me31B shows clear downregulation (Figure 5C').

Overall, based on these two main reasons, I hypothesized that me31B potentially might be a regulator of Notch signaling. In the next chapter, to test this hypothesis I performed some experiments to further confirm Me31B as a Notch signalling component and to study the role of Me31B in the Notch Pathway.

CHAPTER THREE

NOTCH ANALYSIS

3.1 Reporter Analysis

3.1.1 Knockdown of *me31B* Causes Defected Notch Pathway in *Drosophila* FC

In a search for the signaling pathways that are supposedly regulated by Maternal expression of 31B (*Me31B*), we performed some reporter analysis experiments in the *Drosophila* egg chambers and wing imaginal discs. In early stages of the egg chambers (before stage 7), follicle cells express the immature cell fate marker Cut (Figure 6C). Starting from stage 7, Notch activity is activated in follicle cells, resulting in down-regulation of Cut and up-regulation of Hnt (Figure 6B). Meanwhile, some Notch activity reporters (such as *E(spl):CD2, m7-lacZ, GBE-lacZ*) are also up-regulated from the stage 7, and they are later downregulated after stage 10a in response to the loss of Notch activity that occurs at this stage of egg chamber development (Sun and Deng, 2007).

Cut protein is an evolutionarily conserved protein that contains several DNA binding domains like one Cut homeodomain and three Cut repeats. In *Drosophila* melanogaster, genetic studies indicate that Cut functions as a determinant of cell-type specification in several tissues, such as in wing disc and somatic follicle cells (Nepveu, A. 2001). To maintain the dorsal-ventral (DV) boundary, Notch signaling is activated at the DV border of the wing imaginal disc (de Celis et al., 1996). In follicle cells, Cut is important for maintaining the structural integrity of germline cells and is required for egg chamber encapsulation. It is also found that *cut* genetically interacts with the components of the Notch pathway and with the catalytic subunit of Protein kinase A during egg chamber development (Jackson and Blochlinger, 1997). In addition, it is known that Cut also regulates cell cycle progression in the *Drosophila* follicle cells (Sun and Deng, 2005). As we mentioned previously, during the mid-oogenesis Cut expression is downregulated by the Notch pathway in follicle cells and this downregulation is required for proper entry of follicle cells into the endocycle and for their differentiation

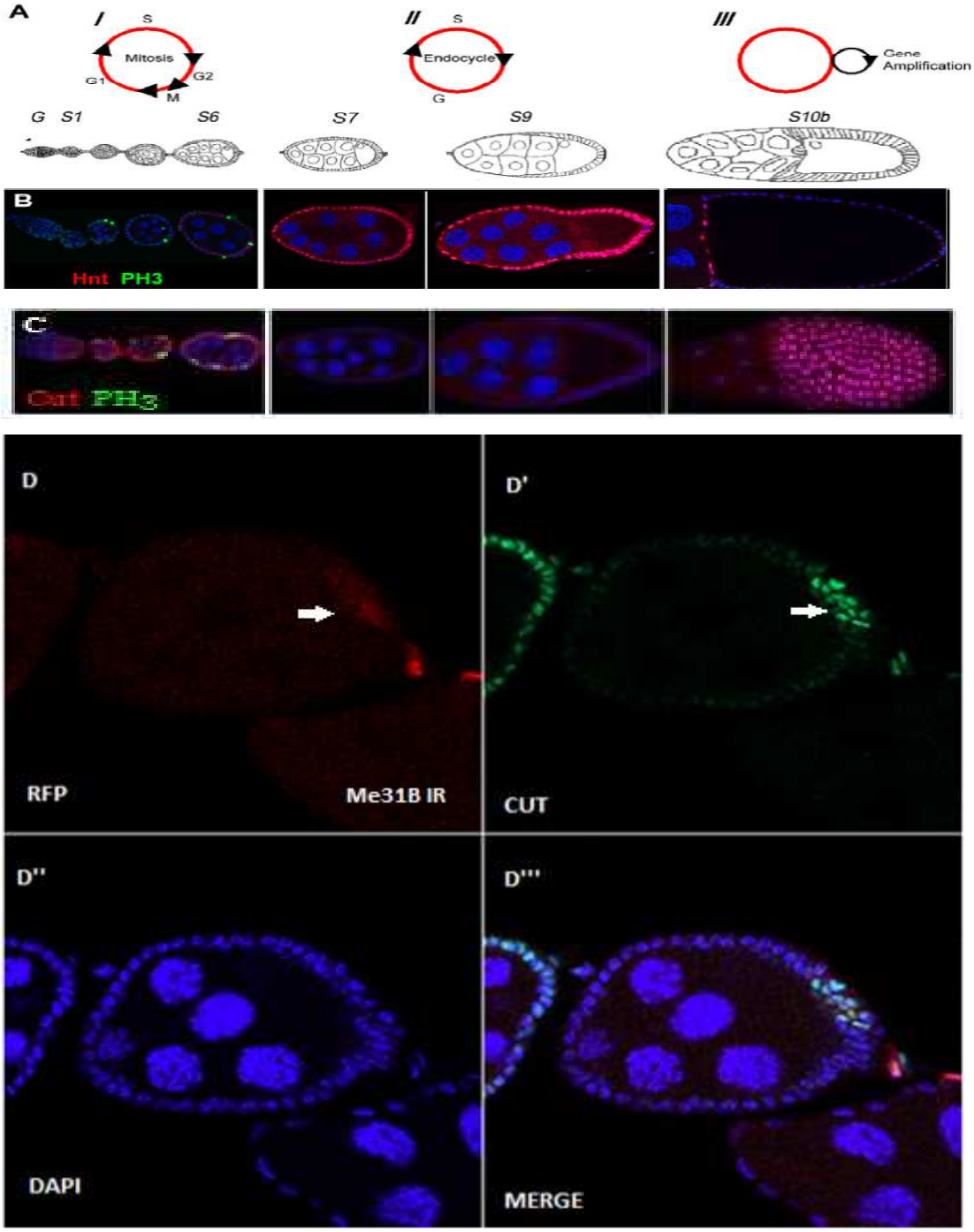


Figure 6. Cell cycle during Drosophila oogenesis and Cut phenotype in *me31B* KD flies (A) Switch of Cell cycle programs and Hnt (B), Cut (C) expression pattern in follicle cells during Drosophila oogenesis(Adepted From Sun and Deng, 2005). (D) RFP marking the expression of *me31B* RNAi causing up-regulation of Cut (D') in the middle stages of oogenesis. (D'') DAPI staining shows smaller nuclei compare to neighboring wild type cells which indicating clone cells remaining in mitotic stages. These findings are suggesting that Notch signaling is disrupted in the clone cells.

Since *cut* is a target gene of Notch and its proper expression indicates proper Notch signaling and proper entering to endocycle stage, to identify whether Me31B affects Notch signaling we examined cut expression in *Drosophila* oogensis by using Me31B RNAi lines available from the Bloomington Stock Center.

For the analysis of these target genes, I crossed *Me31B* RNAi male flies with *hsFlp,GFP:stau;; act>CD2>Gal4, UAS-RFP/TM3,Sb* virgin females. By combining Flip-out and *GAL4-UAS* RNAi techniques we created clonal *me31B*-knockdown cells. After that, expression level of these genes was examined by using antibodies.

As I mentioned previously, the expression of Cut is normally downregulated by Notch signaling during mid-oogenesis, but upregulated during early and late oogenesis. When *Me31B* RNAi is expressed, Cut is upregulated during mid-oogenesis (Figure 6D'). RFP clone cells (D) show upregulation of Cut and small nuclei, raising the possibility that *Me31B* might be involved in the Notch pathway. To confirm this finding, I also examined the expression of Hnt in follicle cells in which Me31B gene is knocked down.

Hindsight is a zinc finger transcription factor and mediates the role of Notch in regulating cell differentiation and the switch of cell-cycle programs. Hindsight, as well as another zinc-finger protein, Tramtrack, downregulates Hedgehog signaling through transcriptional repression of Cubitus interruptus (Sun and Deng, 2007). According to one study, the hindsight (hnt) gene is expressed in all photoreceptor cell precursors and regulates cell morphology, cell fate specification, planar cell polarity and epithelial integrity in the *Drosophila* eye during the retinal development (Pickup et al., 2002). On the other hand, another study demonstrates that Hnt is upregulated by Notch signaling and plays a role in the Notch-dependent downregulation of Cut, thus promoting the M/E switch and cell differentiation (Sun and Deng, 2007).

Consistent with the Cut phenotype, in RFP clone cells (Figure 7A) Hnt expression was downregulated during mid-oogenesis (Figure 7A') even though in wild type its expression is seen in all middle stages.

The results we got from these experiments showed us two target genes of Notch, cut and hnt, show defective phenotypes in UAS-Me31B IR flies. This is consistent with our hypothesis because if Me31B is involved in Notch signaling pathway, we may expect to see the phenotypes with its downregulation.

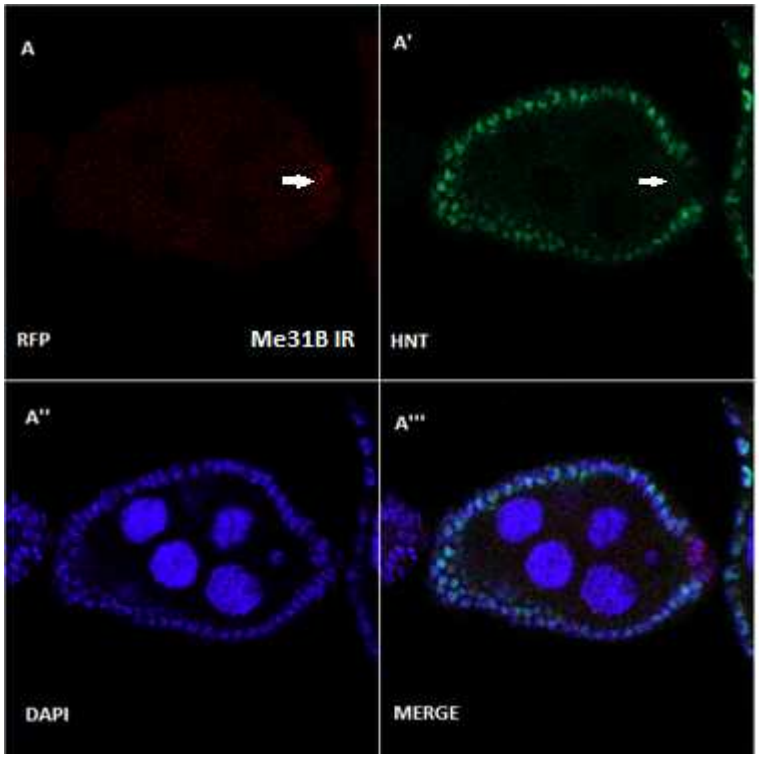


Figure 7. Downregulation of Hnt in middle stages. Knock down of *me31B* in Drosophila follicle cell cause downregulation of HNT(A') in RFP clone cells(A). DAPI is shown in blue (A''). These findings are suggesting that Notch signaling is disrupted in the clone cells.

3.1.2 *me31B* KD Also Causes Defective Notch Signaling in the Wing Disc

Besides the follicle cells we also examined Notch signaling in the *Drosophila* imaginal disc. The dorsal/ventral (d/v) boundary and wing margin patterning during the development of the imaginal wing disc is an important morphogenetic process in which Notch is involved. The formation and maintenance of the d/v boundary requires the locally restricted activation of proper Notch signaling (Irvine and Vogt, 1997). Notch activation at the d/v boundary is required for the localized expression of different genes involved in the formation of the d/v boundary and wing margin patterning, such as wingless (wg) and cut (Micchelli et al., 1997- Couso et al., 1995).

Because proper expression of these localized genes requires Notch activation in wing discs, to determine whether Notch signaling is disrupted we examined expression of wg and cut in the Me31B IR line.

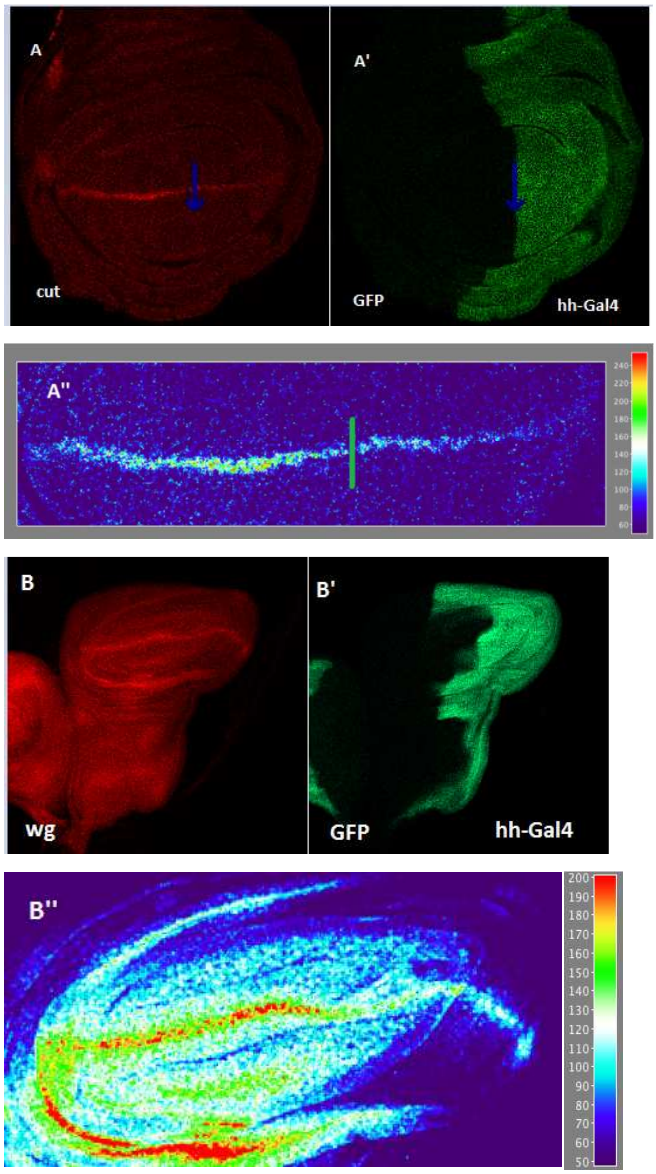


Figure 8. Reducing Cut and Wg expression in the *me31B* knockdown wing imaginal disc. (A) Hh-Gal4 driving *me31BIR* in the posterior compartment of wing disc (GFP) results in Cut (A) disruption. (A") Intensity analysis representing the reduced Cut expression in posterior compartment. (B) shows disrupted wg expression. GFP (B') expressed in dorsal site. Intensity analysis clearly shows how wg expression is reduced (B'').

RNAi can be induced in a stage and tissue specific fashion. This elegant method allows the reduction of gene functions in a tissue specific manner, which is not feasible with ubiquitous gene interference or loss of function mutations. In our experiment we used hh-Gal4-driven Me31B RNAi to detect cut and wg expression in imaginal wing disc. To do so we crossed

Me31B RNAi male flies with hh-Gal4, UAS-CD8-GFP virgin females. When the progenies reached wandering 3rd instar larval stage, we picked and dissected them. Then we performed immune-histostaining for Cut and Wg according to the Deng lab staining protocol.
Cut and wg expression in the d/v boundary are reduced in the posterior site of wing disc in which Me31B is downregulated (Figure 8A-8B). To show cut and wg reduced expression more clearly we used imaging software that is called Fiji, which is a distribution of the popular open-source software ImageJ focused on biological-image analysis (Schindelin et al., 2012). By using this software we made an image that shows intensity of expressed genes (Figure 8A''-8B'')
Based on the results obtained from these experiments we conclude that knocked down of Me31B disrupts Notch signaling not only in follicle cells but also in imaginal wing disc.

3.2 Clonal Analysis of Mutated *me31B*

3.2.1 *me31B* is Required for Proper Expression of Notch Target Genes in FC

To exclude any potential off target artifacts caused by the RNAi experiments and to ascertain that *me31B* is required for regulation of Notch signal, we analyzed three *me31B* null alleles: *P{lacW}me31B*^{k06607} (Bloomington stock), *P{lacW}me31B*$^{\Delta 1}$ and *P{lacW} me31B*$^{\Delta 2}$ kindly gifted from Dr. Akira Nakamura from Kumamoto University, Kyusyu, Japan.
Clonal analysis, also known as mosaic analysis, refers to the production of genetically mutant cells in a heterozygous background. Clonal analysis is a very useful method in studying lethal mutations. According to flybase.org, these tree *me31B* alleles are recessive lethal. To confirm this information, we crossed these three alleles with deficiency lines from Bloomington.

Deficiency line #9503 : *w[1118]; Df(2L)BSC143/CyO* Break points or insertion site : 31B1;31D9, 2L:10209408;10333704 (R5 flank)

Deficiency line #9504 : *w[1118]; Df(2L)BSC144/CyO* Break points or insertion site :31B1;31E5, 2L:10227504;10457595 (R5 flank;R3 author statement->R5)
Both of these deficiency lines include break points or insertion site in *me31B* alleles (2L:10,239,309..10,239,309). Hypothetically, if these mutated alleles are homozygote lethal

then when we cross these alleles with deficiency lines, all the living progenies should have Cyo phenotype, in other words those offspring that have break points or insertion sites in their two alleles should not live. In fact, the larvae that had *P{lacW}me31B*^{k06607} allele and deficiency line break point did not show recessive lethal phenotype, but the other two did show it when the progenies were first or second instar larvae.

w1118 ;Df(2L)/Cyo Xw ; 40AP/Cyo ⟶ *w;40AP/Df(2L)* (Viable)

w1118 ;Df(2L)/Cyo Xw;me31B$^{\Delta 1}$ *FRT/Cyo* ⟶ *w;Df(2L)/ me31B*$^{\Delta 1}$ *FRT* (lethal)

w1118 ;Df(2L)/Cyo Xw;me31B$^{\Delta 2}$ *FRT/Cyo* ⟶ *w;Df(2L)/ me31B*$^{\Delta 2}$ *FRT* (lethal)

Because of the viable progeny we got from *P{lacW}me31B*^{k06607} allele, we decided to not use it in our clonal analysis experiment. Even though we expected to see lethal larvae, we saw viable progeny which made us not trust this allele.

To see and confirm the results we got from the RNAi experiment, we crossed *me31B*$^{\Delta 1}$ *FRT40A/Cyo* and *me31B*$^{\Delta 2}$ *FRT40A/Cyo* male flies, respectively, with *fsFlp; ubi-GFPFRT40A/Cyo* virgin flies. When new progeny came out from their pupae, we picked suitable ones for our future heat shock, dissection and immunostaining (Br, Cut, and Hnt).

♀ *hsflp; 40AGFP/Cyo X w; me31B*$^{\Delta 1\text{-}2}$ *FRT/Cyo* ♂

hsflp;40AGFP/ me31B$^{\Delta 1\text{-}2}$ *FRT* *40AGFP/ Cyo* *me31B*$^{\Delta 1\text{-}2}$ *FRT/Cyo* *Cyo/ Cyo*

↓

Heat shock (2 times 50 minutes), dissection and staining (Br,Cut and Hnt)

Mutant clones were induced through the FRT/FLP method of mitotic recombination (Xu and Rubin, 1993). In order to generate mutant clones, some genetic tools are needed to activate mitotic recombination within the somatic cells of a heterozygous animal. These necessary tools, containing the yeast target sequence (FRT) and enzyme (FLP), were integrated into the *Drosophila* genome via P element mediated transformation . The FRT sites are required to be located on the same arms of the two homologous chromosomes where the mutation site of interest resides. Moreover, FRT sites are located near the centromere for high recombination rate. The enzyme necessary for mitotic recombination, Flipase (FLP), could be expressed under any heterologous promoter of choice. In our experiment, FLP is driven by Hsp70 promoter which is sensitive to heat. Taken together, FRT/FLP technique enables us to create mutant clone cells via mitotic recombination. In combination with GFP marker, as in our experiment, wild

type cells are marked with bright GFP, heterozygous cells with weak GFP, while homozygous mutant cells are indicated by the absence of GFP.

To test the effect of *me31B* null mutation on Notch signaling, we used FLP/FRT system to monitor developmental defects and to detect the expression of Notch pathway. Br and Hnt were downregulated in the absence of *me31B* in GFP negative mutant clones.

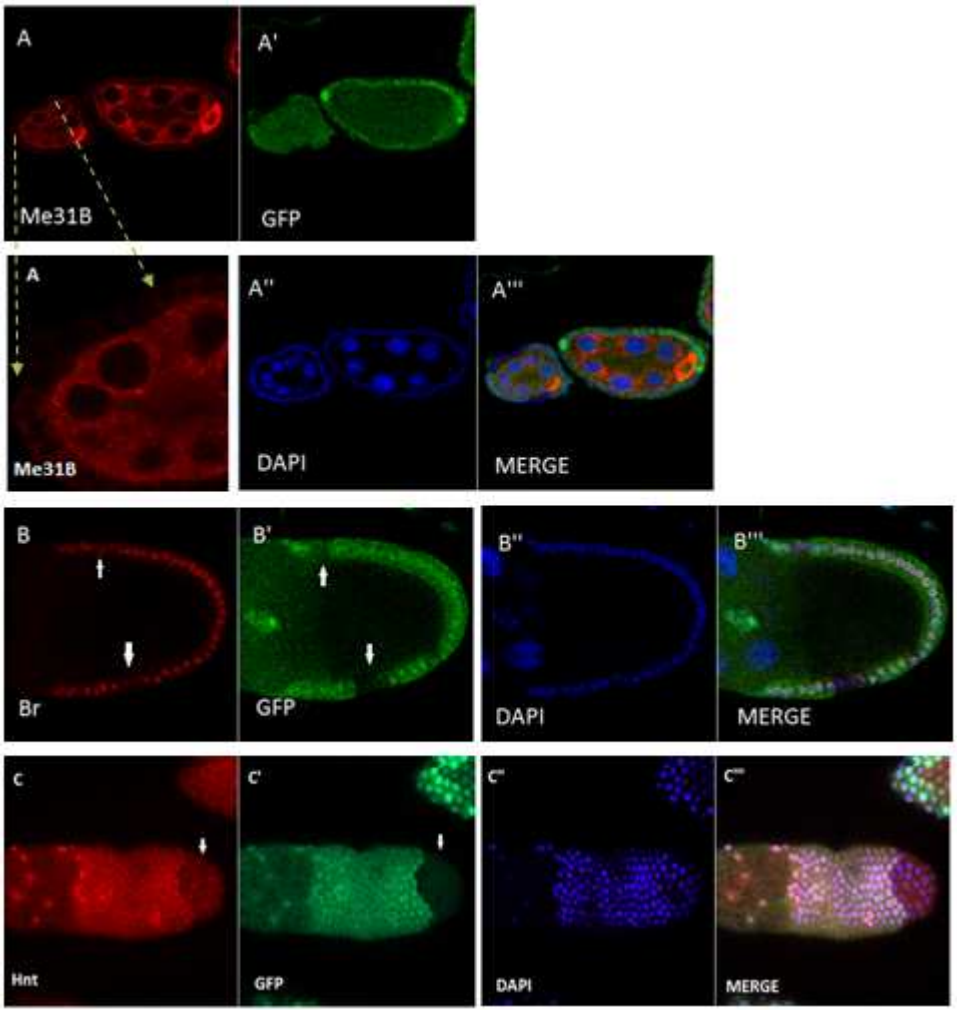


Figure 9 Expression of Me31B and Notch target genes in GFP negative clones. Immunostaining of Me31B GFP negative clones(A') clearly showed demolished expression(A). Br is downregulated (B') in GFP negative clones (B') in stage late 9 or 10a. DAPI (B'') shows nucleus and (B''') merge. Hnt is reduced (C) in GFP negative mutant clone (C').

Consisted with our previous results from RNAi experiment we found that Br and Hnt showed downregulation (Figure 9B-9C), in the middle stage of oogenesis with *me31B* knowdown. In GFP negative mutant clone Me31B is demolished (Figure 9A). Based on these results we

conclude that in Br and Hnt downregulated clones Notch is still not active and accordingly there is a defect in M/E switch and cell proliferation(Sun and Deng, 2007). However, it needs to be done more experiment to ascertain about effects of *me31B* IR on Notch signaling in the mutated me31B lines.

3.3 Genetic Interactions Tests

3.3.1 Depletion of *me31B* Leads to Enhanced Phenotype in Notch Heterozygote Background in *Drosophila* Eye and Wing

A genetic interaction is defined as an unexpected phenotype for a combination of mutations given each mutation's individual effect (Mani et al, 2008) and it provides valuable information about gene function and is useful for studying the organization of biological processes in the cell. Moreover it can show functional relationships between genes and pathways. A double mutant with a more extreme phenotype than expected is defined as a synergistic (enhancement) interaction between the corresponding mutations (synthetic lethality, in the extreme case). In this interaction, two genes may act at the same step of a pathway or in two parallel different pathways.

In order to determine whether there is a genetic interaction between Notch signaling and *me31B* we examined how *me31B* is related to the Notch pathway during the wing and eye development where Notch is known to play an important role (Artavanis-Tsakonas and Muskavitch, 2010). I used GMR-Gal4 to drive me31B RNAi in *Drosophila* eye. The *GMR> me31B IR* flies that grew in 29℃ showed rough eye phenotype (Figure 10C) and when crossed with heterozygote Notch mutant flies, their offspring showed enhanced rough eye phenotype (Figure 10A).

It is known that under so many cases Notch signalling is predicted to delay cell differentiation, keeping cells units to give response to later triggering signals (Coffman et al., 1993). Therefore we believe that these rough eye phenotype in *Drosophila* might be because of these undifferentiated cells.

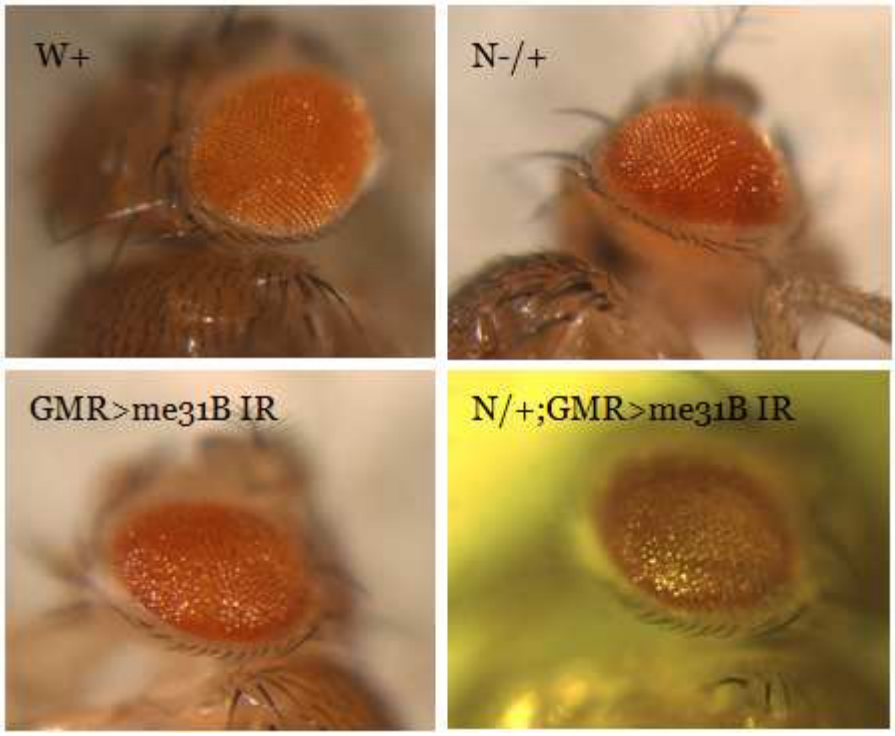


Figure 10. Genetic interaction of *me31B* on eye under W and N heterozygote mutant background. Only *GMR-Gal4* under wild type (D) and Notch -/+ (B) background in 29 C degree. *me31B* IR under wild type background showed rough eye phenotype (C). Rough eye phenotype is enhanced under Notch heterozygote background (A).

Experimentally it was shown that the loss of one copy of the Notch ligand Delta and therefore a reduction in Notch signaling, results in a strong enhancement of the rough eye phenotype (Schreiber et al, 2002). Since reduction of Notch signaling can cause rough eye phenotype, based on our previous finding and the genetic interaction in *Drosophila* eye we hypothesize that knock-down of *me31B* affects proper Delta expression and accordingly reduces Notch signaling, resulting in rough eye phenotype.

On the other hand to determine whether the genetic interaction between the Notch pathway and *Me31B* is restricted in the eye, we extended our investigation to examine how *me31B* is related to Notch pathway during the wing development. We performed same kinds of genetic interaction experiments in the *Drosophila* wing to see whether *me31B* can modulate Notch phenotype during the wing development by using *en-Gal4* and *c96-Gal4*. *en> me31B IR* and *c96> me31B IR* in *Drosophila* wing caused lethal progenies indicating me31B in wing disc is important for development. Most of the larvae did not have a wing disc and those that had wing disc showed disrupted cut (Figure 11B) and wg expression.

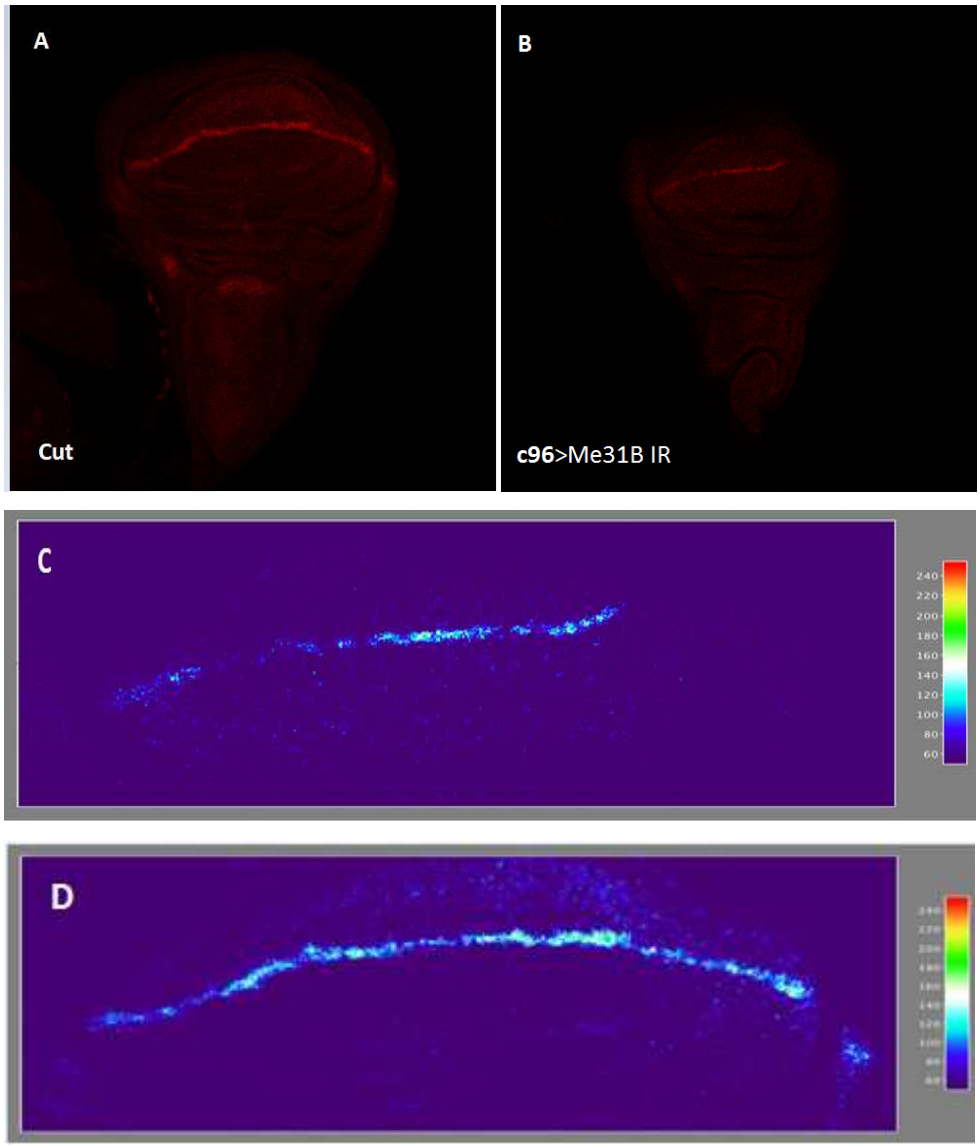


Figure 11. Reducing Cut expression in the *me31B* knockdown wing imaginal disc. The *me31BIR* overexpression by c96-Gal4, expressing in wing disc D-V border, causes reduced Cut level (B) compared to wild type Cut pattern (A). Intensity analysis shows more clearly the reduced Cut expression in posterior compartment (C). Intensity analysis of cut expression in wild type (D)

We conclude these genetic interaction tests as follows. First, *me31B* might act upstream of the Notch pathway and knock down of *me31B* might affect Delta expression. Accordingly, disrupted Delta expression reduced Notch signaling and this cause enhanced rough eye phenotype (Schreiber et al, 2002). Second, synthetic lethality and disrupted cut expression suggests that Me31B expression in wing disc is important for development in *Drosophila.*

3.4 Rescue Experiments

3.4.1 Over Expression of *UAS-N* (full length), *UAS-NICD* and *UAS-Su(H)vp16* Rescue the *me31B* Knockdown Phenotype in Follicle Cells

To determine at which level in the Notch pathway Me31B acts, we preformed rescuing experiments using UAS-N (full length), UAS-NICD and UAS-Su(H)vp16. If expression of N (full length), NICD and Su(H)vp16 can reduce or eliminate the Notch defects caused by mutation or knock down of *me31B*, it, then, would indicate that loss of *me31B* disrupts or reduce Notch activity upstream of these three components.

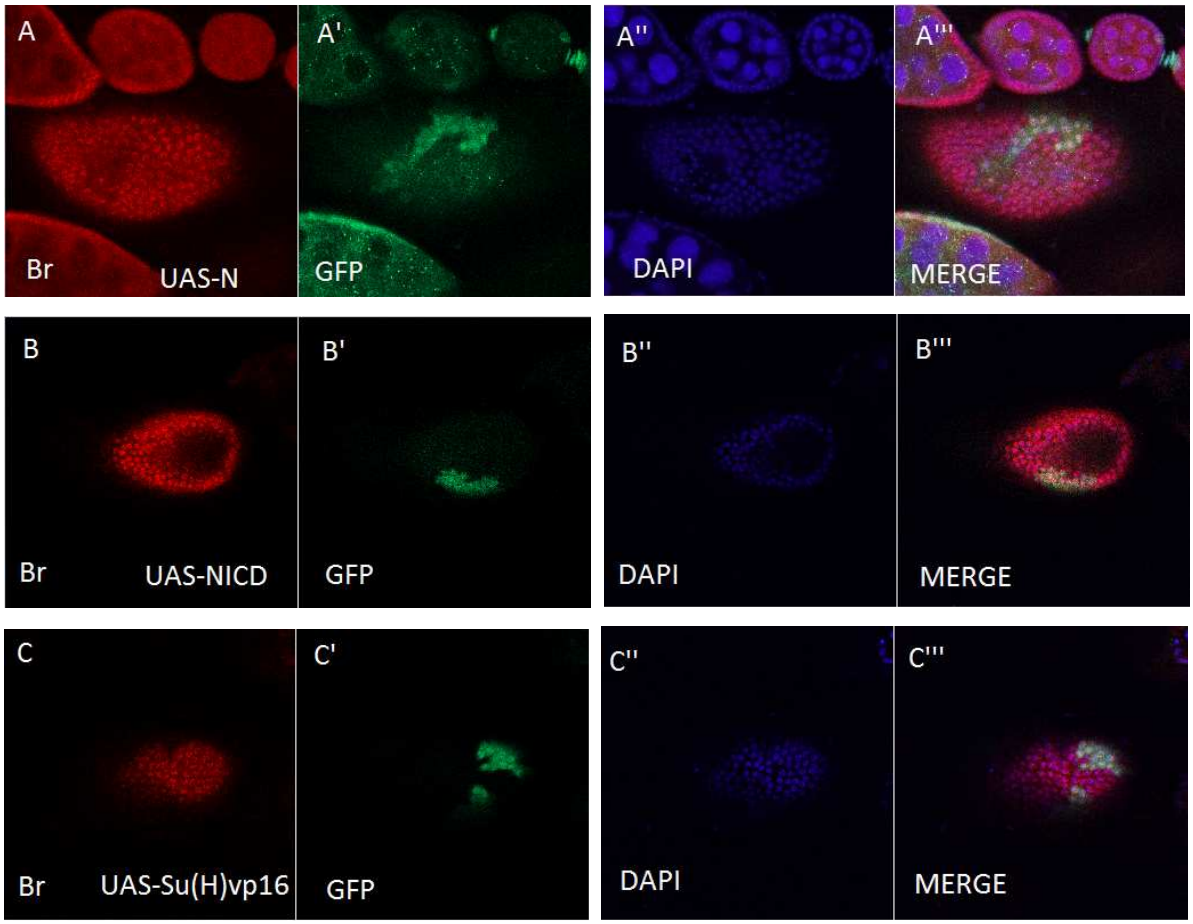


Figure 12. Expression of *UAS-N, UAS-NICD, UAS-Su(H)vp16* to determine whether it can rescue the *me31B* knockdown phenotype in follicle cells. Broad expression (A, B, C) *of UAS-N, UAS-NICD* and *UAS-Su(H)vp16* in GFP clones (A', B', C') seems to be rescued. DAPI staining (A'',B'',C'') shows normal nucleus size.

In contrast with first situation if these three components cannot restore proper Notch activity in *Me31B* expression reduced or null cells, it would suggest that loss of *me31B* affects Notch signaling downstream of them. 13% of GFP clones in *UAS-N* s showed down regulation of Broad (10 out of 65). 9.75% of GFP clones in *UAS-Su(H)vp16* (8 out of 87) and 11% of GFP clones in *UAS-NICD* (13 out of 84) showed down regulation of Br expression. In previously done Br immunosatining in Me31B KD egg chambers, 44% of clones showed Br phenotype. Based on the data we found, over expression of *UAS-N, UAS-NICD* and *UAS Su(H)vp16* rescue to Broad phenotype in *me31B* knock down cells (Figure 13)

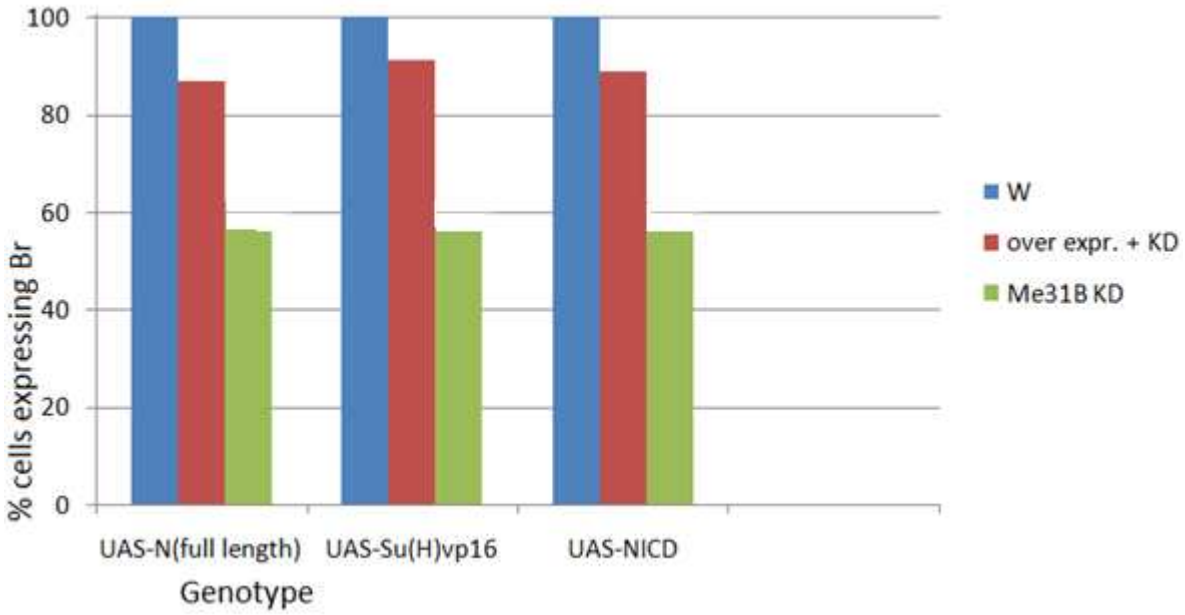


Figure 13. Quantitative analysis of middle stage clone cells that show Br expression. Wild type cells are supposedly taken 100%. Over expression of *N* (full length), *NICD* and *Su(H)vp16* seems to rescue Br phenotype in *me31B* KD clones

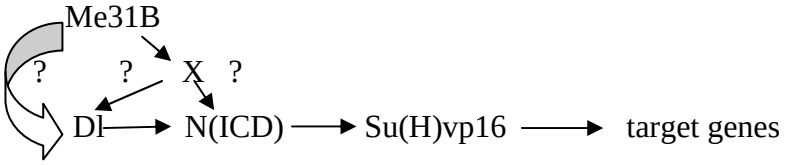
Because expression of these three component of Notch pathway rescued the Br phenotype and because of the fact that only downstream of a pathway can rescue to upstream, we can conclude that Me31B acts upstream of Notch pathway.

CHAPTER FOUR

MIRNA ANALYSIS

4.1 *me31B* Acts Upstream of Notch (N) and Regulates Delta in Both Follicle Cells and Wing Disc.

As our finding suggests that knock down of *me31B* in both follicle cells and wing disc cause disruption of Notch signaling, we decided to make rescuing experiments to determine at which level in the Notch pathway Me31B acts. Based on the results from UAS-Notch, UAS-NICD and UAS-Su(H)vp16 over expression, we concluded Me31B acts upstream of Notch itself because of the fact that only downstream elements of the pathway can rescue upstream defects.



Since Me31B acts upstream of Notch itself, the target point can be either Delta (Dl) or another gene that acts on Dl or Notch. In follicle cells, it is already known that Dl is an important miRNA target and regulates timing of Notch signaling (Poulton and Deng., 2011). Researchers also found that Me31B is a component of P bodies (Hillebrand and Ramaswami., 2007) and required for translational repression of maternal and miRNA-target mRNAs (Hillebrand and Ramaswami., 2010). These observations suggest that knocked down of *me31B* causes a defect in the miRNA pathway and leading to upregulation of Delta in follicle cells and accordingly a defect in Notch signaling.

To test this hypothesis, we examined Dl expression at the follicle cells in *me31B* knocked down flies by doing Dl immunostaining.

Consistent with our hypothesis, in follicle cells, RFP clones (Figure 14A) indicated me31B KD showed Dl upregulation (Figure 14A').it is clearly seen in MERGE figure (Figure 14A''') every RFP clones matches with Dl upregulation. Overexpression of Dl in FC (follicle cell) may cause disrupted Notch signaling in two different ways.

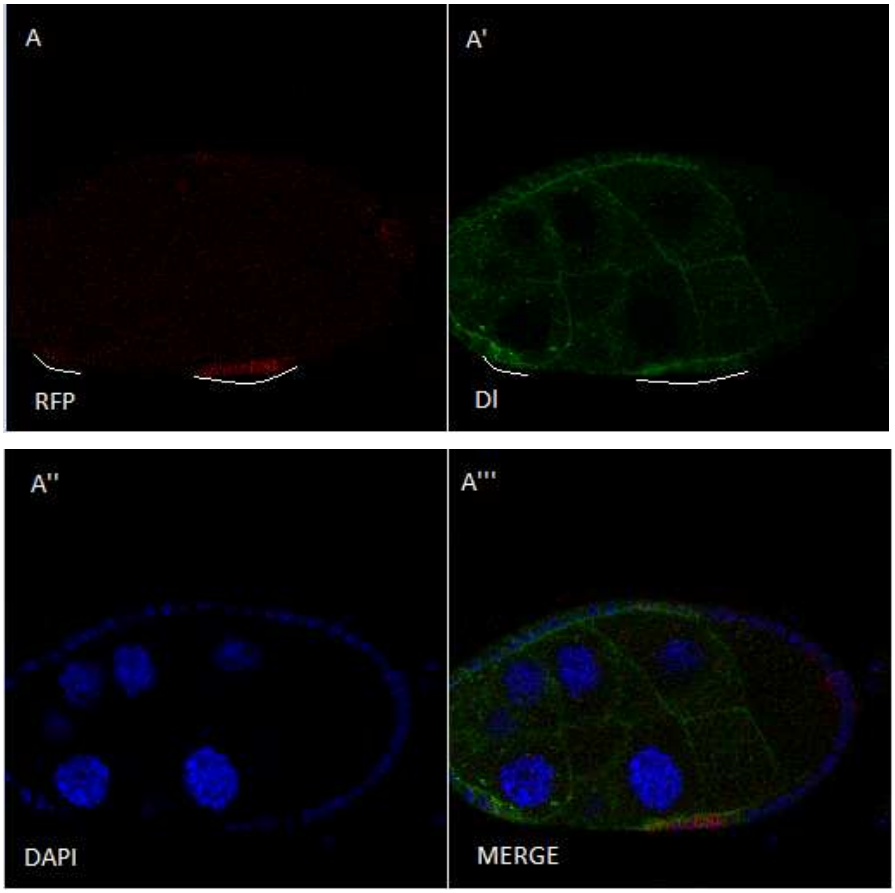


Figure 14. Dl immunostainig in *Drosophila* egg chambers. RFP clones(A) were generated with flp-out technique and include *me31B* KD. In RFP clones Dl is upregulated(A').DAPI staining is showed in (A'') and MERGE is by (A''')

This result might due to *cis* inhibition of Notch by Delta in the same cell (de Celis and Bray., 1997). As Delta can interact Notch in a neighboring cell, it can also interact with Notch in the same cell (Sprinzak and Elowitz., 2010). The ratio of Delta to Notch is important in determining whether a cell is in either a sending state or receiving state. If cis-interaction is higher than trans-interaction, it inhibits Notch signaling in the same cell and disrupted Notch causes defect in target gene expressions.

The other way for disrupted Notch signaling connected with overexpression of Dl in follicle cells is Delta-Delta homotypic interaction. According to one hypothesis, these homotypic interactions may play a negative role in the Notch signalling pathway by reducing the levels of Delta available to bind to receptor (Parody, 1998).So, if delta is upregulated in follicle celss, then it means that there will be much more homotypic interaction with Delta from germline cells and

eventually this situation will reduce the level of Delta available to interact with Notch on follicle cells. As a result of that Notch signal is reduced.

On the other hand, we also examined Dl expression on wing disc to explore if Me31B target the Dl in wing disc too.

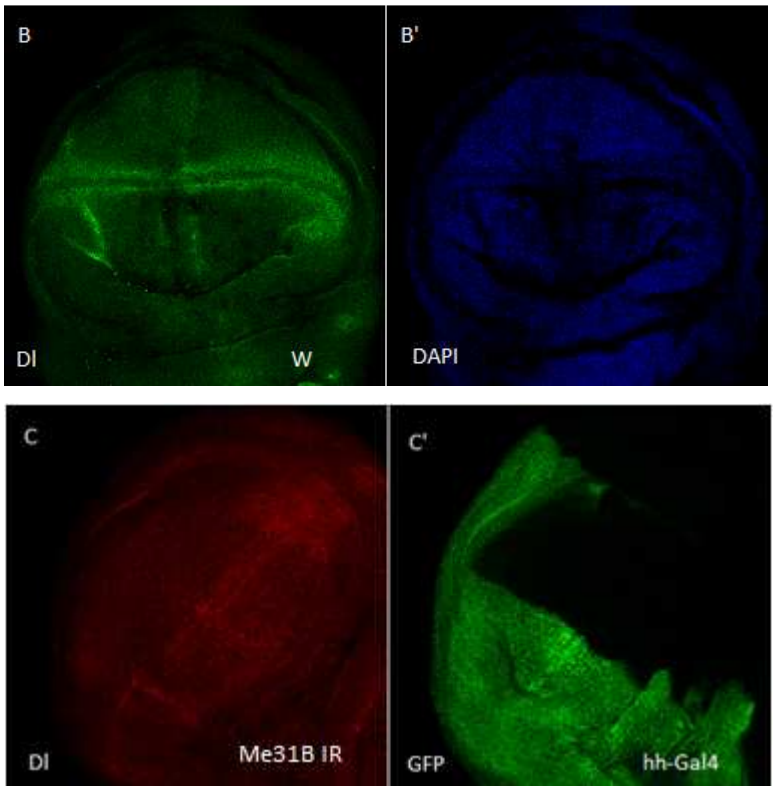


Figure 15. Delta expression on both wild type and *me31B* KD wing disc. (B) shows wild tpe expression pattern and (B') DAPI staining. Dl expression is reduced (C) in posterior region of hh-Gal4>Me31B IR (C')

The result from the Dl immunostaining expreiment by using hh-Gal4 showed that knocked down of *me31B* in wing disc disrupted Dl expression (Figure 15C). In comperison with wild type (Figure 15B) Dl protein expression is reduced in the GFP positive region. Based on this result, we conclude that Dl may not be a direct target of Me31B in wing disc. Instead it may target another gene that regulates Delta in wing disc. Since some studies say that *Apterous* is required for the expression of Delta during wing development (Klein and Arias., 1998), *it* would be good candidate to put between Dl and Me31B. However, that remains to be tested.

Bases on this prediction relationship between me31B, miRNA and Notch signaling in wing disc can be as follows:

me31B(miRNA) → X (*Apterous* ?) → Dl → N(ICD) → Su(H)vp16 → target genes

In any case disrupted Dl expression causes defect in Notch signaling and it cause defect in expression of target genes as previously shown.

We then tested more the idea that *Delta* is an important and related miRNA target in follicle cells, and confirm it is downstream of *me31B* by assaying Notch activation in *me31B, Delta* double-knockdown clones.

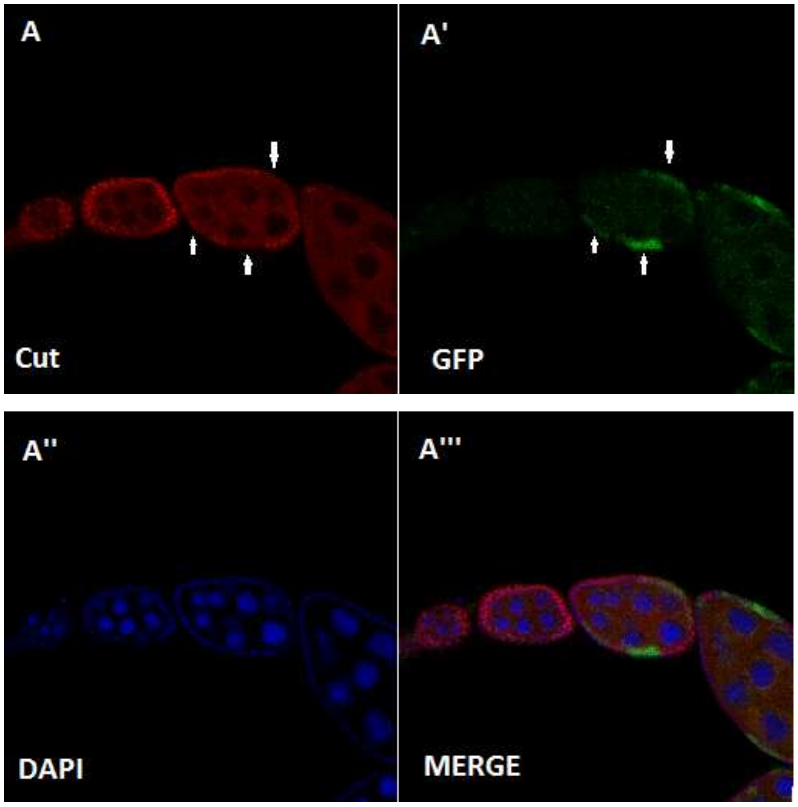


Figure 16. Early downregulation of Cut in *me31B*, Delta double knockdown clones. (A) shows early downregulation of Cut in GFP clones (A'). Nucleus is shown with DAPI (A'') and MERGE is (A''').

Therefore, I came up with an idea that if the loss of miRNAs caused by mutated or knocked down *me31B* results in inhibition of Notch activity through elevated Delta protein levels in follicle cells, then decreasing Delta in follicle cells should reduce cis-inhibition and the dominant phenotype in these cells should be premature Notch activation.

Consistent with this hypothesis that *Delta* is an important miRNA target, downstream of *me31B* and repressor of Notch in follicle cells, we found that the *Delta, me31B* double-knockeddown cells show early downregulation of cut in other words premature Notch activity.

Because knockdown of *me31B* cause upregulation of Delta in follicle cells, sand defective Notch signaling, and because double knockdown of *Delta, me31B* leads to premature Notch activity, we conclude that me31B regulates Notch signaling by targeting *Dl* through miRNA pathway.

CHAPTER FIVE

MATERIAL AND METHODS

5.1 Fly Stock

The following mutant alleles were used in this study: *P{lacW}me31B*^{k06607} **(**Bloomington Stock Center), *me31B*$^{\Delta 1}$ *FRT40A/Cyo* and *me31B*$^{\Delta 2}$ *FRT40A/Cyo* (kindly gifted from Dr. Akira Nakamura from Kumamoto University, Kyusyu, Japan). For more detailed information of these alleles, please visit Flybase at www.flybase.net.

The following stocks were used to analyze rescue phenotypes: *UAS-N(full length), UAS-NICD, UAS-Su(H)vp16 and hsFLP;AY<GFP/Cyo;UAS-me31B/TM3*

The following stocks were used to analyze KD follicle cell clones: *hsFLP;actin5C<CD2<Gal4,UAS-RFP/TM3,Sb* and UAS-me31B IR. Follicle cell clones were generated by heat shock inducible

Flippase (hsFLP) as previously described (Yu et al., 2008).

The following stocks were used to analyze wing disc clones: *UAS-me31B* and *fsFLP; sp/Cyo;hh-Gal4,UAS-CD8-GFP/TM6B* and *w,UAS-Dcr2;c96-Gal4*

The following stocks were used to analyze genetic interaction in eye and wing disc: UAS-me31B, N/FM7C, GMR-Gal4/Cyo;Dr/TM6B and en-Gal4/Cyo;Dr/TM6B

5.2 Generation of Follicle Cell Clones

Mutant clones were generated using the FLP/FRT system (Xu and Rubin, 1993) with heat-shock flipase (hsFLP) and were marked by absence of ubiGFP. To generate follicle cell mutant clones, flies containing the mutant allele (*me31B*$^{\Delta 1}$ *FRT40A,* for example) were crossed to the corresponding FRT marker lines (*FRT40A ubiGFFP*). When the larvae from this cross matured to third instar, they were heat shocked for two hours two consecutive days at 37°C. When adults emerged, female progeny with the correct genotype were selected, placed in yeasted vials, and dissected two days later

Knock down clones were generated using the *flip-out act<Gal4/UAS* system (Pignoni and Zipursky, 1997). Flies with the genotype *hsFLP GFP:Stau;; act<CD2<Gal4, UAS RFP were* crossed to *UAS-me31B RNAi* to knock down gene *me31B* . Heat-shocking was performed on 2 to

5-day old females at 37°C for 45 minutes. After 2 days of recovery, female flies were placed in yeasted vials. Their ovaries were dissected two days following yeasting. Clones were marked by coexpression of RFP.

5.3 Immunocytochemistry

Ovaries were dissected in 1×PBS and were fixed for 10 minutes in 5% formaldehyde in PBS. Ovaries were then washed in PBT (1×PBS plus 0.2% Triton X-100) three times for 10 minutes and then pre-blocked with PBTG (PBT, 0.2% BSA, and 5% normal goat serum, NGS) for one hour at room temperature. Ovaries were incubated with rotation with primary antibodies overnight at 4°C. Ovaries were washed four times in PBT and then incubated with secondary antibodies for two hours at room temperature. Ovaries were washed with PBT twice for 10 minutes each, incubated in DAPI solution (5 μg/ml) for 15 minutes, washed again once with PBT for 10 minutes, and washed once with PBS. Finally, the ovaries were mounted in mounting solution with 80% glycerol and n-propyl gallate (20 mg/ml).

The following primary antibodies were used: mouse anti-Me31B, mouse anti-Dl 1:20, mouse anti-BrC 1:60, mouse anti-Cut 1:15, mouse anti-Hnt 1:15, mouse anti-Wg(1:10) (all from Developmental Study Hyberdermal Bank)

The following secondary antibodies were used at a 1:400 dilution: Alexa 546 goat anti-mouse, Alexa 633 goat anti-mouse, Alexa 488 goat anti-mouse. Images were acquired with a Zeiss LSM 510 confocal microscope.

CHAPTER SIX

CONCLUSION

The developing egg chamber provides an ideal model to study cell-cell signaling. Proper timing of activation and/or suppression of signaling within a pathway is essential to producing normal eggs. In this thesis, I present data to show how Notch activation is regulated by me31B. Knockdown of Me31B, a putative RNA helicase belonging to the DEAD-box family, resulted in disruption of the Br early expression pattern during the endocycle stages. In addition, we found that Hindsight and Cut, in follicle cells, Cut and Wg, in wing disc, are also disrupted when *me31B* is knocked down, suggesting a potential role of Me31B in Notch signaling.

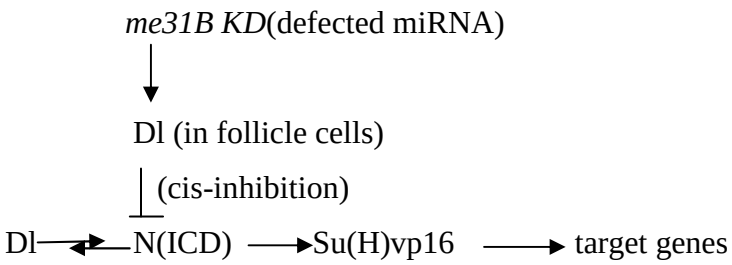
To exclude any potential off target artifacts caused by the RNAi experiments and to ascertain that *me31B* is required for regulation of Notch signal, we analyzed three *me31B* null alleles. Consisted with our previous results from the RNAi experiment, *me31B* knockdown resulted Br and Hnt downregulation (Figure 9B-9C) in the middle stage of oogenesis.

In order to determine whether there is a genetic interaction between Notch signaling and *me31B,* we examined how *me31B* is related to the Notch pathway during the wing and eye development where Notch is known to play an important role (Artavanis-Tsakonas and Muskavitch, 2010). As a result of this experiment I determined that knock down of *me31B,*in a heterozygouse Notch mutant background, enhanced the rough eye phenotype suggesting genetic interaction between Notch and *me31B* in other words me31B might act on same pathway with Notch.

Once we found me31B and N have genetic interaction, to determine at which level in the Notch pathway Me31B acts, I preformed rescue experiments with using UAS-N (full length), UAS-NICD and UAS-Su(H)vp16. Expression of these three components of the Notch pathway rescued the Br phenotype. Because only downstream elements of a pathway can rescue upstream elements, we can conclude that Me31B acts upstream of Notch.

Since Me31B acts upstream of Notch itself, we thought that target point can be either Delta (Dl) or another gene that acts on Dl or Notch. In follicle cells, it is already known that Dl is an important miRNA target and regulates timing of Notch signaling (Poulton and Deng., 2011). Researchers also found that Me31B is a component of RNP that found in P bodies (Hillebrand

and Ramaswami., 2007) and plays important role for translational repression of maternal and miRNA-target mRNAs (Hillebrand and Ramaswami., 2010).



This suggests the hypothesis that knock down of *me31B* causes a miRNA defect and disrupted miRNA leads to upregulation of Delta in follicle cells and accordingly a defect in Notch signaling. To test this hypothesis, we examined Dl expression at the both follicle cells and wing disc in *me31B* knock down flies by doing Dl immunostaining and we found that Dl expression is regulated by me31B. Defected Notch signaling by upregulated Delta in *me31B* KD cells and premature Notch activation in *me31B, Dl* double-knockdown cells confirmed our hypothesis that *me31B* regulates Notch signaling by targeting *Dl* through the miRNA pathway.

REFERENCES

Andrei, M.A., Ingelfinger, D.,Heintzmann, R., Achsel, T., Rivera-Pomar R., Luhrmann, R.(2005). A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies RNA.11, 717–727.

Artavanis, S., Tsakonas, Rand M.D., and Lake. R. J.(1999). Notch signaling: cell fate control and signal integration in development. Science, 284, no.541.

Artavanis-Tsakonas S, Muskavitch, MA. (2010) Notch: the past, the present, and the future. *CurrTop DevBiol*92, 1-29.

Ashraf, S.I., McLoon, A.L., Sclarsic, S.M., Kunes, S.(2006). Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. Cell, 124, 191–205.

Barbee, S. A., Estes, P. S., Cziko, A. M., Hillebrand, J., Luedeman, R. A., Coller, J. M., Johnson, N., Howlett, I. C., Geng, C., Ueda, R., Brand, A. H., Newbury, S. F., Wilhelm, J. E., Levine, R. B., Nakamura, A., Parker, R., and Ramaswami, M. (2006). Staufen- and FMRP-containing neuronal RNPs are structurally and functionally related to somatic P bodies. Neuron52, 997–1009.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-297.

Bray, S. J. (2006). Notch signaling: a simple pathway becomes complex. Nature Rev. Mol. Cell Biol. 7, 678–689.

Bushati, N. and Cohen, S. M.(2007). MicroRNA functions. Annual Review of Cell and Developmental Biology, vol. 23, 175–205.

Carpenter, AT. (1975). Electron microscopy of meiosis in *Drosophila melanogaster* females. I. Structure, arrangement, and temporal change of the synaptonemal complex in wild-type. Chromosoma51,157–182.

Chu, C. Y., and Rana, T. M. (2006). Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. PLoSBiol4, e210. doi: 10.1371/journal.pbio.0040210.

Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993).Expression of an extracellular deletion of Xotch diverts cellfate in Xenopus embryos. Cell 73, 659-671.

Coller, R. Parker.(2005).General translational repression by activators of mRNA decapping Cell, 122, 875–886.

Cordle, J., Redfieldz, C., Stacey, M., Merwe, van der. PA, Willis AC, Champion BR, Hambleton S, Handford.(2008). PA.Localization of the delta-like-1-binding site in human Notch-1 and its modulation by calcium affinity. J BiolChem b;283:11785–11793.

Couso, J. P., Knust, E. and Martinez Arias, A.(1995). Serrate and wingless inDrosophilawing development. Current Biology 5, 1437-1448.

D'Souza, B., Meloty-Kapella, L. &Weinmaster.G. (2010).Canonical and non-canonical Notch ligands.Curr.Top. Dev. Biol. 92, 73–129.

De Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. Development 124, 3241 -3251.

De Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996).Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. Development 122,359 -369.

deCelis, JF., Bray, SJ.(2000). The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe. Development;127, 1291–1302.

deFougerolles, A., Vornlocher, HP., Maraganore, J., Lieberman, J .(2007). Interfering with disease: a progress report on siRNA-based therapeutics. Nat. Rev. Drug Discov. 6, 443-453.

Deng, W. M., Althauser, C. and Ruohola-Baker, H. (2001). Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in *Drosophila* follicle cells.Development 128, 4737-4746.

DeValoir,T., Tucker, M.A., Belikoff, E.J., Camp, L.A., Bolduc, C.and Beckingham, K.(1991). A second maternally expressed Drosophila gene encodes a putative RNA helicase of the DEAD box family. Proc. Natl. Acad. Sci. USA .88, 2113-2117.

E.V. Koonin. (1993). A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication, Nucleic Acids Res. 21, 2541– 2547.

Fehon, R. G.Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A., Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila.Cell 61, 523–534.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditiselegans.Nature 391, 806–11

Fiuza, U. M. and Arias, A. M. (2007).Cell and molecular biology of Notch. Journal of Endocrinology194, 459–474.

Gorbalenya AE, Koonin EV, Donchenko AP, Blinov VM. (1989). Two related super families of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res. 17 (12),4713–30.

Grammont, M. and Irvine, K.D. (2001). Fringe and Notch specify polar cell fate during Drosophila oogenesis. Development, vol. 128, no. 12, 2243–2253.

Hillebrand J., Pan K., Kokaram A., Barbee S., Parker R., Ramaswami M. (2010). The Me31B DEAD-Box helicase localizes to postsynaptic foci and regulates expression of a CaMKII reporter mRNA in dendrites of Drosophila olfactory projection neurons. Front Neural Circuits. 4:121

Hillebrand, J., Barbee, S.A., and Ramaswami, M. (2007) P-body components, microRNA regulation, and synaptic plasticity.TheScientificWorldJOURNAL7(S2), 178–190. DOI 10.1100/tsw.2007.206

Horne, S and Bilder, D. (2005).Mass transit: epithelial morphogenesis in the Drosophila egg chamber. Developmental Dynamics, vol. 232, no. 3, 559-574.

Irvine, K. D., Vogt, T. F. (1997). Dorsal-ventral signaling in limb development. CurrOpin Cell Biol 9:867–876.

Jackson, S. M. and Blochlinger, K. (1997).cut interacts with Notch and protein kinase A to regulate egg chamber formation and to maintain germline cyst integrity during*Drosophila oogenesis.* Development 124,3663 -3672.

Jackson, S. M. and Blochlinger, K. (1997). Cut interacts with Notch and protein kinase A to regulate egg chamber formation and to maintain germline cyst integrity during*Drosophila* oogenesis. *Development* 124,3663 -3672.

Klein T., Arias AM. (1998).Interactions among Delta, Serrate and Fringe modulate Notch activity during Drosophila wing development. Development, Aug;125(15):2951-62

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A. &Tuschl, T. (2003).New microRNAs from mouse and human. RNA9, 175–179.

Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 21, 4663–4670.

Linder, P.; Lasko, P. F.; Ashburner, M.; Leroy, P.; Nielsen, P. J.; Nishi, K.; Schnier, J.; Slonimski, P. P. (1989). Birth of the D-E-A-D box. Nature 337 (6203), 121–122.

Mani, R., St Onge, RP. , Hartman, JLT., Giaever, G. , Roth FP. (2008) Defining genetic interaction. ProcNatlAcadSci U S A 105: 3461.6 doi: 10.1073/pnas.0712255105.

Micchelli CA, Rulifson EJ, Blair SS. (1997) The function and regulation of cut expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. Development 124: 1485–1495.

Miller, A. (1950). The internal anatomy and histology of the imago of *Drosophila melanogaster*.In: Biology of Drosophila, 2nd ed. M. Demerec, ed. Hafner, New York, pp. 420-534.

Mumm JS, Schroeter EH, Saxena MT, Griesemer A, Tian X, Pan DJ, RayWJ&Kopan R. (2000). A ligand-induced extracellular cleavage regulates g-secretase-like proteolytic activation of Notch1. Molecular Cell 5, 197–206.

Nakamura, A., Amikura, R., Hanyu, K., and Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. Development128, 3233–3242.

Nepveu, A. (2001). Role of the multifunctional CDP/Cut/Cuxhomeodomain transcription factor in regulating differentiation.Cell growth and development. *Gene* 270,1-15.

Nichols JT, Miyamoto A, Olsen SL, D'Souza B, Yao C &Weinmaster G. (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. Journal of Cell Biology 176, 445–458

Nystul, T. and Spradling, A.(2010).Regulation of epithelial stem cell replacement and follicle formation in the drosophila ovary. Genetics, vol. 184, no. 2, 503–515.

Parody, T. R. (1998). Structure-Function Analysis of Delta in vivo. Ph.D. Thesis, Indiana University, Bloomington, IN.

Pickup, AT,.Lamka, ML,. Sun, Q., Yip, MLR., Lipshitz, HD. (2002) Control of photoreceptor cell morphology, planar polarity and epithelial integrity during *Drosophila* eye development.Development 129, 2247–2258

Poulton, J. S., Huang, Y. C., Smith, L., Sun, J. J., Leake, N., Schleede, J., Stevens, L. M. and Deng, W. M. (2011). The microRNA pathway regulates thetemporal pattern of Notch signaling in Drosophila follicle cells. *Development* 138, 1737-1745.

Ranganathan, P, Weaver, K. L., and Capobianco, A.J. (2011).Notch signaling in solid tumours: a little bit of everything but not all the time. Nature Reviews Cancer, 11, no. 5.

Raya, A., Kawakami, Y., Rodriguez-Esteban, C., Ibanes M, Rasskin-Gutman, D., Rodriguez-Leon, J., Buscher, D., Feijo, JA.,Izpisua Belmonte, JC.(2004). Notch activity acts as a sensor for extracellular calcium during vertebrate left-right determination. Nature;427:121–128.

Rhoades, M.W., Reinhart, B.J., Lee P. L., Burge C.B., Bartel, B. and Barte, D.P.(2002). Prediction of plant microRNA targets. Cell 110, 513-520.

Roth S, Lynch JA.(2009).Symmetry breaking during Drosophila oogenesis. Cold Spring Harbor Perspect Biol. 1:a001891. doi: 10.1101/cshperspect.a001891.

Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Preibisch S., Rueden C., Saalfeld S., Schmid B., Tinevez J., Hartenstein V., Eliceiri K., Tomancak P., Cardona A. *(*2012*)* . Fiji: an open-source platform for biological-image analysis._ Nature Methods 9(7), 676-682 PMID: 2274772

Schratt, G.M., Tuebing, F., Nigh, E.A., Kane, C.G., Sabatini, M.E., Kiebler, M., Greenberg, M.E. (2006). A brain-specific microRNA regulates dendritic spine development. Nature, 439, 283–289.

Schreiber, S., Preiss, A., Nagel, AC., Wech, I. and Maier, D. (2002). Genetic screen for modifers of the rough eye phenotype resulting from overexpression of the Notch antagonist Hairless in Drosophila. Genesis 33, 141–152.

Shomron, N. and Levy, (2009). C. MicroRNA-biogenesis and Pre-mRNA splicing crosstalk. J Biomed Biotechno l594678.

Song, X., Call, G. B., Kirilly, D.andXie, T.(2007). Notch signaling controls germline stem cell niche formation in the Drosophila ovary. Development, vol. 134, no. 6, 1071–1080.

Sprinzak, D., Lakhanpal, A., Lebon, L., Santat, LA., Fontes, ME., Anderson, GA., Garcia-Ojalvo, J., Elowitz, MB. (2010). Cis-interactions between Notch and Delta generate mutually exclusive signalling states. Nature 465, 86–90.

Struhl G & Adachi A. (1998).Nuclear access and action of notch in vivo. Cell 93, 649–660.

Sun J, Deng WM. (2005). Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in Drosophila follicle cells *Development* 132, 4299-4308.

Sun J, Smith L, Armento A, Deng WM. (2008). Regulation of the endocycle/gene amplification switch by Notch and ecdysone signaling *The Journal of Cell Biology.* 182(5),885-96.

Sun, J., Deng, WM.(2007). Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation *Developmental Cell.* 12(3), 431-42.

Tzolovsky, G., W.M. Deng, T. Schlitt, and M. Bownes.(1999).The Function of the broad complex during Drosophila melanogaster oogenesis. *Genetics* 153,1371-1383.

Vachias, C., Couderc, J. L. and Grammont, M. (2010).A two-step Notch-dependant mechanism controls the selection of the polar cell pair in Drosophila oogenesis. Development, vol. 137, no. 16, 2703–2711.

Ward, E. J. ,Shcherbata, H. R., Reynolds, S. H. , Fischer, K. A. , Hatfield, S. D. and Baker, H. Ruohola. (2006).Stem cells signal to the Niche through the Notch pathway in the Drosophila Ovary. Current Biology, vol. 16, no. 23, 2352–2358.

Wolfe MS. (2006). The g-secretase complex: membrane-embedded proteolytic ensemble. Biochemistry 45, 7931–7939.

Xu, Jingxia and Gridley, Thomas.(2012). Notch signaling during Oogenesis in Drosophila melanogaster.Staff Bibliography, 177.

Xu, T. and Rubin, G.M. (1993).Analysis of genetic mosaics in developing and adult Drosophila tissues. Development 117, 1223-1237.

Ye, W., Lv, Q., Wong, CK., Hu, S., Fu, C., Hua, Z., Cai, G., Li, G., Yang, BB. , Zhang, Y. (2008). The effect of central loops in miRNA: MRE duplexes on the efficiency of miRNAmediated gene regulation. PLoS One 3: e1719.

Yip, M.L., Lamka, M.L., and Lipshitz, H.D. (1997).Control of germband retraction in Drosophila by the zinc-finger protein HINDSIGHT.Development 124, 2129–2141.

Yu J, Poulton J, Huang YC, Deng WM. (2008). The Hippo Pathway Promotes Notch Signaling in Regulation of Cell Differentiation, Proliferation, and Oocyte Polarity. *PLoS ONE.* 3(3): e1761.doi:10.1371/journal.pone.0001761

Zamore, PD., Tuschl, T., Sharp, PA. , Bartel, DP. (2000). RNAi: double-stranded RNA directs the ATPdependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25–33

Zhang, L., Zhao, J.H., and Edenberg, H.J. (1999).A human Raf-responsive zinc-finger protein that binds to divergent sequences. Nucleic Acids Res. 27, 2947–2956

BIOGRAPHICAL SKETCH

Muhammed Soylemez

Education: 2004-2008 Adnan Menderes University B.S Aydın, Turkey
2011-2013 Florida State University Master candidates in Dr. Wu-Min Deng's lab, Biological Science

Awards Received: 2009 Fellowship of Republic of Turkey, Ministry of Education
2008 Outstanding Student Award Adnan Menderes University

Teaching Experience: 2008-2009 Ministry of Education Mus/ Turkey
2012 Fall Semester in General Genetic Course
2013 Spring Semester in Experimental Biology Lab

Memberships: Turkish National Biologists Union
Genetic Society of America

Publications: 1) M. Soylemez, A. Ayaz, B. Ateslier, C. Unsal, K. Metin, M. Balkaya, "Evaluation of Effects of a Herb from Aristolochiaceae Family on Recovery of Wistar Rats Induced by Streptozootosin, " 14th *National Congress of Biology Student, Konya*, Turkey, 2007

2) Soylemez, M., Jia, D., and Deng, W.M. "Identification of me31B from an in vivo RNAi screen as a potential regulator of Notch Signaling". 54th Annual Drosophila Research Conference, Washington, DC, USA, 2013